Nitric Oxide

Biology and Pathobiology



Edited by

Louis J. Ignarro



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Edited by

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To my wife, Sharon Williams Ignarro, M.D., who was willing to spend even more time away from her husband

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Preface

Nitric oxide (NO) is one of the smallest and simplest of biologically active molecules in nature. Moreover, NO appears to be one of the most ubiquitous substances in mammalian species. As one of the most widespread signaling molecules in the mammal, NO is a major player in controlling nearly every cellular and organ function in the body. NO is the only endogenous molecule that functions as a neurotransmitter, autacoid, constitutive mediator, inducible mediator, cytoprotective molecule, and cytotoxic molecule. Certain physiological functions of NO, such as vasodilation and smooth muscle relaxation, are mediated by multiple mechanisms of NO release and NO action. For example, NO can be released as a neurotransmitter, generated as an autacoid by vascular endothelial cells, or produced by an inducible enzyme. Furthermore, NO can promote biological actions by cyclic GMP-dependent and cyclic GMP-independent molecular mechanisms. Because NO plays multiple physiological roles in regulating numerous and diverse organ functions, defects in the NO pathway lead to the development of many different pathophysiological states. These disorders include hypertension, atherosclerosis, coronary artery disease, cardiac failure, pulmonary hypertension, stroke, impotence, vascular complications in diabetes mellitus, gastrointestinal ulcers, asthma, and other CNS and systemic disorders. It is no surprise, therefore, that nearly all small and large pharmaceutical companies have established research and development programs for discovery of novel therapeutic strategies for NO-based disorders.

The number of annual publications dealing with NO has increased steadily from about 20 in 1986 to over 6000 in 1999. Although numerous reviews, treatises, and books on NO have been published, I felt that there was a need to publish a comprehensive textbook addressing the basic principles of all aspects of the field of NO research. To this end, I presented my thoughts and requests to many investigators and received overwhelming support to embark on the time-consuming and arduous task of preparing a textbook on NO. The authors of this text are internationally recognized and represent numerous and diverse basic and clinical fields. The challenging task for the authors was to prepare a comprehensive yet terse chapter on the current state of knowledge in the field of their expertise and to write the treatise in an easy, readable textbook style that focuses on principles. One of my tasks was to integrate the chapters to present a unified textbook on the biology and chemistry of NO. I believe that we all succeeded. Our intention has been to make this book an informative resource not only for the basic scientists in the field but also for all clinicians interested or participating in the care of patients with underlying disorders in NO physiology.

This book has 58 chapters that are divided into three major sections that lead from the principles of basic science to clinically applied science. The book begins with an overview of basic principles and then takes us through a more focused view of chemical biology, NO production, NO transport, oxidative stress, and signal transduction mechanisms. The book continues with in-depth coverage of the principles of NO pathobiology in the nervous system, cardiovascular system, and 16 chapters on numerous disease states and pathophysiological disorders involving NO. The final four chapters of the book address important issues on the clinical significance of NO.

Bringing together so many experts from all over the globe was no easy task. I am grateful to the authors, who worked very hard to meet the objectives, and to do so on time. I am also grateful to Dr. Jasna Markovac and to Jenny Wrenn of Academic Press, without whose continuous support this project would have never been launched or completed.

Louis J. Ignarro

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SECTION I

Overview of Basic Principles

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CHAPTER 1

Introduction and Overview

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Historical Perspectives

Nitroglycerin has been used clinically for well over 100 years to treat angina pectoris but only recently has its mechanism of action been elucidated and attributed to nitric oxide (NO) and cyclic GMP (cGMP). The field of cGMP research preceded the field of NO research. Long before the first significant biological experiments with NO were conducted, the presence of cGMP in mammalian urine was reported, and this led to a widespread interest in the possible biological roles of this new cyclic nucleotide. Up until 1969, cyclic AMP (cAMP) was the only cyclic nucleotide known to play important biological roles in cellular function. In 1969, however, cGMP was detected in mammalian tissues and guanylate cyclase was first discovered and partially characterized. Guanylate cyclase catalyzes the conversion of GTP to cGMP plus inorganic pyrophosphate in a reaction that requires a divalent metal cation such as magnesium. The first insight into the possible importance of endogenous cGMP in biological function was the finding that the decrease in cardiac contractility mediated by acetylcholine is associated with a concomitant and rapid accumulation of cardiac cGMP. Much of the early work involving cGMP suggested that it might function to mediate or signal cellular processes that are ultimately antagonistic or opposite in direction to those mediated by cAMP. This yin–yang hypothesis of biological control signified a type of control mechanism mediated through dual, opposing actions of cGMP and cAMP in bidirectionally regulated systems. Some of the best examples of the dualism theory of biological control were provided by studies on circulating cells such as neutrophils, lymphocytes, and macrophages and on related cells such as mast cells. In general, increases in cGMP production were associated with stimulation of cell function such as phagocytosis and discharge of lysosomal enzymes, whereas increases in cAMP production were associated with inhibition of these functions.

Research involving cGMP was well under way before the connection between NO and cGMP was made in the mid to late 1970s. Before this time, cAMP was believed to mediate the smooth muscle relaxant responses to certain catecholamines, prostaglandins, and adenosine, whereas there was no link between cGMP and smooth muscle function. The first clue that cGMP might be involved in smooth muscle relaxation was discovered in 1975, when sodium azide, hydroxylamine, and sodium nitrite, which were known to be smooth muscle relaxants, were reported to elevate tissue levels of cGMP and to activate guanylate cyclase. Subsequently, nitroglycerin was shown to elevate cGMP levels in rat myometrium and canine femoral artery, and sodium nitroprusside was demonstrated to increase cGMP levels in rat ductus deferens independently of calcium. An important report then appeared showing that nitroglycerin, sodium nitroprusside, and other nitro compounds increased cGMP levels in bovine tracheal smooth muscle, and this was associated with smooth muscle relaxation. Other reports appeared confirming these novel early observations and supporting the hypothesis that cGMP might be involved in mediating smooth muscle relaxation.

Prior to the evidence that cGMP might be involved in smooth muscle relaxation, cGMP was implicated in smooth muscle contraction. Numerous diverse chemical agents elevated cGMP levels in a calcium-dependent manner in smooth muscle tissues, which also contracted in response to such added agents. Additional experiments revealed that there was no direct relationship between smooth muscle

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contraction and cGMP accumulation and that inhibition of cGMP accumulation actually enhanced contractile responses. Therefore, experimental evidence for any physiological role of cGMP in smooth muscle function was controversial. These latter observations were actually consistent with the developing hypothesis that cGMP may be associated with smooth muscle relaxation. The earlier studies showing that nitro compounds stimulated cGMP accumulation in and relaxation of smooth muscle tissues suggested that NO might somehow be involved because certain nitro compounds were known to decompose to NO. The first experiments showing that NO directly relaxes vascular smooth muscle came in 1979, and relaxation was associated with guanylate cyclase activation and cGMP formation. Added chemical analogs of cGMP also caused vascular smooth muscle relaxation. NO gas caused a transient but concentration-dependent relaxation of isolated strips of bovine coronary artery that was blocked by added hemoproteins or methylene blue. Other nitro compounds, including nitrosoamines that generate NO, were shown to elicit similar effects on vascular smooth muscle. Even tobacco smoke, which contains approximately 800 ppm of NO gas, activated guanylate cyclase and caused vascular smooth muscle relaxation. These *in vitro* effects were not artifacts of *in vitro* isolated preparations, as marked hypotensive responses were also observed *in vivo*.

The unequivocal demonstration that nitrovasodilators elicit vascular smooth muscle relaxation via the actions of NO came in 1981, when organic nitrate and nitrite esters, inorganic nitroso compounds, and nitrosoamines were shown to react with thiols to form intermediate *S*-nitrosothiols, which were unstable and decomposed with the liberation of NO. The liberated NO then activated guanylate cyclase and elevated smooth muscle levels of cGMP, resulting in smooth muscle relaxation. A series of *S*-nitrosothiols were synthesized and found to be excellent NO donor molecules both *in vitro* and *in vivo*. The *S*-nitrosothiols, first described in 1980, represented the first known biologically active NO donor agents.

At about the time that the nitrovasodilators were shown to work via a NO mechanism, NO was first shown to inhibit platelet aggregation. Both the initial observation in the laboratory that sodium nitroprusside inhibited platelet aggregation and the obvious question of whether NO could be responsible for this action of sodium nitroprusside prompted this study. NO and other NO donor agents all inhibited platelet aggregation via NO and cGMP-dependent mechanisms.

As evidence that NO stimulated cGMP production, promoted smooth muscle relaxation, and inhibited platelet aggregation accumulated, studies were under way to elucidate the mechanism by which NO activates guanylate cyclase. Earlier studies with relatively crude preparations of soluble guanylate cyclase suggested, and subsequent studies with purified enzyme demonstrated, that heme is required for activation of soluble guanylate cyclase by NO and nitro compounds that generate NO. NO reacts with reduced heme iron, bound as a prosthetic group on guanylate cyclase, to form the nitrosyl–heme adduct, resulting in enzyme activation. The activation of guanylate cyclase occurs as a 100- to 200-fold increase in the V_{max} and a three- to four-fold decrease in the K_m for MgGTP substrate. Enzyme activation by NO can be blocked by hemoglobin or myoglobin by virtue of the capacity of hemoproteins to compete with guanylate cyclase for NO. Oxyhemoproteins can catalyze the rapid oxidation of NO to nitrate anion (NO_3^-) before the NO binds to the guanylate cyclase and thereby prevent enzyme activation.

The discovery of endothelium-dependent vascular smooth muscle relaxation and endothelium-derived relaxing factor (EDRF) occurred just as investigators were elucidating the mechanism of action of nitrovasodilators and the mechanism by which NO activates guanylate cyclase. The identification of EDRF took about 6 years after its initial discovery in 1980. The early experiments concerned with the identification of EDRF were varied in design and led to different conclusions, none of which indicating that EDRF might be NO. Suggestions ranged from arachidonic acid metabolites to carbonyl compounds. In 1984, we reported that methylene blue inhibits the capacity of acetylcholine (EDRF) to stimulate vascular cGMP production and also inhibits endothelium-dependent relaxation. Methylene blue was shown earlier to inhibit the capacity of NO to stimulate vascular cGMP production and, therefore, also relaxation. These observations suggested that EDRF closely resembles NO, but this conclusion was not drawn in our original publication in 1984. It became obvious to us soon after the publication of these data that EDRF might very well be NO. Experiments were designed to ascertain whether EDRF, like NO, could activate cytosolic guanylate cyclase. Bovine arterial or venous rings with intact endothelium were individually incubated in small test tubes containing purified soluble guanylate cyclase, and acetylcholine, bradykinin, or calcium ionophore was added to the reaction mixtures. The result was activation of the purified soluble guanylate cyclase. Endothelium-denuded rings did not liberate a factor that activated guanylate cyclase. Addition of methylene blue or hemoglobin blocked the activation of guanylate cyclase. These studies suggested that EDRF from artery and vein liberate NO, although this conclusion was omitted from the paper, pending additional evidence that EDRF is NO.

The next experiment, which was more selective than the former, revealed for the first time that EDRF might be NO. This experiment was essentially the same as that described above except that two forms of soluble guanylate cyclase were used. One form was the native heme-containing guanylate cyclase and the other form was heme-deficient enzyme. Only the heme-containing form of guanylate cyclase was activated by the endothelium-derived factors released from artery and vein by the endothelium-dependent vasorelaxants. The most definitive experiment that we conducted, showing that EDRF is NO, came from a spectral assay designed to determine whether the EDRF released from isolated fresh bovine aortic endothelial cells could mimic NO in its reaction with deoxyhemoglobin to form the characteristic nitrosyl–heme adduct of hemoglobin. NO characteristically

reacts with deoxyhemoglobin to cause a leftward shift in the Soret absorption maximum from 433 to 406 nm. Only NO causes this characteristic shift in spectrum. When the untreated endothelial cells were mixed with the endothelial cells, no change in spectrum was observed. However, when calcium ionophore was added to the mixture of endothelial cells and deoxyhemoglobin, a shift in the Soret absorption maximum from 433 to 406 nm promptly occurred, thereby indicating that EDRF was NO.

The discovery that EDRF is NO triggered an avalanche of independent studies by hundreds of different investigators in many diverse directions. The discovery implicated that vascular endothelial cells can synthesize NO from some unknown precursor linked to an interaction between a calcium ionophore and the endothelial cell membrane. This is all that was known before arginine was discovered as the substrate for the synthetic reaction and NO synthase was discovered as the enzyme system responsible for the conversion of arginine to NO.

Mechanism of Action of Nitrovasodilators

The mechanism of vasodilator action of nitroglycerin and other organic nitrate esters remained completely unknown from its first clinical use in the 1870s until 1981. The earliest mechanistic studies on nitroglycerin in the 1970s, conducted by Needleman and colleagues, revealed that persistent administration of high doses of drug to animals resulted in the development of tolerance, which was correlated with gradual depletion of tissue thiols. After additional experiments, the authors concluded that nitroglycerin required the presence of free thiol sulfhydryl groups in order to relax vascular smooth muscle. They thought that certain critical ''thiol receptors'' were required for interaction with nitroglycerin to cause vasorelaxation and that repeated exposure to nitroglycerin somehow caused depletion of these critical thiols.

Murad and colleagues discovered that nitroglycerin and other nitro compounds were activators of guanylate cyclase and could stimulate cGMP formation in tissues including smooth muscle. When we repeated similar experiments, we noted that nitroglycerin was only a weak activator of guanylate cyclase but that the addition of thiols such as cysteine to enzyme reaction mixtures markedly enhanced enzyme activation by nitroglycerin. Moreover, we found that thiols enhanced the capacity of various nitro compounds to activate guanylate cyclase and that nitroglycerin specifically required the presence of cysteine to cause enzyme activation. These observations began to reveal some similarities to those earlier experiments conducted by Needleman's group. Not only were thiols required for sustained vasorelaxation, but thiols were also required for activation of guanylate cyclase by nitroglycerin and related organic nitrate esters. What was the connection? Subsequent experiments revealed that the nitro compounds including nitroglycerin reacted with thiols to form intermediate *S*-nitrosothiols, which were chemically unstable and could be readily converted to NO under various conditions. These observations explained why tolerance readily develops to nitroglycerin but not to other nitrovasodilators. Repeated administration of nitroglycerin causes thiol depletion by utilizing all the stores of available thiol to generate *S*-nitrosothiol. In the absence of thiol, there can be no formation of *S*-nitrosothiol and, therefore, NO. Other nitrovasodilators do not absolutely require the presence of free thiol in order to liberate NO, although added thiol can enhance liberation of NO. The conclusion from these experiments was that thiols facilitate the activation of soluble guanylate cyclase by nitrovasodilators via mechanisms that involve liberation of NO from the parent molecules. Figure 1 is a schematic illustration of the mechanism of action of various nitrovasodilators.

Based on these studies, a series of *S*-nitrosothiols were synthesized and found to be good NO donor agents both *in vitro* and *in vivo.* Two of these, *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione (GSNO), have become popular NO donor agents in the laboratory. These two *S*-nitrosothiols are relatively stable in the solid form but decompose readily in aqueous solution. Perhaps more importantly, *S*-nitrosothiols can liberate NO much faster in the presence of tissue or biological components such as cations, heme iron, and thiol-containing proteins. For example, the nitroso moiety of an *S*-nitrosothiol readily undergoes transfer

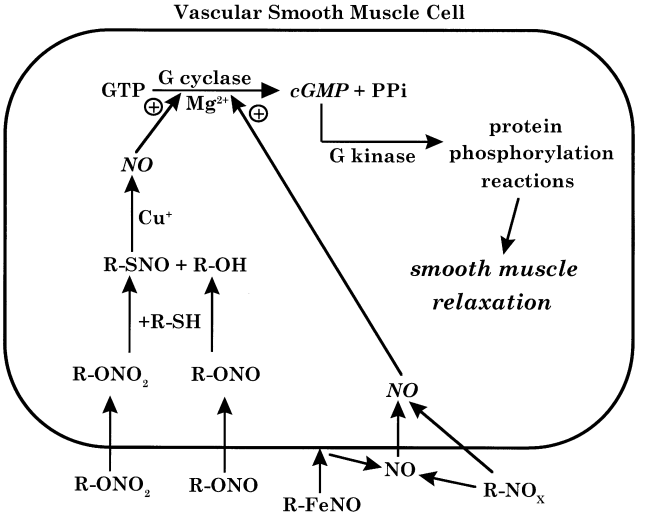


Figure 1 Schematic illustration of mechanism of vascular smooth muscle relaxant action of nitrovasodilators. Organic nitrate esters such as nitroglycerin ($R\text{-}ONO_2$) and organic nitrite esters such as isoamyl nitrite (R-ONO) permeate cells and react with thiols (R-SH) to form intermediate *S*-nitrosothiols (R-SNO) plus the corresponding alcohol (R-OH). R-SNO decomposes, especially in the presence of copper (Cu^+), to form nitric oxide (NO). Nitroprusside (R-FeNO) is an inorganic molecule that does not permeate cell membranes but undergoes decomposition to yield NO, which is lipophilic and readily enters cells. $R\text{-}NO_X$ represents other types of NO donor molecules that may or may not enter cells but decompose to yield NO. NO activates cytosolic guanylate cyclase (G cyclase), which catalyzes the conversion of GTP to cGMP plus inorganic pyrophosphate (PP_i) in the presence of magnesium (Mg^{2+}). cGMP promotes the activation of a protein kinase (G kinase), resulting in the phosphorylation of several proteins, which in turn leads to vascular smooth muscle relaxation.

to other molecules like proteins that contain free sulfhydryl groups. This transnitrosation reaction is relatively rapid and may be biologically important in the transport and actions of NO.

Antiplatelet Action of NO

By the mid 1970s it was known that endothelial denudation leads to rapid platelet adhesion, aggregation, degranulation, and recruitment of additional platelets that ultimately result in thrombus formation. Platelets aggregate in response to a number of agents, such as ADP, epinephrine, serotonin, thrombin, vasopressin, arachidonic acid metabolites, subendothelial matrix components, immune complexes, viruses, and bacteria. Platelet aggregation includes two phases: (1) a primary reversible phase, during which the discoid platelets change their shape and adhere to the exposed subendothelial tissue, and (2) a second, irreversible phase, which causes platelet secretion, release of platelet components, and recruitment of additional platelets. Patients suffering from cardiovascular and cerebrovascular disorders have an increased susceptibility to platelet aggregation, and factors such as dietary lipids, smoking, and stress appear to alter normal platelet aggregation. By the late 1970s the role of cAMP in platelet function was well established, but that of cGMP was uncertain. A number of contradictory reports showed that arachidonic acid metabolites, serotonin, ADP, or collagen caused an increase in platelet cGMP levels. One important observation made at that time was the fact that cAMP elevation inhibited both platelet aggregation and degranulation, whereas cGMP accumulation was associated only with inhibition of platelet aggregation. At the same time it was reported that sodium nitroprusside and nitroglycerin produced accumulation of cGMP in vascular and nonvascular smooth muscle cells. Other studies indicated the ability of sodium nitroprusside and nitroglycerin to inhibit platelet aggregation by a mechanism that was unrelated to cAMP accumulation.

The first report that correlated the antiaggregatory effects of NO, sodium nitroprusside, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, and cigarette smoke with the inhibitory role of guanylate cyclase activation and cGMP accumulation in platelets was published in 1981. All the discordant and contradictory information up to that time was explained. The authors also showed that NO and the nitro compounds disaggregated the platelets already aggregated by ADP and that this effect was mediated by cGMP. In addition to NO, antiaggregatory effects were also found with 8-bromo-cGMP (more lipophilic analog of cGMP) and relatively selective inhibitors of cGMP phosphodiesterase. To further confirm the involvement of the NO–cGMP signal transduction pathway in the inhibition of platelet aggregation by nitro compounds, the authors used hemoproteins to inactivate NO, and this abolished the antiaggregatory effects. A series of *S*-nitrosothiols, all potent activators of guanylate cyclase, showed a marked elevation of platelet cGMP levels that was correlated with the inhibition of platelet aggregation. In a study published in 1983, the same authors used synthetic *S*-nitrosothiols to show their heme-dependent activating effects on a partially purified soluble fraction of guanylate cyclase from human platelets.

By the mid 1980s it was already known that platelet activation played an important role in atherosclerosis, but most of the attention in clinical research was directed toward the cAMP elevating agents that inhibited platelet aggregation. In 1986, when EDRF was identified as NO, a timely shift took place in viewing the importance of NO in vascular disorders. In addition to NO, prostaglandin I_2 (prostacyclin) is another endothelium-derived vasodilator that also inhibits platelet aggregation. However, NO, but not prostacyclin, also inhibits platelet adhesion to the intimal lining of blood vessels. The platelet antiaggregatory effect of prostacyclin is mediated by cAMP. Therefore, at least two distinct, endothelium-derived, signal transduction pathways function to inhibit platelet function and thrombosis. The distinction between these two pathways, however, may be more apparent than real. For example, there may be a close regulatory link between NO and cAMP. NO can activate cyclooxygenase by cGMP-independent mechanisms and thereby enhance the production of prostacyclin, which acts via cAMP. This action of NO to stimulate cAMP production may account for the synergistic effects of NO and prostacyclin on platelet function. Thus, the loss of both endothelial products that regulate normal blood flow in vascular disorders, such as atherosclerosis and ischemia–reperfusion injury, will have profound pathophysiological consequences. This further underlies the importance of the clinical use of NO donors to restore vascular and platelet function in disorders involving endothelial dysfunction.

Red blood cells apparently contribute to vascular and platelet homeostasis in a new and unexpected manner. Red blood cells have been shown to contain a cytosolic, constitutive, calcium-dependent endothelium type of NO synthase. Human and rat red blood cells are capable of inhibiting platelet aggregation and degranulation. This effect is prevented by preincubation of red blood cells with NO synthase inhibitors, indicating that NO released from red blood cells plays a role in the antiplatelet action of red blood cells. The contribution of plasma, blood cellular components, and blood vessels to the available pool of NO reveals the complexity of the biological and chemical microenvironment that ultimately determines platelet function.

NO acts in an autocrine or paracrine manner to modulate platelet function via cGMP-dependent and cGMP-independent mechanisms. In the first case, cGMP carries out its effects by targeting three types of effector molecules: (1) cGMP-dependent protein kinases, (2) cGMP-gated cation channels, and (3) phosphodiesterases. cGMP-dependent protein kinases are the primary mediators of cGMP inhibitory action on platelet aggregation. They act by inhibiting agonist-elicited elevation of intracellular Ca^{2+} by blocking the formation of phospholipase C-mediated inositol 1,4,5-trisphosphate (IP_3), a Ca^{2+}-mobilizing intracellular messenger, from membrane phospholipids. In platelets, the

Ca^{2+}-regulating action of cGMP-dependent protein kinases is mimicked by cAMP-dependent protein kinases. Cytoskeleton-associated, vasodilator-stimulated phosphoprotein (VASP), a modulator of P-selectin and the GP IIb–IIIa complex involved in platelet adhesion and recruitment, appears to be the common target for both cGMP- and cAMP-dependent protein kinases. cGMP-gated ion channels belong to the cyclic nucleotide-gated ion channel family. Allosteric interaction of cGMP with cation channels has been documented in a number of cell types and may also inhibit platelet activity. Phosphodiesterases are enzymes that hydrolyze and inactivate cGMP and cAMP. cGMP can utilize the cAMP pathway by binding to and inhibiting specific phosphodiesterases to modulate cellular cAMP levels and thus affect platelet function. Two of the nine phosphodiesterases currently known, phosphodiesterase II and phosphodiesterase III, are regulated by cGMP. Phosphodiesterase III, which is present in platelets and hydrolyzes cAMP, is inhibited by cGMP in a process that results in intraplatelet accumulation of cAMP and inhibition of platelet aggregation.

There may also be cGMP-independent regulatory effects of NO on platelet function. Cyclooxygenase activation by NO causes cAMP-mediated antiplatelet effects, as discussed above. Other mechanisms, such as NO-mediated inhibition of cytochrome P-450 (responsible for metabolism of a variety of molecules including prostaglandins) and modulation of intracellular iron status are possible. Under pathophysiological conditions, NO may produce nitrosative and oxidative stress, resulting in reduction of intracellular thiols and inactivation of membrane receptors, ion transporters, and key metabolic enzymes, all of which can alter platelet and vascular functions. Platelets, just other cell types, are influenced by NO in a complex manner, and awareness of the multiple mechanisms of action of NO in physiology and pathophysiology is important.

Mechanism of Guanylate Cyclase Activation by NO

As discussed above, NO was discovered to activate the soluble isoform of guanylate cyclase in the mid 1970s, although the mechanism was not understood. In the absence of NO, guanylate cyclase still displayed catalytic activity in converting GTP to cGMP plus inorganic pyrophosphate (Fig. 2). The K_m for MgGTP substrate (or GTP in the presence of excess magnesium), is approximately 100 μM and the V_{max} of the enzymatic reaction is approximately 0.1 μmol/min/mg enzyme protein. Addition of NO to enzyme reaction mixtures lowers the K_m for MgGTP from 100 to about 30 μM and increases the V_{max} from 0.1 to about 10 to 20 μmol/min/mg enzyme protein. This represents about a threefold increase in the apparent affinity of guanylate cyclase for MgGTP substrate and a 100- to 200-fold increase in V_{max} or specific activity of guanylate cyclase.

Several studies in the late 1970s suggested that heme might be necessary for NO to activate guanylate cyclase. This hypothesis was based on some interesting experiments showing that the addition of heme or hemoglobin to relatively crude tissue preparations containing guanylate cyclase resulted in greater enzyme activation by NO and nitro compounds thought to liberate NO. These observations suggested that heme might be a bound component such as a prosthetic group on guanylate cyclase, which served to attract and bind NO to allow it to activate guanylate cyclase. Accordingly, two laboratories set out to ascertain whether or not soluble guanylate cyclase was a hemoprotein that could bind NO. The results clearly indicated that soluble guanylate cyclase purified from bovine lung contained stoichiometric quantities of bound heme. In other experiments, it was possible to detach the heme from guanylate cyclase without denaturing the enzyme protein or decreasing its basal catalytic activity. Such heme-deficient guanylate cyclase preparations failed to undergo activation in the presence of added NO. However, addition of heme or hemoglobin back to enzyme reaction mixtures completely restored the capacity of NO to activate the enzyme. The heme-deficient enzyme could be readily and easily reconstituted with heme by incubation of enzyme with heme or hematin under mild reducing conditions (2 mM dithiothreitol) at 37°C for 20 min. Heme-reconstituted guanylate cyclase behaved just like native enzyme with respect to activation by NO.

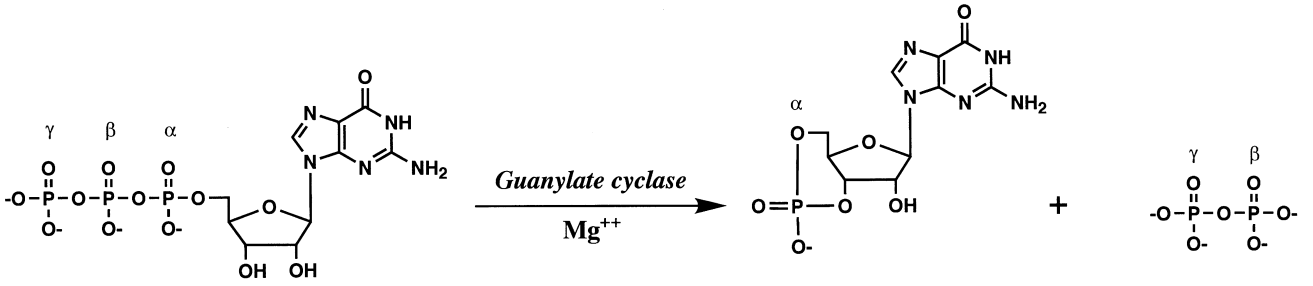


Figure 2 Guanylate cyclase enzymatic reaction. Guanylate cyclase catalyzes the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3',5'-monophosphate (cyclic GMP) plus inorganic pyrophosphate. The terminal two phosphate groups (β and γ) are cleaved as pyrophosphate, and the α phosphate cyclizes to the 3' position on the ribose yielding cyclic GMP.

These studies indicated that soluble guanylate cyclase contains heme and provided the basis for arguing that guanylate cyclase contains a high affinity binding site for NO. Indeed, the heme iron of guanylate cyclase can be regarded as the intracellular receptor site for NO. The question of mechanism of activation of guanylate cyclase by NO still remained. What occurs after NO binds to the heme iron on guanylate cyclase? In order to address this critical mechanistic question, we examined the role of the heme iron itself in enzyme activation by NO. Accordingly, protoporphyrin IX, which is essentially heme without iron, was tested for its binding affinity for purified guanylate cyclase. When protoporphyrin IX was mixed with heme-deficient guanylate cyclase and subsequently chromatographed on a gel filtration column to remove all traces of unbound protoporphyrin IX, some of the porphyrin remained bound to the guanylate cyclase, as determined by spectral analysis. Interestingly, the porphyrin-bound guanylate cyclase recovered from the column was found to be in the fully activated state. That is, its catalytic activity resembled that found on addition of NO to heme-containing guanylate cyclase. Subsequent experiments revealed consistently that protoporphyrin IX activates heme-deficient guanylate cyclase and even heme-containing enzyme by mechanisms involving the displacement of enzyme-bound heme and its replacement with protoporphyrin IX.

A series of enzyme kinetic experiments revealed that protoporphyrin IX activates guanylate cyclase by mechanisms that could not be distinguished from the mechanisms by which NO causes enzyme activation. Protoporphyrin IX was capable of activating either heme-containing or heme-deficient guanylate cyclase, whereas NO required the absolute presence of heme to cause enzyme activation. When the nitrosyl–heme complex itself was prepared and added to enzyme reaction mixtures, we noted that, like protoporphyrin IX, nitrosyl–heme activated both heme-deficient and heme-containing preparations of guanylate cyclase. These observations prompted an in-depth comparative analysis of the kinetic mechanisms by which protoporphyrin IX and nitrosyl–heme activate guanylate cyclase. The results of the experiments revealed that identical mechanisms were involved in guanylate cyclase activation by protoporphyrin IX and nitrosyl–heme. After considerable thought and reflection, we proposed a mechanism that might explain these observations and, therefore, how NO might activate guanylate cyclase. This mechanism is illustrated schematically in Fig. 3. In essence, the hypothesis is that the binding of NO to enzyme-bound heme iron causes a conformational change in the metalloporphyrin attributed to cleavage of the axial ligand between the iron and the enzyme protein. The maintenance of five-coordinate chemistry before and after NO binding indicates that the axial ligand must break. If the process in guanylate cyclase is similar to that in hemoglobin, the heme iron will protrude out of the planar porphyrin ring configuration and expose the underlying region of the protein. The hypothesis is based on the speculation that the catalytic site for MgGTP binding in guanylate cyclase lies in close proximity to the site of axial ligand interaction. When heme is bound to guanylate cyclase, the axial ligand inflicts steric hindrance to the binding of substrate and accounts for the relatively low basal catalytic activity. When NO binds to heme iron, however, breakage of the axial ligand and consequent exposure of the catalytic site to more substrate results in a markedly increased catalytic rate (greater V_{max}). The fa-

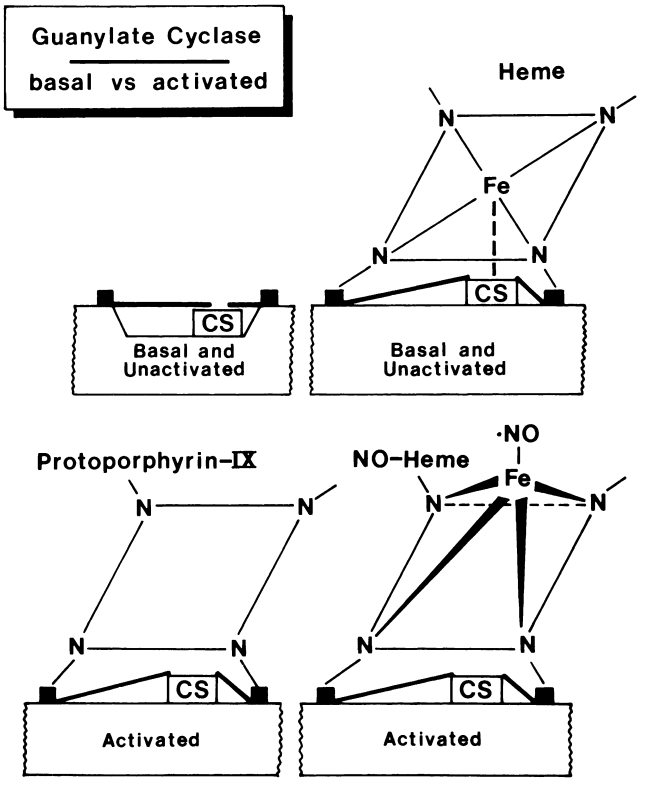


Figure 3 Mechanism of activation of guanylate cyclase by NO. The native enzyme contains bound heme as a five-coordinate complex. The heme iron is coordinated to each of the four pyrrole nitrogen atoms and forms an axial ligand with a histidine residue in the enzyme protein. The heme or porphyrin binding site is hypothesized to be adjacent to the substrate binding site or catalytic site (CS). Binding of heme to guanylate cyclase may cause a conformational change at the substrate binding site, resulting in exposure of the catalytic site to the external environment containing the substrate. The axial ligand, however, may sterically interfere with substrate binding, allowing only a slow catalytic rate. This is designated as basal or resting activity and the enzyme is in the unactivated state. Detachment of heme from guanylate cyclase does not cause any appreciable change in catalytic activity. NO binds to the heme iron to form the nitrosyl–heme or NO–heme complex. The NO–heme complex remains five-coordinate, signifying that the original axial ligand is severed, allowing the iron to protrude outward away from the plane of the porphyrin ring structure. The hypothesis is that such exposure of the catalytic site (CS) allows more efficient substrate binding and a markedly increased catalytic rate. Protoporphyrin IX activates guanylate cyclase by a similar mechanism. Porphyrin binding, whether by protoporphyrin IX or heme, may cause a similar conformational change at the substrate binding site, resulting in exposure of the catalytic site to substrate. Since there is no iron in protoporphyrin IX, there is no axial ligand to sterically interfere with substrate binding. Therefore, binding of protoporphyrin IX causes enzyme activation by increasing exposure of the catalytic site to substrate. This is essentially the same mechanism by which NO–heme causes activation of guanylate cyclase.

cilitation of substrate binding probably accounts for the lower K_m value observed for the activated enzyme.

The previous observations have been confirmed by several investigators. The actual heme content of soluble guanylate cyclase is debatable, with different investigators reporting anywhere from less than 1 to 1.5 mol of heme per mole of the heterodimeric enzyme. This debate will be settled when the crystal structure of the enzyme is obtained. The fact that protoporphyrin IX causes heme-independent activation of guanylate cyclase by kinetic mechanisms indistinguishable from those of the nitrosyl–heme complex helped in the elucidation of the mechanism by which NO activates guanylate cyclase. In the unactivated state, the heme of guanylate cyclase is five-coordinate with the histidine-105 of the β subunit bound to the fifth coordination position of the heme iron. This species has an absorption maximum at 430 nm. NO binds to the sixth coordination position of heme. However, the heme still remains five-coordinate because in the process of NO binding, the histidine to iron coordinate bond breaks. This species has an absorption maximum at 398 nm. It is thought that this loss of histidine–iron bond facilitates the interaction of the porphyrin portion of the complex with the enzyme to cause activation. The association of NO to a five-coordinate iron is known to be nearly diffusion controlled. The dissociation of NO from the complex is also rapid when compared to other hemoproteins. Moreover, NO dissociation increases by about 40- to 50-fold in the presence of the substrate MgGTP, giving a half-life for the nitrosyl adduct of about 5 s at 37°C. This half-life is probably fast enough for rapid deactivation of guanylate cyclase in biological systems.

Copper may also be bound to soluble guanylate cyclase under physiological conditions. The role of the copper is unknown but one possibility might be to facilitate the activation of guanylate cyclase by endogenous *S*-nitrosothiols. Copper (Cu^+) catalyzes the decomposition of *S*-nitrosothiols with the liberation of NO. This might be the mechanism by which *S*-nitrosothiols activate guanylate cyclase by kinetic mechanisms that closely resemble those for NO. Moreover, this would be a convenient mechanism to allow both free NO and *S*-nitrosothiols to activate guanylate cyclase similarly.

Biosynthesis of NO

The recognition of NO as a signaling molecule, the biosynthesis of NO and its regulation, and subsequent cloning and characterization of the enzymes responsible for its production stem from three separate but merging lines of investigation. The search for the endogenous vasodilator agent produced by the vascular endothelium, the investigations into the mechanisms of excitatory neurotransmission, and the cytotoxic actions of macrophages would all converge on NO. Research into the vascular actions of NO began with an apparent experimental paradox. Despite the potent vasodilating action of acetylcholine (ACh) *in vivo,* it did not always produce relaxation of isolated preparations of blood vessels *in vitro.* For example, in helical strip preparations from rabbit thoracic aorta, ACh at relatively high concentrations caused contractions. This contractile effect was mediated by muscarinic receptors. It was observed that if a ring preparation was made, ACh produced marked relaxation at concentrations much lower than those required to produce contraction in the helical strip preparation. It was discovered that the loss of relaxation caused by ACh in the helical strip was the result of unintentional rubbing of its intimal surface during its preparation. If the intimal surface was preserved during preparation, the tissue, whether ring or strip, always exhibited relaxation to ACh. This implied a role for endothelial cells, found in the intimal surface. ACh, acting on muscarinic receptors of the endothelium, stimulates the release of EDRF, which causes relaxation of the vascular smooth muscle. Like ACh, bradykinin was also known to elicit the relaxation of certain isolated arterial rings. Following the example set by acetylcholine, researchers discovered that after removing the endothelial cells from the vessel wall by rubbing the intimal surface, canine arteries failed to relax upon the addition of bradykinin. As with rabbit thoracic aorta, endothelial cells of canine arteries stimulated with bradykinin appeared to release a substance mediating vascular smooth muscle relaxation. Several other substances were found to elicit the release of EDRF from the endothelium and cause subsequent relaxation in specific vascular tissues from different animals. These elicitors included ACh, bradykinin, the calcium ionophore A23187, ATP and ADP, substance P, bradykinin, histamine mediated by H_1-receptors, thrombin, serotonin, and norepinephrine mediated by α_2 receptors.

After the identification of EDRF as NO, studies revealed that histamine, ACh, bradykinin, and the calcium ionophore A23187 all enhanced NO release from endothelial cells perfused with L-, but not D-arginine. The release of ^{15}NO, from L-[*guanido*-$^{15}N_2$]arginine was determined by mass spectrometry, indicating that NO was formed from one of the chemically equivalent guanidino nitrogens of L-arginine. The inhibitor, L-N^G-methylarginine, but not D-N^G-methylarginine, inhibited the generation of NO by endothelial cells in culture. Both of these effects were reversed by the addition of excess L-arginine. These data indicated that L-arginine is the precursor for the formation of NO by vascular endothelium.

In the central nervous system (CNS), activity in excitatory pathways is correlated with subsequent increases in the levels of cGMP. Accordingly, exogenous stimulation of the brain with the excitatory neurotransmitter, glutamate, was found to cause a striking increase in cGMP levels in brain tissue. Brain tissue was found to contain an endogenous activator of guanylate cyclase activity. This activator was both acid soluble and removed by cation-exchange chromatography, suggesting a basic compound. The activator was then purified from rat brain and identified as L-arginine by ^{13}C- and ^1H-NMR spectroscopy and by paper chromatography. L-Arginine, at a concentration of 10–20 μ*M,* stimulated guanylate cyclase activity 15- to 25-fold, whereas D-arginine

and other basic L-amino acids were ineffective. The authors suggested that the activation of neuronal guanylate cyclase by L-arginine was similar to earlier reports that guanylate cyclase is activated by nitroso compounds. Not until several years later would the assumption that arginine could function as a direct activator of guanylate cyclase be disproved. Arginine failed to activate purified guanylate cyclase.

As an excitatory neurotransmitter, glutamate functions through both ionotropic (*N*-methyl-D-aspartate, NMDA) and metabotropic (kainate) receptors. The activation of NMDA receptors in rat cerebellum leads to the release of EDRF, by this time identified as NO, a direct activator of guanylate cyclase. L-Arginine, but not D-arginine, augmented the response to NMDA. The results indicated that stimulation of NMDA receptors results in the activation of an enzyme that catalyzes the formation of NO from L-arginine. Similar results were found with the activation of kainate receptors in rat cerebellum. The response in slices was Ca^{2+} dependent, augmented by L-arginine, and reversibly inhibited by L-N^G-methylarginine. It was concluded that stimulation of kainate receptors also leads to activation of an enzyme that synthesizes NO from L-arginine and that activation of soluble guanylate cyclase by the released NO accounts for cGMP generation.

While researchers were elucidating the role of NO biosynthesis in vascular endothelium and studying glutamate signaling in the brain, others were following a different line of investigation that would also lead to NO. It was observed that both humans and germfree rats excreted more nitrate than was ingested, suggesting endogenous biosynthesis of nitrate. The generation of nitrate increased significantly in infected animals, but it was difficult to exclude a possible role for the infectious agent in producing the observed increase in nitrate levels. This problem was simplified when it was reported that injection of an immunogenic bacterial cell wall constituent, lipopolysaccharide (LPS), could induce nitrate biosynthesis. Thus, intraperitoneal injection of LPS led to a transient increase in nitrate levels in blood and urine. Furthermore, peritoneal macrophages cultured from susceptible mice produced nitrite and nitrate when cultured with LPS, implicating the immune system in the observed phenomenon. LPS-induced nitrate synthesis was also observed with nude mice, indicating that neither functional T lymphocytes nor LPS-responsive B lymphocytes were required for the response *in vivo.* This suggested that macrophages were responding to LPS independent of other immune cells. The researchers responsible for this discovery suggested that nitrite synthesis is significant because of its potential involvement in the chemistry of macrophage-mediated cytotoxicity. They confirmed their own suspicions a few years later while investigating the RAW 264.7 macrophage cell line. When activated by LPS plus interferon-γ (IFN-γ) these cells synthesized high levels of nitrite (NO_2^-) and nitrate (NO_3^-). Changing the medium after activation enabled them to show that L-arginine was the only amino acid essential for this synthesis. Also, the stereoisomer D-arginine would not substitute for L-arginine. When morpholine (tetrahydro-1,4-oxazine) was added to the culture medium of the activated macrophages, N-nitrosation occurred, generating *N*-nitrosomorpholine. Gas chromatography–mass spectrometry (GC/MS) experiments using L-[*guanido*-$^{15}N_2$]arginine established that both NO_2^- and NO_3^- and the nitrosyl group of *N*-nitrosomorpholine were derived exclusively from one of the chemically equivalent guanidino nitrogens of arginine. Chromatographic analysis showed that the other product in the conversion of L-arginine to NO_2^- and NO_3^- was L-citrulline. The enzymatic conversion of L-arginine to L-citrulline plus NO_2^- and NO_3^- is oxidative but was found to require reducing equivalents in the form of NADPH. When subcellular fractions containing the enzyme activity were incubated with L-arginine and NADPH, transient generation of NO was observed. As with other experiments, the formation of NO_2^- and NO_3^- was inhibited by L-N^G-methylarginine, but so was the generation of NO, suggesting that NO is an intermediate in the biosynthesis of NO_2^- and NO_3^-. Furthermore, when incubated with L-[*guanido*-$^{15}N_2$]arginine, NO was ^{15}N-labeled. The results clearly demonstrated that NO is an intermediate in the conversion of L-arginine to NO_2^-, NO_3^-, and citrulline.

Once established that NO was endogenously produced in vascular endothelium, brain, and the immune system, the quest to isolate the responsible enzyme and to characterize its properties was under way. Early attempts to isolate NO synthase were problematic due to the difficulty in monitoring the generation of a transient species such as NO. This problem was averted when investigators followed the stoichiometric conversion of L-arginine to L-citrulline that accompanied NO synthesis, rather than monitoring NO generation. Following the transformation of [^{3}H]arginine to [^{3}H]citrulline became a simple technique for monitoring NO synthase activity, and enzyme was first purified to homogeneity using this monitoring technique. Following homogenization of rat cerebellum, a two-column purification utilizing diethylaminoethyl (DEAE) cellulose followed by 2',5'-ADP agarose resulted in 6000-fold purification of NO synthase. The kinetic values determined for NO synthase included a K_m for L-arginine of 1.5 μM; a V_{max} of 0.96 mmol min^{-1} mg^{-1}; and a K_i of 1.4 μM for L-N^G-methylarginine. In addition, the purified brain enzyme required that calcium (Ca^{2+}) and calmodulin be added back in order to observe enzymatic activity. The purified brain NO synthase migrated as a single 150-kb band on SDS-PAGE. The purification of NO synthase from cultured macrophages was also achieved by utilizing 2'5'-ADP column chromatography and monitoring the purity of the eluate fractions by testing for the conversion of [^{14}C]arginine to [^{14}C]citrulline. Using ^{18}O-labeled dioxygen ($^{18}O_2$), it was determined by mass spectrometry that the ureido oxygen of L-citrulline derives from O_2 and not water as some researchers had earlier proposed. In contrast to the purified brain enzyme, calcium and calmodulin were not required for full activity of the purified macrophage NO synthase. From these publications a remarkable distinction could be seen in the regulation of NO synthesis, one purified enzyme that required Ca^{2+}/calmodulin for activity, and one that did not. Apart from distinctions based on tissue distribution, subcellular localization, and physiologi-

cal or pathophysiological roles of different NOS isoforms, the difference between calcium-activated and calcium-independent isoforms remains perhaps the most fundamental to enzymologists.

Following purification of macrophage inducible NO synthase (iNOS), enzyme activity required the addition of L-arginine, NADPH, and cytosolic cofactors including tetrahydrobiopterin. Researchers investigating other possible requirements for macrophage NO synthase discovered that flavin adenine dinucleotide (FAD) was needed for full enzyme activity. The addition of reconstituted cytosolic fractions to purified iNOS significantly enhanced the rate of NO synthesis. FAD alone increased activity more than twofold and suggested that NO synthase is a flavoprotein. NADPH was not required to regenerate tetrahydrobiopterin, and neither $NADP^+$ nor NADH or NAD^+ could substitute for NADPH. These findings suggested that NO synthesis by a macrophage NO synthase is an NADPH-utilizing FAD flavoprotein. Murine macrophage NO synthase ran as a single band with a relative molecular mass of 130 kDa. Gel filtration experiments estimated the native molecular mass to be 260 kDa, indicating that the native enzyme exists as a dimer. Also, fluorescence studies demonstrated the presence of one bound FAD and one bound flavin mononucleotide (FMN) per subunit.

The flavin requirement of NO synthase was confirmed when purified rat cerebellar NO synthase was cloned by screening a rat brain cDNA library. The resulting clones had an identical open reading frame of 4287 bases encoding a protein of 1429 amino acids and a relative molecular mass of 160 kDa, roughly corresponding to the size of the purified cerebellar enzyme. Sequence analysis revealed putative binding sites for calmodulin, NADPH, FAD, and FMN. Computerized sequence alignment with a FASTA program revealed remarkable similarity between cloned rat neuronal NO synthase (nNOS) and both cytochrome P-450 reductase and monooxygenase. The assay for detection of cytochrome P-450 monooxygenase (P-450) is spectroscopic, with an absorbance maximum near 450 nm for the carbon monoxide-reduced iron–heme component of the enzyme. Purified rat cerebellar NO synthase, like P-450, has a reduced CO absorbance maximum near 445 nm. A similar finding was also published for macrophage NO synthase, with a reduced CO absorbance maximum of 447 nm. Similar to the P-450BM3 isoform from bacteria, NO synthase is biochemically unique in that it contains both a reductase and a heme domain encoded in the same protein.

The characterization of the reductase and oxygenase domains of NO synthase was enabled first through their physical separation. This was accomplished by limited digestion with trypsin, which cuts the NO synthase monomer at one conserved site. Reconstitution of NO synthesis activity from isolated oxygenase and reductase domains of iNOS then provided important insights into the chemistry of catalysis. Although NO was not produced from L-arginine following reconstitution, it could be formed from the reaction intermediate N^G-hydroxy-L-arginine (L-NOHA). It was shown that the isolated iNOS reductase and oxygenase domains each retained their separate catalytic functions but interacted to catalyze only the second step of NO synthesis. The tryptic fragments were still able to interact and transfer electrons, despite a requirement for exceptionally high 6:1 reductase: oxygenase stoichiometry for optimal catalysis. Electron transfer, being inefficient in the reconstituted enzyme, was likely limited to single electrons. However, the conversion of L-arginine to L-NOHA by NO synthase is hypothesized to be analogous to a mixed function oxygenase, in that it requires two electrons derived from NADPH. Such electron transfer was unlikely in the reconstituted enzyme, which explains why L-NOHA, but not L-arginine, could act as a substrate for NO generation.

The heme environment in cloned, expressed nNOS has been examined and the chemistry compared to the P-450 class of enzymes. The electron density marker line in the Raman spectrum of ligand-free Fe^{II} nNOS has a low frequency (1347 cm^{-1}), indicating a thiolate axial ligand on the heme. Also, the heme in resting nNOS is five-coordinate high spin and thereby differs from the resting state of most P-450s, which are predominantly six-coordinate low spin. Such differences in heme coordination make NO synthase a unique enzyme, which may explain its equally unique and elusive catalytic process.

The molecular cloning of macrophage NO synthase was accomplished by screening a cDNA library made from LPS-stimulated RAW 267.4 macrophages. The macrophage enzyme displays only 50% sequence identity to nNOS. Like nNOS, macrophage NO synthase has recognition sites for FAD, FMN, and NADPH and also has a consensus calmodulin-binding site. Macrophage NO synthase mRNA is very inducible; it was undetectable in quiescent macrophages but prominent 2–6 hr after LPS treatment.

NO synthase in vascular endothelium shares common biochemical and pharmacological properties with nNOS, including constitutive expression and Ca^{2+}/calmodulin-dependent activity. However, cloning and molecular characterization of NO synthase from bovine endothelial cells indicated specific differences. NO synthase was purified from bovine brain and tryptic peptides were sequenced. The sequence data was utilized to isolate clones encoding NO synthase from a bovine aortic endothelial cell cDNA library. A full-length NO synthase cDNA clone was isolated, representing a protein of 1205 amino acids with a molecular mass of 133 kDa. The deduced amino acid sequence of the endothelial NO synthase (eNOS) cDNA differs at numerous residues from the sequence determined for NO synthase purified from bovine brain (bovine nNOS). Endothelial NOS shares only 50–60% sequence identity to either the iNOS clone obtained from murine macrophages or the nNOS clone obtained from rat cerebellum. One of the striking differences between eNOS and other isoforms is myristoylation of the protein. This was demonstrated by the incorporation of [^{3}H]myristic acid into eNOS, and autoradiography of a partially purified protein run on SDS-PAGE.

NO synthase in the endothelium is a particulate enzyme, whereas other isoforms of NO synthase are soluble. The incorporation of myristate to eNOS makes the enzyme

particulate because fatty acid anchors the enzyme into the membrane. Through this lipid anchor, eNOS is associated with the Golgi in both cultured endothelial cells and intact blood vessels. Immunofluorescence studies using monoclonal antibodies have shown that eNOS is localized in a perinuclear region in both human and bovine endothelium, consistent with Golgi distribution. Colocalization with a Golgi-specific marker and immunoperoxidase studies demonstrated expression of eNOS on the cytoplasmic face. In relating this subcellular localization to functional significance it was noticed that a wild-type eNOS released several-fold more NO than cells expressing a mutant, nonacylated enzyme. Thus, acylation and association with the Golgi is important for eNOS function. In addition, palmitoylation of eNOS also regulates subcellular distribution and function, but in a manner that is more dynamic and responsive to extracellular signals.

NO synthase isolated from neurons (nNOS) or endothelium (eNOS) are both calmodulin dependent. Calmodulin binds reversibly to neuronal NO synthase in response to elevated Ca^{2+} and initiates the conversion of L-arginine to L-citrulline plus NO. Calmodulin binding allows NADPH-derived electrons to pass onto the catalytic heme. It has been demonstrated that calmodulin-triggered electron transfer to heme is independent of substrate binding and is required for NO synthesis. Inducible NO synthase isolated from cytokine-induced macrophages contains tightly bound calmodulin and catalyzes spontaneous electron transfer to the heme. This is consistent with the hypothesis that calmodulin binding regulates electron transfer in neuronal and endothelial isoforms and is a molecular switch that regulates catalytic activity on the basis of intracellular Ca^{2+} levels.

The primary sequence map of the three isoforms of human NO synthase is illustrated in Fig. 4. The structure of NO synthase has been investigated for both the murine inducible and bovine endothelial isoforms. Crystal structures for dimerized oxygenase domains from murine iNOS (residues 1–498) with different cofactors and molecules bound at the active site have recently been obtained at a resolutions near 2.6 Å. Tetrahydrobiopterin binding was essential for assembly, as it was determined to fold the central interface region, create a 30-Å-deep active-site channel, and tilt the heme for likely interactions with the reductase domain. Proximity of heme with H_4B and L-arginine suggests that tetrahydrobiopterin has electronic influences on heme-bound oxygen. L-Arginine binds to glutamic acid and stacks with heme in a hydrophobic pocket to prevent activated dioxygen from contacting water during the two distinct steps in NO synthesis.

Crystal structures of the oxygenase domain of bovine eNOS have also been recently obtained, and the description contrasts strikingly with that for murine iNOS. Crystals for eNOS in tetrahydrobiopterin-free and tetrahydrobiopterin-bound forms have been derived at resolutions of 1.95 and 1.9 Å, respectively. In both structures a zinc ion is tetrahedrally coordinated to pairs of symmetry-related cysteine residues at the dimer interface. This contrasts with iNOS, in which these conserved cysteines were reportedly involved in disulfide bonding between the dimers. The eNOS crystal structure suggests that the zinc–sulfur cluster plays a structural role in maintaining the integrity of the tetrahydrobiopterin-binding site. Such an intimate role for zinc in determining tetrahydrobiopterin binding disputes the reported structural importance of tetrahydrobiopterin in the murine iNOS model. Also, in the eNOS model, L-arginine was observed at the tetrahydrobiopterin site, suggesting a possible involvement of tetrahydrobiopterin in catalysis. The authors proposed a model in which a positively charged pterin radical participates in the catalytic cycle. The elucidation of NO synthase biochemistry and catalysis remains a center of sci-

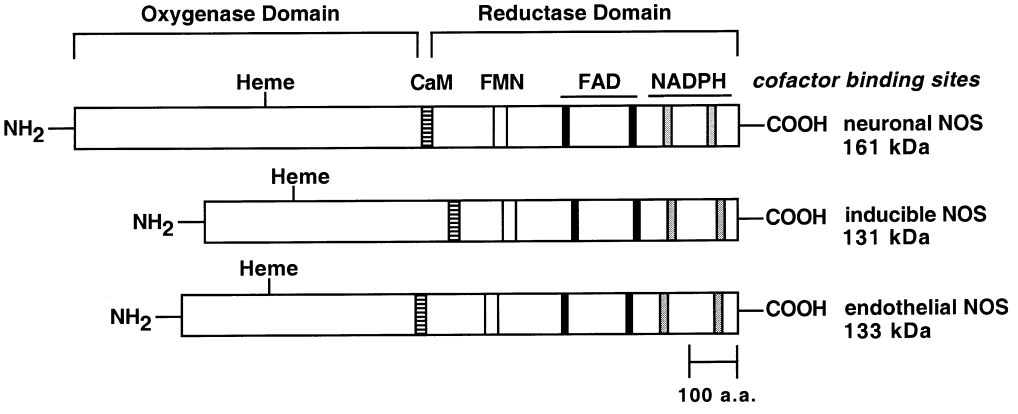


Figure 4 Primary sequence map of the three isoforms of human NO synthase and comparison to human NADPH–cytochrome P-450 reductase. All NO synthase isoforms are flavo-hemoproteins that utilize NADPH as a substrate for reducing equivalents. Illustrated in the diagram are binding sites for the cofactors including NADPH, FAD, FMN, and Ca^{2+}/calmodulin. Calmodulin is constitutively bound to the iNOS isoform and no Ca^{2+} is required for binding. The residues that bind tetrahydrobiopterin are not illustrated. The oxygenase domain of NO synthase contains a conserved cysteine residue that acts as an axial ligand for the heme iron.

entific debate. Future studies will undoubtedly clarify the roles of tetrahydrobiopterin and zinc in all three NO synthase isoforms.

Inactivation of NO

The inactivation of NO is unique with respect to other signaling molecules in that its inactivation is dependent solely on its nonenzymatic, chemical reactivity with other molecules. This section addresses the well-known oxidative mechanisms by which NO is inactivated. Reductive mechanisms, however, may also play a role in the inactivation of NO, but such reductive mechanisms are not well characterized.

NO readily reacts with oxyhemoglobin or oxymyoglobin to give nitrate (NO_3^-) and the oxidized hemoproteins, methemoglobin and metmyoglobin [Eq.(1)].

$$\text{Hb(Fe-O}_2) + \cdot\text{NO} \rightarrow \text{metHb(Fe(III))} + \text{NO}_3^- \qquad (1)$$

This reaction may be the primary mechanism by which the movement and concentration of NO are controlled *in vivo.* Due to the high concentrations of oxyhemoglobin in the body, its reaction with NO may be the primary metabolic as well as the primary detoxification mechanism for NO. Indeed, the measurement of NO_3^- in blood or urine is often used as an indirect indicator of *in vivo* NO production. In addition to the interaction of NO with oxyhemoglobin or oxymyoglobin, NO can react with the met and deoxy forms of hemoglobin or myoglobin [Eqs. (2) and (3)].

$$\text{metHb(Fe(III))} + \cdot\text{NO} \rightarrow \text{deoxyHb(Fe(II))} + \text{NO}_2^- + 2\text{H}^+ \qquad (2)$$

$$\text{deoxyHb(Fe(II))} + \cdot\text{NO} \rightarrow \text{Hb(Fe(II))–NO} \qquad (3)$$

NO is capable of reducing Fe(III) to Fe(II) resulting in the formation of nitrite (NO_2^-) and deoxyhemoglobin or deoxymyoglobin. The reaction of Fe(II) heme in deoxyhemoglobin or deoxymyoglobin with NO leads to an essentially irreversible Fe(II)–NO complex. The physiological relevance of these reactions, however, is unclear.

NO interacts with superoxide radical, the one-electron reduction product of O_2 in a near diffusion controlled reaction to form peroxynitrite ($^-$OONO) [Eq. (4)].

$$\cdot\text{NO} + \text{O}^{2\cdot-} \rightarrow {}^-\text{ONOO} \qquad (4)$$

Peroxynitrite has only a fraction of the biological activity of NO and, therefore, this reaction represents an inactivation mechanism for NO. Peroxynitrite, however, is extremely electrophilic and can, therefore, behave as a potent oxidant. Under pathophysiological conditions involving mitochondrial dysfunction, where high concentrations of superoxide may be formed, the reaction of NO with superoxide to form peroxynitrite may lead to the oxidation of a variety of biological molecules and modification of cellular function. Under these conditions peroxynitrite formation should not be seen as a mechanism of inactivation of NO. In most cases, the physiological concentrations of superoxide are very low due to the high levels of superoxide dismutase in cells. Under physiological conditions, therefore, the reaction of NO with superoxide leads to peroxynitrite levels that probably have little or no effect on cellular function. Under these conditions, peroxynitrite formation can be considered as a mechanism of inactivation of NO.

Although the Lewis dot formalism does not indicate it, molecular oxygen (O_2) contains two unpaired electrons and is therefore a diradical. NO, itself being a radical, reacts with O_2 to produce NO_2 [Eq. (5)]. NO_2 can then dimerize to form N_2O_4 or react with another NO molecule to form N_2O_3 [Eqs. (6) and (8)]. Both N_2O_4 and N_2O_3 are hydrolytically unstable and will react with water to give $NO_2^- + NO_3^-$ in the case of N_2O_4 or 2 NO_2^- in the case of N_2O_3 [Eqs. (7) and (9)].

$$\cdot\text{NO} + \text{O}_2 \rightarrow \cdot\text{ONOO}$$
$$\cdot\text{ONOO} + \cdot\text{NO} \rightarrow \text{ONOONO} \rightarrow 2\text{NO}_2 \qquad (5)$$

$$2\text{NO}_2 \rightarrow \text{N}_2\text{O}_4 \qquad (6)$$

$$\text{N}_2\text{O}_4 + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \qquad (7)$$

$$\text{NO}_2 + \cdot\text{NO} \rightarrow \text{N}_2\text{O}_3 \qquad (8)$$

$$\text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow 2\text{NO}_2^- + 2\text{H}^+ \qquad (9)$$

In aerobic aqueous solutions NO_2^- is the predominant reaction product of the reaction between O_2 and NO. Therefore, it is reasonable to assume that the mechanism involving N_2O_3 formation is predominant in the inactivation of NO by O_2. It was previously thought that this chemistry involving NO and O_2 was irrelevant *in vivo* because the reaction is second order with respect to NO and therefore NO would not reach high enough concentrations in the cell for this reaction to be physiologically important. However, recent studies have shown that because of the lipid solubility of NO, it preferentially partitions into lipid membranes leading to locally high concentrations of NO that likely make this chemistry physiologically relevant.

Endogenous *S*-Nitrosothiols

Thiols are by far the most abundant electrophile present *in vivo,* reaching concentrations greater than 1 m*M.* NO and related species have been shown to react readily with both protein and low-molecular-weight thiols to form *S*-nitrosothiols. An increasing number of proteins such as albumin, $p21^{ras}$, glyceraldehyde 3-phosphate dehydrogenase, and hemoglobin as well as the low molecular weight thiols, cysteine and glutathione, have been found to be S-nitrosylated *in vivo.* In addition, many proteins have been shown to be *S*-nitrosylated in cultured cells and in purified enzyme systems. It is becoming evident, with the discovery of new S-nitrosylated molecules, that the formation of *S*-nitrosothiols may be important in a variety of biologically diverse processes.

Although sufficient evidence exists that EDRF is NO, the argument has been advanced that EDRF might be a more chemically stable adduct of NO, such as an *S*-nitrosothiol.

NO appears to circulate in blood primarily as the *S*-nitroso adduct of serum albumin and may act as a reservoir for storing NO. Serum albumin contains a single free sulfhydryl group and is the most abundant source of reduced thiol in the plasma because the plasma concentration of serum albumin is approximately 0.5 m*M*. Moreover, the free sulfhydryl group in serum albumin is particularly susceptible to S-nitrosylation. *S*-Nitroso-albumin is relatively stable when compared to NO, having a half-life in phosphate buffer, pH 7.6, of approximately 24 hr. The concentration of free NO in plasma is approximately 4 n*M,* whereas the concentration of *S*-nitroso-albumin is 5 μ*M* (about 80% of the total plasma nitrosothiols), a difference of four orders of magnitude. In animals, administration of exogenous *S*-nitroso-albumin leads to vasorelaxation, decreased mean arterial blood pressure, and inhibition of platelet aggregation, whereas administration of NO synthase inhibitors leads to decreased concentrations of plasma nitrosothiols, vasoconstriction, and increased mean arterial blood pressure. This suggests not only that *S*-nitroso-albumin possesses the properties of NO but also that the formation of *S*-nitrosothiols in the plasma is dependent on NO synthase activity.

The hypothesis has been forwarded that *S*-nitroso-albumin may act as a reservoir for storing or carrying NO. The question arises as to how NO is transferred from *S*-nitroso-albumin to intracellular targets if any appreciable transfer does occur. Large proteins, such as albumin, can not cross the plasma membrane and any free NO released would be scavenged by the iron in hemoglobin. Low-molecular-weight thiols such as cysteine or glutathione may act to transfer NO from *S*-nitroso proteins across the plasma membrane to intracellular targets. *S*-Nitrosocysteine (CysNO) and *S*-nitrosoglutathione (GSNO) have been detected in plasma at concentrations of 0.3 and 0.1 μ*M,* respectively. L-Cysteine enhances the vasodilatory and platelet inhibitory effects of *S*-nitroso-albumin, and when rabbits are infused with *S*-nitroso-albumin, CysNO levels have been shown to increase by as much as 12-fold.

Another protein that has been proposed to act as a carrier of NO in the blood is hemoglobin. *S*-Nitroso-hemoglobin concentrations have been reported to be 0.3 μ*M* in arterial blood and 0.03 μ*M* in venous blood. Oxygenation of hemoglobin promotes the binding of NO to cysteine-β93, forming *S*-nitroso-hemoglobin. Deoxygenation of *S*-nitroso-hemoglobin causes an allosteric change in the protein that promotes NO release. Accordingly, the proposal has been advanced that hemoglobin can sense the physiological oxygen gradient and bring local blood flow in line with oxygen requirements by releasing NO in poorly oxygenated regions, thereby causing vasorelaxation and increased blood flow.

Cell surface protein disulfide isomerase (PDI) has been implicated in the transfer of NO across the plasma membrane. PDI plays an important role in maintaining the redox status of plasma membrane protein thiols, catalyzing protein disulfide bond formation, reduction, and isomerization. PDI is proposed to be both directly and indirectly involved in the cellular entry of NO. PDI may act directly by catalyzing transnitrosation reactions and indirectly by maintaining thiols in their reduced state, which is required for their reaction with NO.

GSNO may act as an intracellular carrier of NO. Glutathione is a tripeptide containing a single sulfhydryl group and is present intracellularly at concentrations greater than 1 m*M*. GSNO may be involved in the transfer of NO from NO synthase to guanylate cyclase. In isolated rat hearts a Cu^{+}-specific chelator prevented bradykinin-induced cGMP accumulation but did not affect cGMP accumulation due to exogenous NO sources. In addition, activation of NO synthase in the presence of glutathione led to the formation of GSNO. Since Cu^{+} is known to catalyze the release of NO from *S*-nitrosothiols, it was concluded that GSNO must be involved in the pathway. In addition to its possible involvement in the NO–cGMP signaling pathway, GSNO has been proposed to act as an intracellular store of NO in brain tissue. GSNO has been detected by HPLC coupled to mass spectrometry in rat brain tissue.

Many cysteine-containing proteins, including enzymes, ion channels, and transcription factors, have been shown to be S-nitrosylated and their function modified. Although there is limited evidence of intracellular S-nitrosylated proteins *in vivo,* the capacity of NO to interact with thiols to form *S*-nitrosothiols lends itself to the possibility that S-nitrosylation could be a global mechanism by which NO regulates protein function. In addition, many proteins have been shown to be S-nitrosylated and their function modified *in vitro,* supporting the idea of a global regulatory role. One of the earliest examples of a protein whose function is modified by S-nitrosylation is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a ubiquitous protein involved in the glycolytic pathway. It contains 16 cysteine residues, four of which are known to be essential for enzymatic activity. Using conditions that produced chronic inflammation in liver it was shown that increases in NOS activity correlated with decreased GAPDH activity. Exposure of purified GAPDH to authentic NO led to the modification of four cysteine residues per molecule. The modification of GAPDH by NO was reversible by addition of the thiol reducing agent, dithiothreitol (DTT), which is consistent with the mechanism of S-nitrosylation. Modification of the cysteine residues by exposure to NO led to decreased enzyme activity, which was also shown to be reversible. Subsequently, S-nitrosylation of GAPDH was shown to facilitate NADH attachment and, consequently, irreversible inhibition of GAPDH activity.

NO is capable of inhibiting seven members of the caspase family via S-nitrosylation. The caspases are a group of cysteine proteases that are involved in apoptotic signal transduction and cytokine maturation and contain an active site cysteine required for enzymatic activity. Of the seven caspases inhibited by S-nitrosylation, caspase-3 has gained the most attention because it is involved in the apoptotic signaling pathway. Both endogenous and exogenous NO were capable of inhibiting apoptosis in hepatocytes, and this correlated with a decrease in caspase-3 activity. As with

GAPDH, purified caspase-3, along with six other members of the caspase family, was inhibited by NO and inhibition was reversed by DTT, suggesting that S-nitrosylation is involved.

NO has been shown to directly activate both the calcium-dependent potassium (K^+_{Ca}) channel in vascular smooth muscle and the cardiac calcium release channel (CRC). The K^+_{Ca} channel is activated by NO concentrations in the order of 0.2 μM. Activation of the K^+_{Ca} channel by NO was blocked by pretreatment with the sulfhydryl-modifying agent *N*-ethylmaleimide. In addition, *N*-ethylmaleimide did not prevent the activation of the K^+_{Ca} channel by other substrates, indicating that *N*-ethylmaleimide did not inactivate the channel itself but rather prevented the activation by NO. This suggests direct modification of cysteine residues in the K^+_{Ca} channels by NO, but S-nitrosylation was not shown to be the definitive mechanism. CRC, when isolated from canine hearts, was shown to contain *S*-nitrosothiol groups, which were eliminated by addition of DTT. CRC contains approximately 84 free thiols and is activated by S-nitrosylation of as few as 12 thiol residues, the activation being reversible by DTT. In contrast, oxidation of up to 24 thiol residues per CRC did not lead to activation of CRC. These observations indicate not only that S-nitrosylation can activate CRC, but that CRC can distinguish nitrosative from oxidative signals.

Another molecule of interest that has been shown to be S-nitrosylated is $p21^{ras}$. NO promotes guanine nucleotide exchange on $p21^{ras}$ by nitrosating a specific cysteine residue in a highly conserved region of the GTP binding site. This leads to downstream signaling via multiple MAP kinase subgroups and translocation of the transcription factor NFκB. Substitution of the cysteine residue with a serine residue prevented S-nitrosylation of $p21^{ras}$ and NO no longer stimulated guanine nucleotide exchange. These findings suggest a role for multiple MAP kinase signaling pathways in the cellular response to NO via a cGMP-independent mechanism.

NO–cGMP Signal Transduction Pathway

The NO–cGMP signal transduction pathway is employed by NO to carry out many of its biological effects. Adding to the intricacy and complexity of this system is the cross talk that occurs between this signal transduction pathway and the other major pathways in the cell including those involving cAMP and phosphoinositides. cGMP was discovered almost four decades ago in rat urine. This was the result of a systemic search for an analog of its better known cousin, cAMP. However, cGMP remained obscure relative to cAMP for many years for a number of reasons. No clear function could be assigned to cGMP in part because of a lack of a selective agonist of soluble guanylate cyclase. However, this changed in the 1970s with the discovery that the so-called nitrovasodilators cause smooth muscle relaxation by activating guanylate cyclase. Then in the mid 1980s came the report that the mediator of the severe secretory diarrhea seen in gastroenteritis caused by heat stable enterotoxin releasing *Escherichia coli* was cGMP. The discoveries of natriuretic peptides, guanylin, and NO as endogenous activators of guanylate cyclase led to the appreciation of cGMP as a second messenger of immense biological significance and potential. cGMP represents a second messenger that acts on three main classes of effector proteins: cGMP-dependent protein kinases (cGK), cGMP-gated ion channels (CNG), and cGMP-regulated phosphodiesterases (PDE). These effector proteins mediate protein phosphorylation, cation influx, and cyclic nucleotide metabolism, respectively. The biological actions of cGMP are terminated by its breakdown by phosphodiesterases.

cGMP, through its effector proteins, mediates several of the physiological functions of NO including regulation of smooth muscle tone, inhibition of platelet aggregation, regulation of neurotransmission, and chloride and water secretion in the intestines. However, as indicated previously and in other chapters in this book, several of the effects of NO are mediated by cGMP- (and guanylate cyclase-) independent mechanisms. These, among other things, include reactions of NO with metal and thiol centers of target molecules. Figure 5 illustrates the signal transduction pathway for the NO–cGMP system and indicates some NO-elicited effects that are not mediated by cGMP. cGMP-dependent protein kinases have emerged as signal transduction mediators of great importance. cGMP was identified in rat urine in 1963, but it was not until 1970 that cGK, the first target of cGMP to be identified, was discovered in lobster tail muscle. Subsequently, PDE and CNG were recognized as additional targets of cGMP. cGK belongs to the large superfamily of protein kinases. These enzymes regulate the activity of their target proteins by catalyzing the transfer of a γ-phosphoryl group of ATP to a hydroxyl group on serine, threonine, or tyrosyl residues present in the target proteins. Within the protein kinase superfamily, cGKs are related to cAMP-dependent protein kinases (cAK). 8-Bromo-cGMP and 8-pCPT-cGMP are potent cGK activators that have been widely used to study the role of cGK in intact cells. These compounds are ideal for this purpose as they permeate cellular membranes to some extent, are relatively resistant to PDE, and are fairly selective for cGK. cGK antagonists such as Rp-8-Bromo-cGMPS and Rp-8-pCPT-cGMPS are important additional chemical probes used to investigate the physiological functions of cGKs.

Two isozymes of cGKs have been identified and cloned in mammalian cells. They exhibit greater than 50% sequence homology and have similar structural organization. However, they differ in their cellular and subcellular distribution and consequently in their assigned biological roles. cGK Type I is predominantly a cytosolic protein, whereas cGK Type II is tightly bound to membranes. Type I is more widely expressed with high levels of the enzyme being found in vascular smooth muscle cells, platelets, aortic and pulmonary endothelial cells, and cerebellum. Type II has a more limited distribution with high levels being expressed in intestinal epithelial cells and also in brain and kidney. These localizations suggest that Type I is more important in the cardiovascular system, whereas Type II plays a more

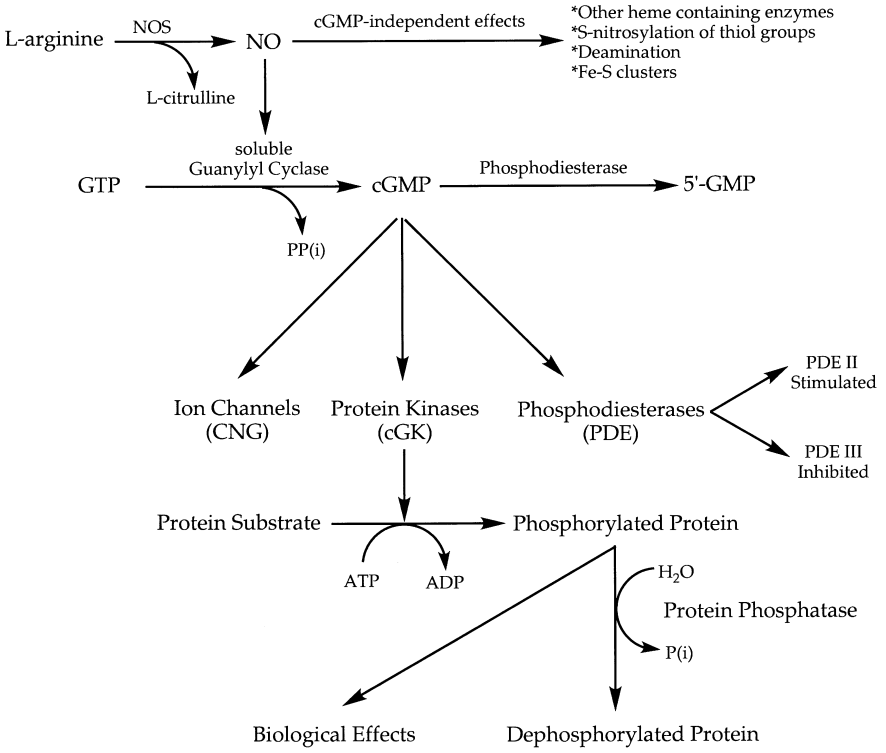


Figure 5 Signal transduction pathway for the NO–cGMP system in mammalian cells. NO generated from L-arginine by NO synthase (NOS) isoforms activates soluble guanylyl (guanylate) cyclase, thereby increasing production of cGMP. The cGMP can be degraded by phosphodiesterase or can bind to other proteins including ion channels, protein kinases, and phosphodiesterases as illustrated. cGMP can either activate or inhibit specific phosphodiesterase isoforms. Binding of cGMP to protein kinases causes their activation and subsequent phosphorylation of proteins. The phosphorylated proteins can cause or lead to a biological effect and are generally inactivated by dephosphorylation caused by protein phosphatases. Although many of the biological actions of NO are mediated by cGMP via this signal transduction pathway, some effects of NO are cGMP independent, such as those illustrated in the upper right corner of the figure.

important role in ion transport in the intestine and perhaps the kidney.

Smooth muscle relaxation is a key physiological function of NO that is mediated by cGMP. Smooth muscle contraction has been shown to be dependent on phosphorylation of the regulatory light chain of myosin by myosin light chain kinase (MLCK). Activation of MLCK is in turn dependent on a rise in free intracellular Ca^{2+} levels. cGK is thought to lower free intracellular calcium levels by multiple mechanisms including inhibition of the phospholipase C (PL-C)/inositol trisphosphate (IP_3) pathway and direct action on various ion channels in the membrane. cGK might also activate a phosphatase that causes dephosphorylation of MLCK. The antiplatelet action of NO represents another major physiological function of NO that is GMP dependent. The inhibition of platelet activation, adhesion, secretion, and aggregation has been shown to be mediated primarily by cGK. A major mechanism by which cGK brings this about is by decreasing intracellular free calcium by some of the mechanisms indicated previously but especially by inhibition of the PL-C/IP_3 pathway.

An important and interesting target of cGK I is a cytoskeletal protein called VASP (vasodilator-associated phosphoprotein). This protein interacts with other cytoskeletal proteins and might be involved in the coordination of different signal transduction pathways. Evidence suggests that VASP, in addition to other things, is important in the enhancement of actin filament formation and inhibition of integrin function. Phosphorylation of VASP might be one of the mechanisms by which cGMP–cGK regulates cell adhesion and migration. Another mechanism might be through regulation (inhibition) of the MLC/MLCK system. MLC phosphorylation leads to assembly of myosin into bipolar filaments that enhance actin tension. This rise in actin tension has multiple effects including clustering of integrin receptors, activation of focal adhesion kinases, and recruitment of various signaling proteins into focal adhesions. By preventing this phosphorylation of MLC or promoting the dephosphorylation of phosphorylated MLC, cGMP–cGK might inhibit cellular adhesion, aggregation, and migration. In addition to being of importance in platelet adhesion and aggregation, the cGMP–cGK pathway may be significant in the regulation of many physiological and pathophysiological processes including embryogenesis, wound healing, tumor invasion and metastasis, and atherosclerosis.

In the intestines, cGMP is involved in the secretion of chloride and water. CFTR, a Cl^- channel encoded by the CF (cystic fibrosis) gene, is thought to play a pivotal role in the secretory response to cGMP. Several mechanisms have been proposed to explain how cGMP might modulate CFTR function. Recent reports suggest that cGK II plays a key role in this transduction pathway.

The other major effector proteins that cGMP acts on are ion channels. These ion channels belong to the cyclic nucleotide gated (CNG) ion channel family. CNG channels are heteromeric proteins that are directly and cooperatively opened by the binding of cyclic nucleotides at a site in their C terminus. Once opened, the channels allow the influx of Na^+ and Ca^{2+} into the cell. This triggers a variety of biochemical cascades with diverse biological effects. Functionally, CNG channels belong to the class of ligand-gated ion channels where the binding of a ligand (cAMP or cGMP) activates them. However, on the basis of their primary sequence, CNG channels structurally belong to the superfamily of voltage-gated ion channels. The CNG channels are a relatively recent discovery. Before this time, cyclic nucleotides were thought to affect ion channel function only through cyclic nucleotide-dependent protein kinases and channel phosphorylation. Direct binding of the cyclic nucleotides is a much faster method of modulating ion channel activity. The first member of the CNG family to be identified was the cGMP-activated cation channel of rod and cone photoreceptors in the retina. A CNG channel with more or less equal sensitivity to both cAMP and cGMP was detected in the membranes of olfactory neurons. Earlier it was thought that the CNG might be limited to the visual and olfactory sensory system but now it is known that CNG channels are much more widespread. More recent findings are the discovery of a related channel in cardiac tissue that appears to generate pacemaker current and the discovery of a similar channel in sea urchin sperm.

Active cross talk occurs between the NO–cGMP signal transduction pathway and other major signal transduction pathways and second messengers. This lends more complexity and diversity to the pathway. One important interaction is between cGMP and cAMP. One of the ways in which cGMP modulates cAMP is by regulating key enzymes called phosphodiesterases (PDE). PDE represent the third major target effector protein for cGMP. They break down the cyclic nucleotides, cAMP and cGMP, thereby terminating their actions in the process. Nine different types of PDEs have been identified to date. They differ in their substrate specificity, sensitivity, distribution, and regulation. Two of these nine PDEs are regulated by cGMP. PDE III is inhibited by cGMP, resulting in higher levels of cAMP. PDE II is stimulated by cGMP, causing the levels of cAMP to go down. Examples of this type of interaction are evident in platelets and smooth muscle cells where cGMP potentiates the effects of cAMP by inhibiting PDE III. Another mechanism by which cGMP can use the cAMP pathway is by cross-activating cAMP-dependent kinases (cAK). This occurs at higher levels of cGMP ($>5\ \mu M$). An example of this is seen in colonic T84 cells. This method of cross-activation has so far been less well-documented in the cardiovascular system. However, the antiproliferative effects of cGMP are thought to be at least partly dependent on cross-activation of cAK. Opposite cross-activation, that is, activation of cGK by cAMP, also occurs and is thought to be partly responsible for the vasorelaxant effects of cAMP. It has also been shown that activated guanylate cyclase can synthesize cAMP.

Potent and selective inhibitors and activators for many of the multiple steps in the NO–cGMP signal transduction pathway are still not available. Hence caution needs to be exercised in assigning biological roles to this pathway. Discovery of new pharmacological tools to manipulate this fascinating signal transduction pathway in a selective manner is of great pharmacological and therapeutic interest, as exemplified by the discovery of the selective PDE V inhibitor sildenafil (Viagra).

Additional Reading

Abu-Soud, H. M., and Stuehr, D. J. (1993). Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10769–10772.

Arnold, W. P., Mittal, C. K., Katsuki, S., and Murad, F. (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various issue preparations. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3203–3207.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Bredt, D. S., and Snyder, S. H. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9030–9033.

Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 682–685.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351,** 714–718.

Butler, A. R., and Rhodes, P. (1997). Chemistry, analysis, and biological roles of *S*-nitrosothiols. *Anal. Biochem.* **249,** 1–9.

Cherry, P. D., Furchgott, R. F., Zawadzki, J. V., and Jothianandan, D. (1982). Role of endothelial cells in relaxation of isolated arteries by bradykinin. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2106–2110.

Cheung, P.-Y., Salas, E., Schulz, R., and Radomski, M. W. (1997). Nitric oxide and platelet function: Implications for neonatology. *Semin. Perinatol.* **21,** 409–417.

Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* **279,** 2121–2126.

Crow, J. P. and Beckman, J. S. (1995). The role of peroxynitrite in nitric oxide-mediated toxicity. *Curr. Top. Microbiol. Immunol.* **196,** 57–73.

Fukuto, J. M. (1995). Chemistry of nitric oxide: Biologically relevant aspects. *Adv. Pharmacol.* **34,** 1–15.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Garthwaite, J., Garthwaite, G., Palmer, R. M., and Moncada, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur. J. Pharmacol.* **172,** 413–416.

Green, L. C., Tannenbaum, S. R., and Goldman, P. (1981). Nitrate synthesis in the germfree and conventional rat. *Science* **212,** 56–58.

Gruetter, C. A., Barry, B. K., McNamara, D. B., Gruetter, D. Y., Kadowitz, P. J., and Ignarro, L. J. (1979). Relaxation of bovine coronary artery and

activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcingenic nitrosoamine. *J. Cyclic Nucleotide Res.* **5,** 211–224.

Gruetter, C. A., Barry, B. K., McNamara, D. B., Kadowitz, P. J., and Ignarro, L. J. (1980). Coronary arterial relaxation and guanylate cyclase activation by cigarette smoke, *N*'-nitrosonornicotine and nitric oxide. *J. Pharmacol. Exp. Ther.* **214,** 9–15.

Haslam, R. J., Davidson, M. M. L., Davies, T., Lynham, J. A., and McClenaghan, M. D. (1978). Regulation of blood platelet function by cyclic nucleotides. *Adv. Cyclic Nucleotide Res.* **9,** 533–552.

Hevel, J. M., White, K. A., and Marletta, M. A. (1991). Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* **266,** 22789–22791.

Ignarro, L. J., Edwards, J. C., Gruetter, D. Y., Barry, B. K., and Gruetter, C. A. (1980). Possible involvement of *S*-nitrosothiols in the activation of guanylate cyclase by nitroso compounds. *FEBS Lett.* **110,** 275–278.

Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J., and Gruetter, C. A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of *S*-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.* **218,** 739–749.

Ignarro, L. J., Wood, K. S., and Wolin, M. S. (1982). Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2870–2873.

Ignarro, L. J., Wood, K. S., and Wolin, M. S. (1984). Regulation of purified soluble guanylate cyclase by porphyrins and metalloporphyrins: A unifying concept. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17,** 267–274.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Ignarro, L. J., Byrns, R. E., Buga, G. M., and Wood, K. S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.* **61,** 866–879.

Ignarro, L. J., Bush, P. A., Buga, G. M., Wood, K. S., Fukuto, J. M., and Rajfer, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.* **170,** 843–850.

Katsuki, S., Arnold, W. P., and Murad, F. (1977). Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. *J. Cyclic Nucleotide Res.* **3,** 239–247.

Keaney, J. F., Jr., Simon, D. I., Stamler, J. S., Jaraki, O., Scharfstein, J., Vita, J. A., and Loscalzo, J. (1993). NO forms an adduct with serum albumin that has endothelium-derived relaxing factor-like properties. *J. Clin. Invest.* **91,** 1582–1589.

Kim, Y. M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6348–6352.

Lancaster, J. R., Jr. (1996). Diffusion of free nitric oxide. *Methods Enzymol.* **268,** 31–50.

Lancaster, J. R., Jr. (1997). A tutorial on the diffusibility and reactivity of free nitric oxide. *NO Biol. Chem.* **1,** 18–30.

Li, J., Billiar, T. R., Talanian, R. V., and Kim, Y. M. (1997). Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem. Bophys. Res. Commun.* **240,** 419–424.

Lincoln, T. M., and Cornwell, T. L. (1991). Towards an understanding of the mechanism of action of cyclic AMP and cyclic GMP in smooth muscle relaxation. *Blood Vessels* **28,** 129–137.

Lincoln, T. M., and Cornwell, T. L. (1993). Intracellular cyclic GMP receptor proteins. *FASEB J.* **7,** 328–338.

Lincoln, T. M., Pryzwansky, K. B., Cornwell, T. L., Wyatt, T. A., and MacMillan, L. A. (1993). Cyclic-GMP-dependent protein kinase in smooth muscle and neutrophils. *Adv. Second Messenger Phosphoprotein Res.* **28,** 121–132.

Lincoln, T. M., Komalavilas, P., and Cornwell, T. L. (1994). Pleiotropic regulation of vascular smooth muscle tone by cyclic GMP-dependent protein kinase. *Hypertension* **23,** 1141–1147.

Liu, X., Miller, M. J., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998). Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2175–2179.

Liu, X., Miller, M. J., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster, J. R., Jr. (1998). Diffusion-limited reaction of free nitric oxide with erythrocytes. *J. Biol. Chem.* **273,** 18709–18713.

Liu, Z., Rudd, M. A., Freedman, J. E., and Loscalzo, J. (1998). S-Transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. *J. Pharmacol. Exp. Ther.* **284,** 526–534.

McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. (1992). Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11141–11145.

Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., and Wishnok, J. S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. *Biochemistry* **27,** 8706–8711.

Mellion, B. T., Ignarro, L. J., Ohlstein, E. H., Pontecorvo, E. G., Hyman, A. L., and Kadowitz, P. J. (1981). Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* **57,** 946–955.

Mellion, B. T., Ignarro, L. J., Meyers, C. B., Ohlstein, E. H., Ballot, B. A., Hyman, A. L., and Kadowitz, P. J. (1983). Inhibition of human platelet aggregation by *S*-nitrosothiols. Heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation. *Mol. Pharmacol.* **23,** 653–664.

Mohr, S., Stamler, J. S., and Brüne, B. (1996). Posttranslational modification of glyceraldehyde-3-phosphate dehydrogenase by S-nitrosylation and subsequent NADH attachment. *J. Biol. Chem.* **271,** 4209–4214.

Pollock, J. S., Klinghofer, V., Förstermann, U., and Murad, F. (1992). Endothelial nitric oxide synthase is myristylated. *FEBS Lett.* **309,** 402–404.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide, and prostacyclin in platelets. *Br. J. Pharmacol.* **92,** 181–187.

Rajfer, J., Aronson, W. J., Bush, P. A., Dorey, F. J., and Ignarro, L. J. (1992). Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, nonholinergic neurotransmission. *N. Engl. J. Med.* **326,** 90–94.

Raman, C. S., Li, H., Martásek, P., Král, V., Masters, B. S., and Poulos, T. L. (1998). Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell* **95,** 939–950.

Robinson, L. J., Busconi, L., and Michel, T. (1995). Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J. Biol. Chem.* **270,** 995–998.

Sakuma, I., Stuehr, D. J., Gross, S. S., Nathan, C., and Levi, R. (1988). Identification of arginine as a precursor of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 8664–8667.

Scharfstein, J. S., Keaney, J. F., Jr., Slivka, A., Welch, G. N., Vita, J. A., Stamler, J. S., and Loscalzo, J. (1994). In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols. *J. Clin. Invest.* **94,** 1432–1439.

Sessa, W. C., García-Cardeña, G., Liu, J., Keh, A., Pollock, J. S., Bradley, J., Thiru, S., Braverman, I. M., and Desai, K. M. (1995). The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J. Biol. Chem.* **270,** 17641–17644.

Simon, D. I., Mullins, M. E., Jia, L., Gaston, B., Singel, D. J., and Stamler, J. S. (1996). Polynitrosylated proteins: Characterization, bioactivity, and functional consequences. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4736–4741.

Stamler, J. S. (1995). *S*-nitrosothiols and the bioregulatory actions of nitrogen oxides through reactions with thiol groups. *Curr. Top. Microbiol. Immunol.* **196,** 19–36.

Stamler, J. S., Jaraki, O., Osborne, J., Simon, D. I., Keaney, J. F., Jr., Vita, J., Singel, D. J., Valeri, C. R., and Loscalzo, J. (1992). Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7674–7677.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stuehr, D. J., and Marletta, M. A. (1985). Mammalian nitrate biosynthesis: Mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7738–7742.

Wang, J., Stuehr, D. J., Ikeda-Saito, M., and Rousseau, D. L. (1993). Heme coordination and structure of the catalytic site n nitric oxide synthase. *J. Biol. Chem.* **268,** 22255–22258.

Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Böhme, E., Schultz, G., and Koesling, D. (1994). Mutation of His-105 in the beta 1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2592–2596.

White, K. A., and Marletta, M. A. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* **31,** 6627–6631.

Wink, D. A., and Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radicals Biol. Med.* **25,** 434–456.

Wink, D. A., Hanbauer, I., Grisham, M. B., Laval, F., Nims R. W., Laval, J., Cook, J., Pacelli, R., Liebmann, J., Krishna, M., Ford, P. C., and Mitchell, J. B. (1996). Chemical biology of nitric oxide: Regulation and protective and toxic mechanisms. *Curr. Top. Cell. Regul.* **34,** 159–187.

Wolin, M. S., Wood, K. S., and Ignarro, L. J. (1982). Guanylate cyclase from bovine lung: A kinetic analysis of the regulation of the purified soluble enzyme by protoporphyrin X, heme, and nitrosyl-heme. *J. Biol. Chem.* **257,** 13312–13320.

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SECTION II

Principles of Biology

CHAPTER 2

The Chemical Properties of Nitric Oxide and Related Nitrogen Oxides

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DUE TO THE DISCOVERY OF ITS ENDOGENOUS BIOSYNTHESIS AND DIVERSITY OF ITS BIOLOGICAL ACTIONS, NITRIC OXIDE (NO) IS A MOLECULE OF EXTREME PHYSIOLOGICAL, PHARMACOLOGICAL, AND PATHOPHYSIOLOGICAL INTEREST. MUCH OF THE BIOLOGICAL UTILITY OF NO IS DUE TO ITS UNIQUE CHEMICAL INTERACTIONS WITH BIOLOGICAL MOLECULES. THUS, AN UNDERSTANDING OF THE BASIC CHEMICAL PROPERTIES OF NO, AND DERIVED SPECIES, WILL PROVIDE INSIGHT INTO THE INTIMATE MECHANISMS OF ITS BIOLOGICAL ACTIVITY. AS A PREFACE TO UNDERSTANDING THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL PROPERTIES OF NO, A DISCUSSION OF SOME OF THE BIOLOGICALLY RELEVANT CHEMISTRY OF NO IS PROVIDED.

Nitric oxide (·NO) is a simple, diatomic molecule possessing unique and fascinating chemistry.[1] Indeed, the utility of ·NO as a biological effector and/or mediator is due to some of its novel chemical properties along with the properties of other nitrogen oxides derived from it. Because the biological and physiological roles of ·NO and related species will be addressed in detail in subsequent chapters, presented herein is a discussion of the physiologically relevant chemical properties of ·NO and other nitrogen oxides and how these properties may be utilized to confer specificity to its biological actions. For more comprehensive treatments of general ·NO and nitrogen oxide chemistry, other reviews are available (Gilbert and Thomas, 1972; Schwartz and White, 1983; Vosper, 1975; Bonner and Hughes, 1988; Ragsdale, 1973; Bonner and Stedman, 1996).

[1] The nomenclature used herein for the nitrogen oxides is somewhat antiquated and can lead to confusion when attempting to assign oxidation states based on suffixes. However, due to the prevalence of these terms in the current biochemical and physiological literature, we will continue to use the outdated terms in this chapter (e.g., nitric oxide instead of nitrogen monoxide, nitrous oxide instead of dinitrogen monoxide). For a treatise on the correct nomenclature for the nitrogen oxides, the reader is referred to the article by Koppenol and Traynham (1996).

·NO and the ·NO Dimer $(NO)_2$

Nitric oxide is a colorless gas at room temperature and pressure (boiling point −151.7°C). The maximum solubility of ·NO (at 1 atm partial pressure) in water at room temperature and pressure is approximately 2 m*M* which is slightly higher than the solubility of dioxygen (O_2) in water. Also, like O_2, ·NO is somewhat lipophilic and possesses 6- to 8-fold higher solubility in nonpolar solvents (Shaw and Vosper, 1977) and lipid membranes compared to water. Thus, the rates of ·NO reactions in a hydrophobic environment can be dramatically increased (with O_2, e.g.) over that found in water due to its increased concentration (Liu *et al.,* 1998).

It becomes immediately evident from a Lewis dot depiction that ·NO has one unpaired electron and thus is formally a free radical species (Fig. 1). The radical nature of ·NO is evidenced by its ability to react with other species with

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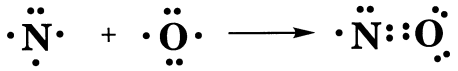


Figure 1 Lewis dot structure of ·NO.

unpaired electrons such as O_2, superoxide ($O_2^{\cdot-}$) and other radical species (the details of these processes will be discussed later). However, at room temperature and pressure, ·NO has little propensity to react with itself in a radical–radical dimerization process. At first glance, this may seem curious since dimerization would lead to a structure (ON−NO) whereby all atoms have a full compliment of eight valance electrons and, therefore, would satisfy the octet rule. In order to reconcile this apparent anomaly, the molecular orbitals of ·NO must be considered.

Simple combination of the nitrogen and oxygen atomic orbitals gives the following set of molecular orbitals (Fig. 2). In order of increasing energy, the molecular orbitals consist of a σ bonding orbital (σ_s^b) and a corresponding σ antibonding orbital (σ_s^*), two degenerate π bonding orbitals (π_{xy}^b), one σ bonding orbital (σ_z^b, only slightly higher in energy than the π bonding orbitals), two degenerate antibonding π orbitals (π_{xy}^*), and a high energy σ antibonding orbital (σ_z^*) (Jorgensen and Salem, 1973). Since the total number of valence electrons in the ·NO molecule is 11 (five from nitrogen and six from oxygen), the low-lying σ bonding and σ antibonding orbitals along with the bonding π and σ orbitals are all filled. However, the last electron must reside in a π* antibonding orbital. In molecular orbital terminology, the electronic structure of ·NO is $KK(\sigma_s^b)^2(\sigma_s^*)^2(\pi_{xy}^b)^4(\sigma_z^b)^2(\pi_{xy}^*)^1$. Thus, ·NO has a net bond order of 2.5 because there is one electron in the π* antibonding orbital. When ·NO forms a dimer (O=N−N=O), the overall bond order for the molecule is 5. Therefore, since there is no net gain in overall bonding when ·NO dimerizes and entropy would favor the monomer, ·NO exists as a monomer at room temperature and pressure.

The above rationale for the observation that ·NO exists primarily as a monomeric species at room temperature and pressure is purely qualitative. This issue has also been examined quantitatively. The experimental dimerization energy for two ·NO molecules has been measured to be in the range of only 2–4 kcal/mol (Hetzler *et al.,* 1991; Dkhissi *et al.,* 1997; Forte and van den Bergh, 1978). In order to understand the low magnitude of this bond energy, one needs to examine the ·NO dimer itself. The ·NO dimer has been the subject of considerable experimental and theoretical study (East, 1998; East *et al.,* 1998; Casassa *et al.,* 1986; Williams and Murrell, 1971; Kukolich, 1982; Lee *et al.,* 1990; Tonner *et al.,* 1983; Jursic, 1995; Casassa *et al.,* 1988). The N−N bond in the dimer is not only unusually weak but is also extremely long (2.263 Å) (McKellar *et al.,* 1995). For comparison, the covalent bond in hydrazine, H_2NNH_2, has an N−N bond dissociation energy of 65.8 kcal/mol and an N−N bond length of 1.45 Å (McMillen and Golden, 1982). The N−N interaction in the ·NO dimer is considerably weaker than a covalent bond and yet cannot be explained entirely by van der Waals interactions, which provide stabilization on the order of only tenths of a kilocalorie per mole. Interestingly, ·NO dimer formation does very little to disturb the nitrogen–oxygen orbital overlap. The experimental N−O bond distance in the dimer is 1.1515 Å (McKellar *et al.,* 1995) whereas the N−O bond distance in the monomer is 1.151 Å (Huber and Herzberg, 1979). The predicted ground state for the ·NO dimer is singlet and a symmetric planar *cis* conformation is favored (Ha, 1981; Skaarup *et al.,* 1976). This more sterically hindered *cis* geometry is lower in energy than the trans configuration, because there is favorable orbital overlap between the oxygens giving a weak O−O bond (Fig. 3). This indicates that the electrons in the N−N σ bond of the dimer are delocalized over the entire dimer structure which would predict a weak N−N bond.

A partial rationale for the unusually long and weak N−N bond in the ·NO dimer may also be found in an examination of the nature of the unpaired electron in the ·NO monomer.

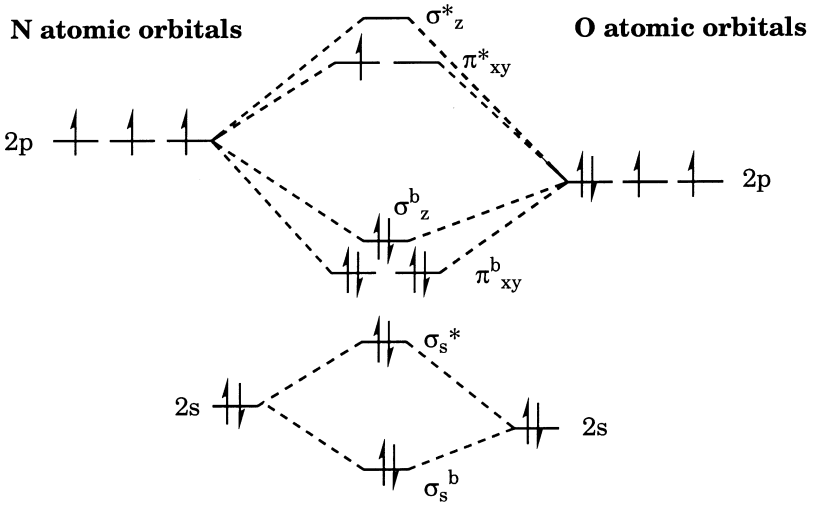


Figure 2 Molecular orbital diagram for ·NO.

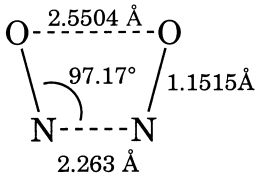


Figure 3 Structure of the ·NO dimer.

Theoretical studies confirm that the unpaired radical electron of nitric oxide resides in a π* orbital (Perez Jigato *et al.,* 1995; East, 1998; Duarte *et al.,* 1998) (also see Fig. 2). The radical does not reside completely on the nitrogen but is shared between the nitrogen and the oxygen (which may explain the existence of a weak O−O bond in the *cis* conformation of the dimer). However, the nitrogen does have a higher orbital coefficient, which explains the observed radical chemistry predominating at the nitrogen (i.e., reaction of ·NO with other radicals occurs through the nitrogen atom). Thus, the weak N−N bond in the ·NO dimer can be attributed to a variety of effects including the weak attractive forces of the delocalized electrons in the π* molecular orbitals as well as electronic repulsion of the lone pair electrons on the adjacent, bonding nitrogens.

·NO and Its Relationship to Other Nitrogen Oxide Species

Within the series of redox related nitrogen oxides, ·NO occupies a central position (Fig. 4). Thus, ·NO can conceivably be either oxidized or reduced to generate a variety of species and, indeed, it is likely that both oxidative and reductive pathways for ·NO exist in a physiological environment.

The Reaction of ·NO with O_2 and O_2-derived Species in Aqueous Solution

Based on the argument above, ·NO is considered a free radical in spite of the fact that it does not have the tendency to dimerize at room temperature and pressure. Nitric oxide will, however, react with other species with unpaired electrons. One of the most biologically relevant reactions of this type is the reaction of ·NO with O_2. As a ground state triplet, O_2 has two unpaired electrons [the electron configuration for O_2 is $KK(\sigma_s^b)^2(\sigma_s^*)^2(\pi_{xy}^b)^4(\sigma_z^b)^2(\pi_{xy}^*)^2$] and therefore possesses some chemical characteristics of a biradical. As such, O_2 is known to react with other radical species. Since O_2 has two unpaired electrons, the product of O_2 and ·NO (and other radicals) still has one unpaired electron which can react further. Thus, the reaction of ·NO with O_2 consumes two equivalents of ·NO to give two equivalents of nitrogen dioxide ($\cdot NO_2$) possibly via the reactions (1), (2), and (3).

$$\cdot NO + O_2 \rightarrow \cdot OONO \quad (1)$$

$$\cdot OONO + \cdot NO \rightarrow ONOONO \quad (2)$$

$$ONOONO \rightarrow 2\cdot NO_2 \quad (3)$$

Although the reaction of 2 ·NO molecules with O_2 to generate 2 $\cdot NO_2$ molecules may occur via the three-step sequence indicated by reactions (1)–(3), it has also been postulated that this process can proceed via reaction of O_2 with an ·NO dimer $(NO)_2$ species (e.g., see Olbregts, 1985). That is, the initial step may involve the generation of an ·NO dimer which, in turn, reacts with O_2 to give two $\cdot NO_2$ molecules. Regardless of which mechanism is in operation, the observed kinetic order of the $\cdot NO_2$ forming reaction would be second order in ·NO and first order in O_2.

Nitrogen Dioxide

Like ·NO, $\cdot NO_2$ also possesses an unpaired electron which can be delocalized throughout the molecule (Fig. 5). As the structures in Fig. 5 demonstrate, assigning a full complement of eight electrons around two of the three atoms in NO_2 leaves the unpaired electron on the remaining atom.

Unlike ·NO, $\cdot NO_2$ is a fairly potent oxidant as indicated by a reduction potential of 1.04 V for the $\cdot NO_2/NO_2^-$ couple (Stanbury, 1989). Thus, the reaction between two relatively innocuous oxidizing agents, O_2 and ·NO, generates a more potent oxidant, $\cdot NO_2$. There are a variety of potential reaction pathways by which $\cdot NO_2$ can cause oxidation: hydrogen atom abstraction, addition to unsaturated bonds, and electron transfer reactions (for an excellent review of $\cdot NO_2$ oxidation chemistry, see Huie, 1994). Generally speaking, H-atom abstraction by $\cdot NO_2$ in solution is extremely slow and probably only relevant to biological systems with activated hydrogen atoms (i.e., weak atom–hydrogen bond). The reversible addition of $\cdot NO_2$ to olefins generates initially a nitroalkyl radical species which can react with another radical species such

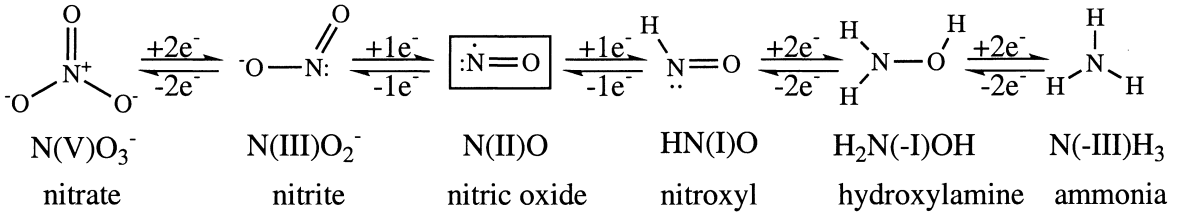


Figure 4 Redox relationship of ·NO to other nitrogen oxide species. The Roman numerals in the chemical formulas refer to the oxidation state of the nitrogen atom. Note: for the sake of simplicity, two prevalent nitrogen oxides that are oxidized relative to ·NO, peroxynitrite (^-OONO) and nitrogen dioxide ($\cdot NO_2$), are not shown in this scheme (and will be discussed later).

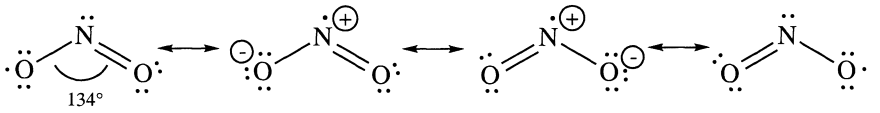


Figure 5 Lewis dot depiction and resonance forms of $\cdot NO_2$.

as a second molecule of $\cdot NO_2$ or O_2. Reaction with $\cdot NO_2$ would result in a dinitro adduct and reaction with O_2 would generate a nitroalkylperoxy radical which can undergo further radical chemistry. Electron transfer reactions are probably the most dominant reaction pathway for direct $\cdot NO_2$-mediated oxidations in biological systems (not including reactions of $\cdot NO_2$-derived species which will be discussed later). Nitrogen dioxide will react reasonably well with, for example, the anions of ascorbate, phenol, or thiols with rate constants of approximately 10^7–10^8 M^{-1} s^{-1} to form NO_2^- and the corresponding radical species. The radical generated from the reaction of $\cdot NO_2$ with phenoxide can either react with another equivalent of $\cdot NO_2$ or dimerize (Fig. 6) (Prutz *et al.,* 1985).

Consistent with an electron transfer mechanism, the oxidation of phenolic compounds by $\cdot NO_2$ exhibits a dramatic pH effect. That is, the second order rate constant for the reaction of $\cdot NO_2$ with, for example, tyrosine is 3.2×10^5 M^{-1} s^{-1} at pH 7.5 and 2.0×10^7 M^{-1} s^{-1} at pH 11.3. Since the pK_a of a phenolic hydroxyl is approximately 10, this indicates an increased rate of reaction for the phenoxide anion. Thiols (or more likely, thiolates) are also oxidized by $\cdot NO_2$ generating, initially, the corresponding thiyl radical and NO_2^- (Prutz *et al.,* 1985) (Fig. 6). In the presence of excess thiolate, the thiyl radical can, among other reactions, combine with thiolate to form the corresponding disulfide radical anion which is a fairly potent one-electron reductant capable of reducing O_2 to $O_2^{\cdot -}$ (e.g., see Winterbourn and Metodiewa, 1995). The oxidation of thiolate by $\cdot NO_2$ has a second order rate constant of 2.4×10^8 M^{-1} s^{-1} (pH 9.2) and, therefore, is significantly faster than the reaction of $\cdot NO_2$ with phenoxide. Also, $\cdot NO_2$ is reported to react with $O_2^{\cdot -}$ by an electron transfer process to give NO_2^- and O_2 with a second order rate constant of approximately 10^8 M^{-1} s^{-1} (Warneck and Wurzinger, 1988).

Dinitrogen Tetroxide, Dinitrogen Trioxide, and Related Species

Since $\cdot NO_2$ is a free radical, it can further react with other radical species including itself or $\cdot NO$. The rapid radical–radical combination reaction of two $\cdot NO_2$ molecules results in the formation of dinitrogen tetroxide (N_2O_4) [reaction (4)]. Due to the delocalization of the unpaired electron, dimerization of $\cdot NO_2$ can occur in a variety of ways. The most stable structure of N_2O_4 in aqueous solution is generally thought to contain a nitrogen–nitrogen bond (Fig. 7) (other forms that contain an N−O bond between two $\cdot NO_2$ molecules can exist as well).

In an aqueous environment N_2O_4 is unstable with respect to hydrolysis, which results in the generation of one equivalent each of nitrite (NO_2^-) and nitrate (NO_3^-) [reaction (5)].

$$\cdot NO_2 + \cdot NO_2 \rightarrow N_2O_4 \quad (k = 4.5 \times 10^8\ M^{-1}\ s^{-1}) \qquad (4)$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (k = 1 \times 10^3\ s^{-1}) \qquad (5)$$

Reaction (5) represents the nitrosation (addition of the equivalent of NO^+) of water by N_2O_4 to give NO_2^-. Dinitrogen

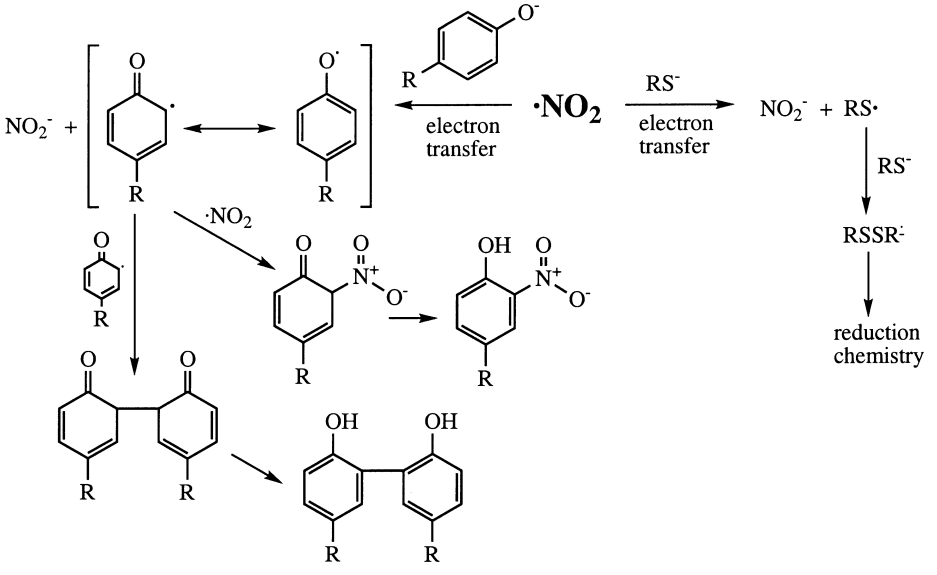
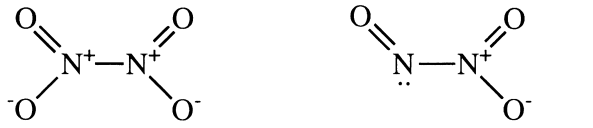


Figure 6 The reaction of $\cdot NO_2$ with phenoxide and thiolate.



Dinitrogen tetroxide (N_2O_4) Dinitrogen trioxide (N_2O_3)

Figure 7 Most stable structures of N_2O_4 and N_2O_3. Both molecules are planar, and only one resonance form for each is shown.

tetroxide is, in fact, a generally good nitrosating agent as demonstrated by its efficient reaction with thiols resulting in the generation of *S*-nitrosothiols [reaction (6)] (Oae *et al.,* 1978).

$$RSH + N_2O_4 \rightarrow RS{-}NO + NO_3^- + H^+ \quad (6)$$

This reaction exemplifies the ability of N_2O_4 to act as a source of nitrosonium ion (NO^+). In fact, N_2O_4 is reported to be in equilibrium with NO^+ and NO_3^- under a variety of conditions. It is not immediately clear how NO^+ and NO_3^- can be in direct equilibrium with the N−N bridged structure of N_2O_4 shown in Fig. 7 since formation of NO^+ and NO_3^- from $O_2N{-}NO_2$ would require not only the cleavage of the N−N bond but also a transfer of an oxygen atom from one nitrogen to the other. However, it should be noted that other structures for the $\cdot NO_2$ dimer are possible (and may be in equilibrium) which may account for the nitrosating ability of N_2O_4. For example, the N−N bonded species shown in Fig. 7, $O_2N{-}NO_2$, may be in equilibrium with an N−O bonded species, $ON{-}ONO_2$, and nucleophilic attack on the trivalent nitrogen atom of $ON{-}ONO_2$ followed by heterolytic N−O bond cleavage would yield directly the observed nitrosated product and NO_3^- (Challis and Kyrtopoulos, 1978). Regardless, the nitrosating ability of N_2O_4 is well established and synthetically useful.

A radical–radical combination reaction between $\cdot NO_2$ and $\cdot NO$ gives dinitrogen trioxide (N_2O_3) [reaction (7)]. As with $\cdot NO_2$ dimerization, the most stable structure of N_2O_3 contains an N−N bond (Fig. 7). Dinitrogen trioxide is also unstable in an aqueous system and hydrolyzes to generate two equivalents of NO_2^- [reaction (8)]. Like N_2O_4, N_2O_3 not only exists as an N−N bonded species, $ON{-}NO_2$, but can also exist as an N−O bonded species, ON−ONO, which may be responsible for most of the nitrosation chemistry (Challis and Kyrtopoulos, 1978). Thus, similar to the reaction of N_2O_4 with water, the reaction of N_2O_3 represents a net nitrosation of water to give NO_2^-. Nitrosation of other nucleophiles such as thiols can also occur via this chemistry and will be discussed later.

$$\cdot NO_2 + \cdot NO \rightarrow N_2O_3 \quad (k = 1.1 \times 10^9\ M^{-1}\ s^{-1}) \quad (7)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (k = 1 \times 10^3\ s^{-1}) \quad (8)$$

Although oxidation of $\cdot NO$ by O_2 results in $\cdot NO_2$ formation [reactions (1–3)] and $\cdot NO_2$ is known to dimerize to N_2O_4 and then hydrolyze to form NO_2^- and NO_3^- [reactions (4) and (5)], air oxidation of $\cdot NO$ in an aqueous environment results in the near exclusive generation of NO_2^- with little or trace amounts of NO_3^-. The lack of NO_3^- formation indicates that N_2O_4 (the $\cdot NO_2$ dimerization product) is not generated in appreciable amounts when $\cdot NO$ is air oxidized in water. The absence of N_2O_4 formation when $\cdot NO$ is air oxidized is due to the fact that any $\cdot NO_2$ formed is rapidly trapped by excess $\cdot NO$ to form N_2O_3 [reaction (7)] which is rapidly hydrolyzed to give exclusively NO_2^- [reaction (8)]. Therefore, the levels of $\cdot NO_2$ under these conditions do not get high enough for the second order dimerization reaction [reaction (4)] to be significant.

The stoichiometric expression for reactions (1)–(3), (7), and (8), the oxidation of $\cdot NO$ by O_2 to NO_2^- in an aqueous system, is written as follows:

$$4\cdot NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (9)$$

The rate expression for the loss of $\cdot NO$ from reaction (9) is thus $-d[NO]/dt = 4k[\cdot NO]^2[O_2]$ with $k = 2 \times 10^6\ M^{-2}\ s^{-1}$ (Ford *et al.,* 1993; Lewis and Deen, 1994). Thus, $\cdot NO$ degradation in an aerobic, aqueous solution is not linear with respect to the $\cdot NO$ concentration. For example, assuming O_2 concentrations around 200 μM, a 10 μM solution of $\cdot NO$ will have degraded to half its original concentration in about 1 minute whereas a 10 n*M* solution will take over 70 hours to degrade to half its original concentration (note that the term ''half-life'' is not used in this context since the decomposition kinetics are not first order and therefore the half-life changes with $\cdot NO$ concentration).

Nitrate and Nitrite

The pK_a of nitric acid (HNO_3) is −1.6 and, therefore, the nitrate ion, NO_3^-, is the near exclusive species present under physiological conditions. Nitrate, though, has little or no physiologically relevant chemistry. However, under certain physiological conditions, NO_2^- chemistry can occur. The pK_a of nitrous acid (HNO_2) is 3.3, much higher than that of nitric acid. Nitrous acid exists in equilibrium with N_2O_3 in water (Williams, 1983) [reaction (10)]. Dinitrogen trioxide can be considered to be the anhydride of nitrous acid.

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad (10)$$

Thus, under slightly acidic conditions, NO_2^- can be protonated to generate N_2O_3. The equilibrium constant for reaction (10) ($[N_2O_3]/[HNO_2]^2$) is reported to be $3.03 \times 10^{-3}\ M^{-1}$ (although there is some discrepancy between the older literature and the more recent reports) (Williams, 1983, and references therein). Nevertheless, N_2O_3 is a potent electrophile capable of nitrosating (adding the elements of ''NO^+'') a variety of nucleophiles [as is evidenced by reaction (8) where H_2O serves as the nucleophile]. Consistent with N_2O_3 as the nitrosating agent, the rate expression for the nitrosation of nucleophiles by acidified NO_2^- is first order in the nucleophile (Nuc) and first order in N_2O_3, rate = $k[Nuc][N_2O_3]$. The rate of nitrosation of amines by acidified NO_2^- can be expressed as rate = $k[amine][HNO_2]^2$, again

consistent with N_2O_3 as the nitrosating species. The rate constants for amine nitrosation by N_2O_3 can be quite high (on the order of 10^8–10^9 M^{-1} s^{-1}). It should be realized, however, that nitrosation by NO_2^- is second order in HNO_2 and would therefore require fairly high concentrations and low acidity to be physiologically relevant. Thiol nitrosation by N_2O_3 may be an important physiological occurrence and will be specifically covered later in the text in a discussion of ·NO–thiol chemistry. Under extremely acidic, nonphysiological conditions, nitrosation of nucleophiles can occur via a process which is first order in HNO_2. Under these conditions, the nitrosating agent is likely to be either $H_2NO_2^+$ or NO^+, both of which will have no appreciable lifetime under physiological conditions.

Of potential physiological and toxicological importance is the reaction of NO_2^- with oxyhemoproteins such as oxyhemoglobin. It has been known for many years that the reaction of NO_2^- with, for example, oxyhemoglobin results in methemoglobin and NO_3^- formation (e.g., Wallace and Caughey, 1975; Doyle *et al.,* 1985; Kosaka *et al.,* 1981). This reaction is multistep, complex, and characterized by a lag phase followed by an autocatalytic phase. The overall mechanism of this reaction is a matter of some speculation although it appears to be generally accepted that the first step involves an electron transfer reaction between NO_2^- and oxyhemoglobin [reaction (11)] (Wallace and Caughey, 1975). This initiation step is slow and most likely accounts for the lag phase of the reaction.

$$HbFe^{II}O_2 + NO_2^- + H^+ \rightarrow HbFe^{III}OOH + \cdot NO_2 \quad (11)$$

It has been proposed that the $HbFe^{III}OOH$ species depicted in reaction (11) can then react with NO_2^- to also generate $\cdot NO_2$ and a high valent metaloxo species that further reacts with NO_2^- to give $\cdot NO_2$ and methemoglobin, $HbFe^{III}$ [reactions (12) and (13)].

$$HbFe^{III}OOH + NO_2^- \rightarrow HbFe^{IV}O + \cdot NO_2 + HO^- \quad (12)$$

$$HbFe^{IV}O + NO_2^- + 2H^+ \rightarrow HbFe^{III} + \cdot NO_2 + H_2O \quad (13)$$

Since the process is autocatalytic, there is a requirement that a step which generates one of the products also generates a reactive, ''chain carrying'' intermediate. This argument has led to the proposal that $\cdot NO_2$ reacts with oxyhemoglobin to generate the perferryl intermediate of reaction (12) [reaction (14)] (Lissi, 1998).

$$HbFe^{II}O_2 + \cdot NO_2 + H_2O \rightarrow HbFe^{II}OOH + NO_3^- + H^+ \quad (14)$$

Although the reaction sequence depicted above accounts for the lag phase and autocatalytic nature of the chemistry, the overall reaction scheme remains speculative and, as yet, unestablished.

The Reaction of ·NO with Superoxide in Aqueous Solution and the Formation of Peroxynitrite ($^-$OONO)

The one-electron reduction of O_2 results in the generation of $O_2^{\cdot-}$. The electron configuration for this species is $KK(\sigma_s^b)^2(\sigma_s^*)^2(\pi_{xy}^b)^4(\sigma_z^b)^2(\pi_{xy}^*)^3$ and therefore $O_2^{\cdot-}$ possesses an unpaired electron occupying a π^* antibonding orbital. Thus, since both ·NO and $O_2^{\cdot-}$ both have unpaired electrons, the reaction between them is extremely facile (Blough and Zafirou, 1985) with a rate constant near the diffusion controlled limit [reaction (15)] (Huie and Padmaja, 1993; Goldstein and Czapski, 1995).

$$\cdot NO + O_2^{\cdot-} \rightarrow {}^-OONO \quad (k = 4\text{–}7 \times 10^9\ M^{-1}\ s^{-1}) \quad (15)$$

In a related reaction, ·NO is also capable of reacting with metal-bound O_2 complexes. For example, oxyhemoglobin and oxymyoglobin react readily with ·NO to give NO_3^- and the oxidized hemoproteins, methemoglobin and metmyoglobin (Doyle and Hoekstra, 1981). The similarity between this reaction and the direct reaction of ·NO with $O_2^{\cdot-}$ is easily seen if one considers that the O_2 complexes of ferrous hemoglobin ($HbFe^{II}$) or ferrous myoglobin are probably more accurately depicted as ferric–$O_2^{\cdot-}$ complexes (i.e., $HbFe^{III}-O_2^{\cdot-}$) [reaction (16)].

$$[HbFe^{II}-O_2 \leftrightarrow HbFe^{III}-O_2^{\cdot-}] + \cdot NO \rightarrow HbFe^{III} + NO_3^- \ (k = 3.7 \times 10^7\ M^{-1}\ s^{-1}) \quad (16)$$

Reaction with oxyhemoglobin or oxymyoglobin serves as a very useful assay for ·NO since the conversion of oxyhemoglobin or oxymyoglobin to methemoglobin or metmyoglobin is easily followed by visible spectroscopy (Murphy and Noack, 1994).

The Chemistry of $^-$OONO

The immediate product from the reaction of ·NO with $O_2^{\cdot-}$ is $^-$OONO [reaction (15)]. The pK_a of HOONO is reported to be 6.8 and, therefore, a significant amount of the protonated species, peroxynitrous acid (HOONO), exists at physiological pH (e.g., see Koppenol *et al.,* 1992). Peroxynitrous acid is a much more potent oxidizing agent than either $O_2^{\cdot-}$ or ·NO and is capable of carrying out a variety of oxidations. Early reports on the chemical nature of HOONO indicated that it was capable of decomposing via homolytic O−O bond cleavage to generate hydroxyl radical (HO·) and $\cdot NO_2$ (e.g., Mahoney, 1970). The calculated homolysis enthalpy for the O−O bond predicts a bond dissociation energy of only 22.5 kcal/mol to give the hydroxyl and nitrogen dioxide radicals (Bartberger *et al.,* 1998). Recombination of these two radical species could then result in NO_3^- formation [reaction (17)].

$$HOONO \rightarrow [HO\cdot + \cdot NO_2] \rightarrow NO_3^- + H^+ \quad (17)$$

The proposed generation of HO· and $\cdot NO_2$ as intermediates in the decomposition of HOONO may account for its oxidizing capability since both species are potent oxidizing agents. The idea that HO· and $\cdot NO_2$ are formed as species along the reaction coordinate of HOONO decomposition to NO_3^- has been disputed (e.g., Koppenol *et al.,* 1992; Pou *et al.,* 1995; Shi *et al.,* 1994; Pryor *et al.,* 1996; Lemercier *et al.,* 1995). Therefore, other reactive intermediates or species have been proposed to account for the oxidizing potential of HOONO.

However, numerous reports have indicated that finite amounts of HO· and ·NO_2 may be formed from $^-$OONO decomposition and that at least some of the oxidation chemistry associated with HOONO may be a result of HO· or ·NO_2 generation (e.g., Pfeiffer *et al.,* 1997; van der Vliet *et al.,* 1994; Merenyi and Lind, 1997, 1998). Theoretical studies support the existence of [HO·—·NO_2] caged radical pairs as possible intermediates in HOONO decomposition (Houk *et al.,* 1996), a concept previously proposed by others (Pryor and Squadrito, 1995). Although the exact nature of the oxidant(s) formed by HOONO is a matter of some controversy, there is no question that HOONO can serve to oxidize a variety of molecules. For example, HOONO is capable of converting methionine to the corresponding sulfoxide via a two electron oxidation or capable of oxidizing methionine by a single electron resulting in the elimination of ethylene (Pryor *et al.,* 1994). In spite of the oxidizing potential of HOONO, the rate constants for reaction with easily oxidizable substrates like ascorbate monoanion ($k = 2.36 \times 10^2$ M^{-1} s^{-1}) (Squadrito and Pryor, 1995; Bartlett *et al.,* 1995) or sulfhydryls ($k = 5.9 \times 10^3$ M^{-1} s^{-1}) (Radi *et al.,* 1991) are surprisingly small.

As indicated above, HOONO is a potent oxidant. However, the conjugate base, $^-$OONO, possesses nucleophilic character as evidenced by its ability to react with electrophiles such as carbon dioxide (CO_2) (Lymar and Hurst, 1995; Uppu *et al.,* 1996). The reaction of $^-$OONO with CO_2 is one of the fastest known reactions for $^-$OONO ($k = 3 \times 10^4$ M^{-1} s^{-1}). In fact, in a physiological milieu, one of the predominant fates of $^-$OONO generation is likely to be reaction with CO_2 due to the relatively high rate constant and the high concentration of CO_2 (>1 mM in plasma). The immediate reaction product from the $^-$OONO/CO_2 reaction is nitrosoperoxycarbonate [reaction (18)].

$$^-\text{OONO} + CO_2 \rightarrow \text{ONOOCOO}^- \quad (k = 3 \times 10^4\ M^{-1}\ s^{-1}) \tag{18}$$

Like HOONO, nitrosoperoxycarbonate is inherently unstable and spontaneously rearranges to the nitrocarbonate anion which can then hydrolyze to generate NO_3^- and bicarbonate (HCO_3^-) [reactions (19) and (20)]. Since HCO_3^- is in equilibrium with CO_2 and H_2O, the process is catalytic with respect to CO_2.

$$\text{ONOOCOO}^- \rightarrow O_2\text{NOCO}_2^- \tag{19}$$

$$O_2\text{NOCO}_2^- + H_2O \rightarrow NO_3^- + HCO_3^- + H^+ \tag{20}$$

Thus, CO_2 can serve as a catalyst for the degradation of $^-$OONO to NO_3^-. Similarly, theoretical studies have postulated that ketones can catalyze the conversion of $^-$OONO to NO_3^- (Bartberger *et al.,* 1998).

Instead of acting merely as a degradation catalyst, CO_2 can also serve to activate $^-$OONO as a nitrating agent. For example, the ability of $^-$OONO to nitrate aromatic rings is significantly increased in the presence of CO_2 (Gow *et al.,* 1996; Denicola *et al.,* 1996). However, other oxidations performed by $^-$OONO (i.e., thiol oxidation, benzoate hydroxylation, etc.) are inhibited by the presence of CO_2 (Denicola *et al.,* 1996; Zhang *et al.,* 1997).

The potential physiological generation of $^-$OONO/HOONO results from the rapid reaction of $O_2^{\cdot-}$ with ·NO [reaction (15)], both known to be generated in significant amounts under certain pathophysiological conditions. However, HOONO can react further with either ·NO or $O_2^{\cdot-}$ to generate, among other things, ·NO_2 [reactions (21) and (22)] (Miles *et al.,* 1996).

$$\text{HOONO} + O_2^{\cdot-} + H^+ \rightarrow O_2 + \cdot NO_2 + H_2O \tag{21}$$

$$\text{HOONO} + \cdot NO \rightarrow \cdot NO_2 + NO_2^- + H^+ \tag{22}$$

Thus, the fate/lifetime of $^-$OONO/HOONO may also depend on the ''flux'' of the two precursors, ·NO and $O_2^{\cdot-}$.

Due to the potential relevance of $^-$OONO to biological systems and reports implicating it as a major factor in a variety of pathophysiological processes, the chemistry and biology of $^-$OONO has been the subject of intense investigation. The treatment of this topic herein is only meant as an introduction to this work and is not intended to be either comprehensive or exhaustive. For a more detailed treatment of this subject the reader is referred to any one of a number of excellent reviews (Pryor and Squadrito, 1995; Beckman *et al.,* 1994; Edwards and Plumb, 1994).

The Free-Radical Chemistry of ·NO

As indicated above, ·NO reacts rapidly via simple radical–radical combination reactions with species possessing unpaired electrons such as ·NO_2, O_2, and $O_2^{\cdot-}$. The ability of ·NO to ''quench'' other radical species also allows it to terminate radical chain reactions. A good example of this phenomenon is the effect ·NO has on the O_2-dependent oxidation of lipids (e.g., Wink *et al.,* 1993; Hogg *et al.,* 1993; Rubbo *et al.,* 1994, 1995; Struck *et al.,* 1995). Due to the fact that many lipids contain ''activated'' allylic C—H bonds, they are susceptible to oxidative damage. Lipid peroxidation results from the net abstraction of an allylic hydrogen atom of the unsaturated fatty acid (Lipid—H) by an initiating radical species (X·) to generate a lipid radical (Lipid·) [reaction (23)]. The lipid radical then reacts with O_2 to generate an alkylperoxy radical (Lipid—OO·) which can further react with another lipid to form another lipid radical that can also react with O_2 [reactions (24–25)]. These two reactions represent chain propagating steps.

$$\text{Lipid—H} + \text{X}\cdot \rightarrow \text{Lipid}\cdot + \text{X—H} \quad (\text{X}\cdot = \text{chain initiating species}) \tag{23}$$

$$\text{Lipid}\cdot + O_2 \rightarrow \text{Lipid—OO}\cdot \tag{24}$$

$$\text{Lipid—OO}\cdot + \text{Lipid—H} \rightarrow \text{Lipid—OOH} + \text{Lipid}\cdot, \text{ etc.} \tag{25}$$

The outcome of this sequence of reactions is that a single initiating event can lead to the destruction/modification of numerous lipid molecules which can result in a loss of

membrane integrity. Lipid peroxidation can be avoided or limited either by inhibiting the generation of the initiating species ''X·'' [reaction (23)] or by quenching one of the chain carrying radical intermediates, Lipid−OO· or Lipid· [reactions (24) and (25)]. Nitric oxide is known to limit lipid peroxidation by acting as a chain terminating species [e.g., reaction (26)].

$$\text{Lipid}-\text{OO}\cdot + \cdot\text{NO} \rightarrow \underset{\text{(chain terminated)}}{\text{Lipid}-\text{OO}-\text{NO}} \qquad (26)$$

Further, ·NO has been reported to inhibit the generation of chain initiating species by altering the reactivity of metals known to serve as catalysts for their generation (this aspect of ·NO chemistry will be addressed in detail later in the discussion of ·NO and metals) (for a general review on ·NO and free radical biology, see Rubbo *et al.,* 1996).

Unlike other radical species such as $\cdot NO_2$, halogen atoms, or HO·, ·NO does not readily abstract hydrogen atoms (S_H2 reaction) or add to unsaturated bonds. The inability of nitric oxide to serve as an oxidizing agent (i.e., via H atom abstraction) can be inferred from its low reduction potential ($E° = 0.39$ V for the NO/$^3NO^-$ couple and −0.35 V for the NO/$^1NO^-$ couple) (Stanbury, 1989). For comparison, the reduction potentials for $\cdot NO_2$, HO·, F·, and Cl· are 1.04, 1.9, 3.6, and 2.41 V, respectively. There are, however, some instances where ·NO has been proposed to abstract H atoms. For example, the reaction of ·NO with hydroxylamine (NH_2OH) gives as final products dinitrogen (N_2), nitrous oxide (N_2O), and water [reaction (27)].

$$2\cdot NO + 2NH_2OH \rightarrow N_2 + N_2O + 3H_2O \qquad (27)$$

The first step of this complex reaction appears to involve the abstraction of a hydrogen atom from the nitrogen of hydroxylamine to generate nitroxyl (HNO) and the corresponding nitrogen-centered radical species (Bonner and Wang, 1986).

The Reactions of ·NO with Metals

Nitric oxide is capable of serving as a ligand in a variety of metal complexes, and the coordination chemistry of ·NO with metals has been examined extensively (for comprehensive reviews see Richter-Addo and Legzdins, 1992; McCleverty, 1979; Eisenberg and Meyer, 1975). Nitrosonium ion (NO^+) is isoelectronic with carbon monoxide (CO), therefore many metals that form carbonyl (CO) complexes are also capable of forming isoelectronic and isostructural nitrosyl (·NO) complexes. However, ·NO differs from CO as a metal ligand in that it can adopt two different binding geometries reflecting different metal–NO bonding interactions. The geometry of the metal–NO bond can either be linear (Fig. 8A), or bent (Fig. 8B).

The linear geometry is the most common bonding mode for metal nitrosyls and, in this situation, the net bonding interaction between the metal and ·NO consists of both σ donation from ·NO to the metal and π backbonding from occupied *d* orbitals of proper symmetry on the metal to the π* antibonding orbital on ·NO (Fig. 8A). In the bent geometry, the metal can be envisioned to donate an electron to ·NO to form NO^-, which then binds the metal in a σ interaction. This leaves an electron pair localized in an sp^2 orbital on the nitrogen atom of the ligand. For ''electron counting'' purposes the nitrosyl ligand in the linear bonding complexes is considered to be nitrosonium ion, NO^+, and in the bent complexes, nitroxyl, NO^-. That is, in the linear bonding geometry, ·NO is envisioned to donate an electron to the metal prior to bonding and thus it is formally NO^+. In the bent geometry, the metal can be envisioned to first donate an electron to ·NO, giving NO^-, which then binds the metal. Using this formalism, ·NO is considered to be a three-electron donor (one-electron donation to the metal to make NO^+ and two-electron donation to form the M−NO bond) in the linear bonding mode, but a one-electron donor (two-electron donation to form the M−NO bond minus one electron donated from the metal to ·NO) in the bent geometry. It should be noted that this assignment is merely a formalism for counting electrons in these complexes and does not always reflect the chemistry or properties of the nitrosyl ligand.

The ability of ·NO to bind metals via the two modes described above makes it an ''amphoteric'' ligand (e.g., see Collman *et al.,* 1987). The conversion of a linear to bent bonding geometry in a metal–nitrosyl complex is equivalent to oxidizing the metal since the bent geometry requires a formal donation of electrons from the metal to the nitrosyl ligand. This may change the overall coordination geometry of the metal. Although biological relevance of this phenomenon has not been reported, the amphoteric nature of ·NO binding to metals is rare among all ligands and would certainly allow unique interactions with metalloproteins not available to other biologically relevant diatomic ligands (CO, O_2, CN^-, etc.).

Coordination of ·NO to a metal as either ''NO^+'' or ''NO^-'' may predict that metal-bound nitrosyls can be either electrophilic or nucleophilic. That is, ·NO ligands bound in a linear fashion to metals might be expected to be subject to nucleophilic attack whereas ·NO bound in a bent fashion might be susceptible to reaction with electrophiles. Although this type of argument is not rigorous since the assignment of the ligands as ''NO^+'' and ''NO^-'' is only a formalism for accounting for electrons, metal bound nitrosyls exhibit both electrophilic as well as nucleophilic character (Cotton and Wilkinson, 1988).

Due to the fact that ·NO is more electronegative than CO and thus a better electron acceptor, it can more efficiently backbond with occupied *d* orbitals on metals. A major portion of the strength of many metal–NO bonds is due to the backbonding phenomenon (Fig. 8A), and since this type of bonding is more prevalent with ·NO compared to CO, the metal–NO bond is generally stronger than the metal–CO bond. Thus, ·NO is typically a better (more stable) ligand for backbonding metals compared to CO. A corollary to this idea is that complexation of ·NO to metals weakens the N−O bond since electrons from the metal are being transferred into an ·NO antibonding orbital.

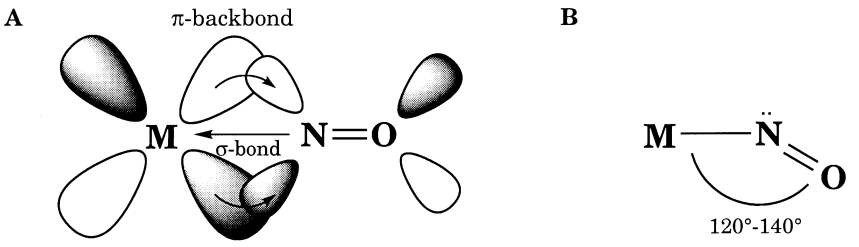


Figure 8 (A) Linear geometry (arrows depict bonding interactions) and (B) bent geometry for M-nitrosyl bond. M = metal ion.

As indicated above, the bonding of ·NO to metals can be envisioned to result in the formation of an electropositive nitrosyl ligand [reaction (28)].

$$M^n + {\cdot}NO \rightarrow [M^n{-}NO \leftrightarrow M^{n-1}{-}NO^+] \quad (M^n = \text{metal ion complex with charge } n) \tag{28}$$

Formally, this can be thought of as an internal electron transfer from ·NO to the metal to give a complex that consists of a reduced metal and an NO^+ ligand. Although it is unwise to assume that this is always the bonding interaction between ·NO in metal complexes (see above), there are cases where the binding to metals makes ·NO susceptible to nucleophilic attack. For example, ligation of ·NO to a synthetic ferric porphyrin species, tetraphenylporphyriniron(III) chloride (Fe^{III}TPPCl), in the presence of a protic solvent such as methanol or water results in the eventual formation of a ferrous nitrosyl adduct (Wayland and Olson, 1974; Chien, 1969). The mechanism for this reductive nitrosylation is multistep and is shown below [reactions (29–31)].

$$Fe^{III}TPPCl + {\cdot}NO \rightarrow [Fe^{III}{-}(NO)TPPCl \leftrightarrow Fe^{II}{-}(NO^+)TPPCl] \quad (TPP = \text{tetraphenylporphyrin}) \tag{29}$$

$$Fe^{II}{-}(NO^+)TPPCl + MeOH \rightarrow Fe^{II}TPP + MeONO + HCl \tag{30}$$

$$Fe^{II}TPP + {\cdot}NO \rightarrow Fe^{II}(NO)TPP \tag{31}$$

The ability of ferricporphyrins to activate ·NO to be a nitrosating agent for nucleophilic species has been further demonstrated with hemoproteins such as myoglobin and catalase, whereby addition of ·NO to the ferric form of these proteins led to the nitrosation of, for example, phenol (Wade and Castro, 1990). This chemistry was further extended to include nitrosation of nucleic acids resulting in the conversion of cytosine to uracil (Castro and Bartnicki, 1994). It also appears that other nonheme metal ion complexes ligate and activate ·NO in a similar manner (Gwost and Caulton, 1973).

Nitric oxide can also perform similar chemistry with certain cupric complexes. For example, it has been reported that synthetic cupric complexes with relatively high reduction potentials can be reduced by bound ·NO via an inner-sphere charge transfer mechanism. Nucleophilic attack by methanol, for example, on the bound nitrosyl ligand (or nitrosonium ligand) ($LCu^I{-}NO^+$) results in the generation of the cuprous complex (LCu^I) and the nitrite ester of methanol (MeONO) (Tran *et al.,* 1998) [reactions (32) and (33)].

$$LCu^{II} + {\cdot}NO \rightarrow [LCu^{II}{-}NO \leftrightarrow LCu^I{-}NO^+], \quad L = \text{ligand system} \tag{32}$$

$$[LCu^{II}{-}NO \leftrightarrow LCu^I{-}NO^+] + MeOH \rightarrow LCu^I + MeONO + H^+ \tag{33}$$

It has been proposed that the ability of ·NO to bind and reduce redox active metals, such as iron, may indirectly lead to the generation of oxygen-derived free radical species via the Fenton reaction (Farias-Eisner *et al.,* 1996) [reaction (34)].

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + HO^- + HO{\cdot} \tag{34}$$

That is, ·NO can reduce ferric ion, Fe^{III} (under slightly acidic conditions), to ferrous ion, Fe^{II}, which in the presence of hydrogen peroxide (H_2O_2) can lead to the generation of potent oxidants such as HO·. However, others have reported that ·NO actually inhibits this chemistry since it can bind Fe^{II} complexes, forming the ferrous nitrosyl complex, which is unable to catalyze reaction (34) and/or changes the course of the reaction giving other, nonoxidizing products [reaction (35)] (e.g., Kanner *et al.,* 1991, 1992; Kanner, 1996, and references therein).

$$X{-}Fe^{II}{-}NO + H_2O_2 \rightarrow X{-}Fe^{III} + NO_2^- + H_2O \quad (X = \text{ligand system}) \tag{35}$$

Thus, ·NO can act as an antioxidant by serving as a radical chain-terminating species [reaction (26)] or by altering the chemistry of catalytic metal species. However, depending on the conditions of the reaction (i.e., pH, ligand system, ·NO concentration, etc.), it is conceivable that ·NO can serve as a pro-oxidant capable of activating catalytic metals that generate reactive oxygen species.

The coordination of ·NO to iron hemoproteins is an important aspect of its physiological utility. In fact, ·NO is an extremely good ligand for ferrous hemes and, unlike other simple diatomic ligands such as O_2 and CO, binds ferric hemes as well (although the affinity of ·NO to ferrous hemes is significantly greater) (e.g., Hoshino *et al.,* 1993). One of

the most important functions of biological ·NO is to serve as an activator of the iron heme-containing enzyme guanylate cyclase (Ignarro, 1989). However, other biologically relevant and similar ligands such as O_2 and CO also bind iron hemoproteins and yet they do not possess the unique biological activity associated with ·NO. Thus, the nature of the interaction between ·NO and hemoproteins is distinct from that of other simple diatomic ligands. One of the ways in which ·NO is distinct from, for example, O_2 and CO, is that its coordination to a ferrous hemoprotein labilizes the trans axial ligand (Fig. 9) (Traylor and Sharma, 1992).

In the case of guanylate cyclase, the trans axial ligand is an imidazole moiety (from a histidine) which, after ·NO assisted release, may serve to increase the catalytic activity of the enzyme (Traylor *et al.,* 1993).

The kinetics of ·NO binding to iron hemoproteins are highly dependent on both the oxidation state and coordination environment of the iron center (Hoshino *et al.,* 1993). For example, the association rate constant for ·NO binding to a synthetic ferric heme, Fe^{III}TPPS (TPPS, *meso*-tetrakis (*p*-sulfonatophenyl)porphyrin), is $7.2 \times 10^5\ M^{-1}\ s^{-1}$ and the rate constant for dissociation is $6.8 \times 10^2\ s^{-1}$. On the other hand, ·NO irreversibly binds to the ferrous form, Fe^{II}TPPS, with a rate constant of $1.8 \times 10^9\ M^{-1}\ s^{-1}$. Metmyoglobin ($MbFe^{III}$) binds ·NO with a rate constant of $1.9 \times 10^5\ M^{-1}\ s^{-1}$ and has a low rate constant of $13.6\ s^{-1}$ for the reverse reaction. Myoglobin ($MbFe^{II}$) itself binds ·NO irreversibly with a rate constant of $1.7 \times 10^7\ M^{-1}\ s^{-1}$.

Ferric cytochrome *c* binds ·NO with a rate constant of $7.2 \times 10^2\ M^{-1}\ s^{-1}$ and ·NO dissociates with a rate constant of $4.4 \times 10^{-2}\ s^{-1}$. Reduced ferrous cytochrome *c* has association and dissociation rate constants of $8.3\ M^{-1}\ s^{-1}$ and $2.87 \times 10^{-5}\ s^{-1}$, respectively. Note that the rate of binding for the ferrous species is much lower than the rate for binding of ferric cytochrome *c,* but the opposite is true for myoglobin and FeTPPS.

The reaction of ·NO with iron–sulfur (Fe–S) centers has received significant attention since this appears to be an interaction of physiological importance (e.g., Hibbs *et al.,* 1988). Generally, ·NO is able to disrupt Fe–S clusters, releasing iron in the form of a dinitrosyl–iron–dithiol complex (e.g., Drapier, 1997; Kennedy *et al.,* 1997). The mechanistic intricacies of this interaction are not yet fully established.

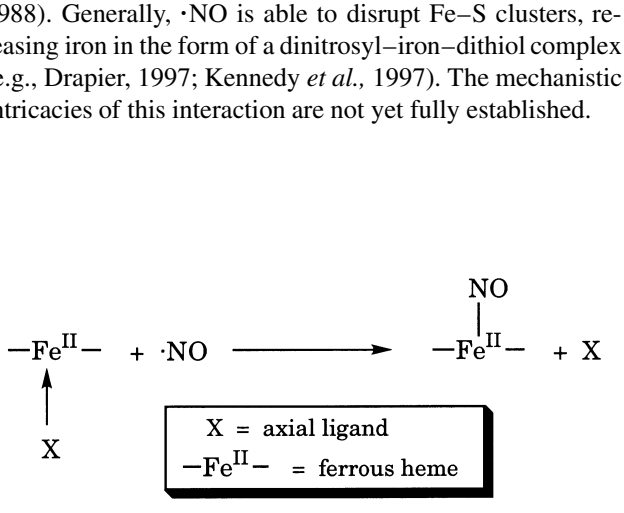


Figure 9 Labilization of trans axial ligand, X, from ferrous heme by coordination of ·NO.

The Reaction of ·NO and ·NO-Derived Species with Thiols

The physiological chemistry of thiols is intimately associated with the physiological chemistry of ·NO. As one of the premier nucleophilic species in biological systems, thiols can react with electrophilic nitrogen oxide species derived from ·NO. For example, air oxidation of ·NO results in the formation of the electrophilic nitrosating species, N_2O_3 [reactions (1–3) and (7), above]. Nitrosation of thiols by N_2O_3 is facile and results in the formation of an *S*-nitrosothiol (RS−NO) [reaction (36)].

$$\begin{aligned} &RSH + N_2O_3 \rightarrow \\ &RS{-}NO + NO_2^- + H^+ \end{aligned} \tag{36}$$

Thiol nitrosation may also occur via the intermediacy of a ferric heme nitrosyl species (or depicted below as a ferrous nitrosonium species) in a similar manner to that shown in reaction (30) [reaction (37)] (Wade and Castro, 1990).

$$\begin{aligned} &RSH + Fe^{II}{-}(NO^+)heme \rightarrow \\ &RS{-}NO + Fe^{II}heme + H^+ \end{aligned} \tag{37}$$

Interestingly, ·NO will also react with thiols under anaerobic conditions. Early studies of this reaction by Pryor and coworkers indicated that thiols (or more likely thiolates, RS^-) will react with ·NO to form intermediates which further decompose to generate N_2O, H_2O, and the corresponding disulfide (Pryor *et al.,* 1982) [reaction (38)].

$$2RSH + 2{\cdot}NO \rightarrow RSSR + N_2O + H_2O \tag{38}$$

The mechanism for reaction (38) has been postulated to occur via initial nucleophilic attack onto the electrophilic nitrogen atom of ·NO by thiolate to form an adduct (RS−N−OH) that further reacts with another ·NO to generate an intermediate species which then decomposes spontaneously to give the observed products (Fig. 10) (DeMaster *et al.,* 1995).

Thus, ·NO can directly oxidize thiols to the corresponding disulfides or can indirectly oxidize thiols to *S*-nitrosothiols via intermediates such as N_2O_3 or metal nitrosyl species.

S-Nitrosothiols are capable of releasing ·NO in the presence of cuprous ion [reaction (39)] (e.g., Williams, 1996; Dicks *et al.,* 1996).

$$RS{-}NO + Cu^I \rightarrow RS^- + {\cdot}NO + Cu^{II} \tag{39}$$

Photolytic homolysis of the RS−NO bond generating both ·NO and the corresponding thiyl radical is also possible and is an issue when working with *S*-nitrosothiols.

S-Nitrosothiols can also undergo transnitrosation reactions whereby the nitroso function is transferred from one thiol to another via nucleophilic attack on the nitrogen atom of the *S*-nitrosothiol [reaction (40)] (e.g., see Barnett *et al.,* 1994a,b).

$$RS{-}NO + R'SH \rightarrow RSH + R'S{-}NO \tag{40}$$

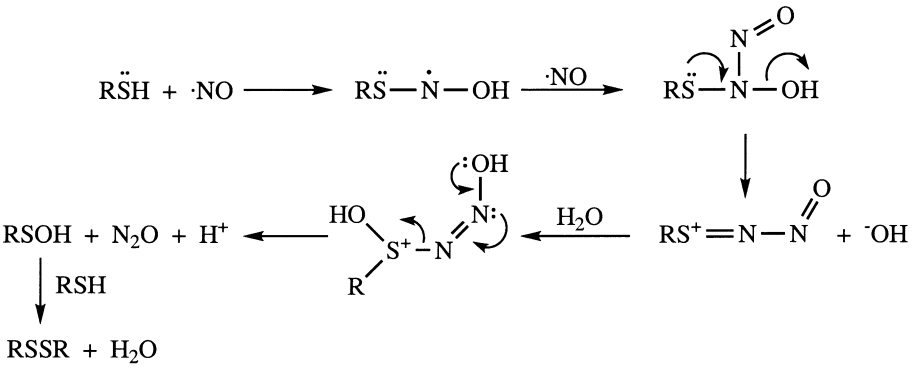


Figure 10 Possible mechanism for the O_2-independent oxidation of RSH.

Alternatively, the thiol can react at the sulfur atom of the *S*-nitrosothiol which would generate the disulfide and HNO [reaction (41)] (e.g., Arnelle and Stamler, 1995; Wong *et al.,* 1998).

$$RS-NO + R'SH \rightarrow RSSR' + HNO \qquad (41)$$

Further reaction of thiols with HNO is possible (and will be discussed later).

Reduced ·NO Species

Except for HNO/NO^-, most all of the ·NO-derived species mentioned thus far are oxidized with respect to ·NO (see Fig. 4). It appears, however, that biological metabolism of ·NO can lead to reduced as well as oxidized nitrogen species. Already mentioned is the possible generation of HNO from degradation of *S*-nitrosothiols [reaction (41)]. Other studies have alluded to possible physiological generation of HNO and other reduced species (e.g., Singh *et al.,* 1996; Hobbs *et al.,* 1994; Schmidt *et al.,* 1996). Thus, it is worthwhile to briefly describe the chemistry of species that are reduced relative to ·NO.

The Chemistry of HNO

Compared to its redox partners, the physiological/bioinorganic chemistry of HNO has been studied much less (for a review on the basic inorganic chemistry of HNO and related species, see Bonner and Hughes, 1988). One reason for this may be due to the metastable nature of HNO. This lack of inherent stability stems from its self-reactivity, that is, one HNO molecule can react with another to initially give hyponitrous acid, which can then dehydrate to give nitrous oxide (N_2O) [reaction (42)]. Thus, detection of N_2O in chemical studies is often used as an indication of HNO intermediacy (although this is not a rigorous proof; e.g. see Fig. 10). The overall rate constant for the dimerization–dehydration process is extremely high (Bazylinski and Hollocher, 1985a).

$$HNO + HNO \rightarrow HONNOH \rightarrow N_2O + H_2O \quad (k = 2\text{–}8 \times 10^9\ M^{-1}\ s^{-1}) \qquad (42)$$

In spite of the high rate constant, N_2O formation via reaction (42) might be extremely limited in biological systems. This is due to the fact that reaction (42) is second order in HNO, and HNO dimerization competes with other biologically relevant HNO reactions.

The potential physiological importance of HNO in mammalian systems has only recently been recognized; hence, past interest in its bioinorganic chemistry has been limited (although much of the basic inorganic chemical properties of HNO has been elucidated). The pK_a of HNO generated by pulse radiolysis is reported to be 4.7 (Gratzel *et al.,* 1970). However, recent calculations indicate that this value may not be correct and the pK_a may be considerably higher (between 7 and 8) (K. Houk, personal communication, 2000). Regardless, HNO can exist in both protonated and unprotonated forms, and the chemistry of both is possible in aqueous systems. Nitroxyl anion is isoelectronic with O_2 and can therefore exist in either the ground state triplet ($^3NO^-$) or the excited singlet ($^1NO^-$) spin state. As with O_2, the reaction chemistry of NO^- will be highly dependent on its spin state (discussed later). The reduction potential for the couple ·NO/$^1NO^-$ is −0.35 V, and for ·NO/$^3NO^-$ is 0.39 V (Stanbury, 1989). Singlet NO^- is, therefore, a much better reducing agent than the triplet form.

Besides its propensity to dimerize and generate N_2O [reaction (42)], NO^- also reacts with its one-electron oxidation product, ·NO, to generate $N_2O_2^-$ [reaction (43)] (Gratzel *et al.,* 1970; Seddon *et al.,* 1973). This species is unstable and can either react with another ·NO to give $N_3O_3^-$ [reaction (44)] or it is proposed that it may spontaneously decompose to generate HO· and N_2O [reaction 45)] (Seddon *et al.,* 1973). $N_3O_3^-$ will decompose to N_2O and NO_2^- [reaction (46)] (Gratzel *et al.,* 1970; Seddon *et al.,* 1973). Thus, at relatively high ·NO concentrations $N_2O_2^-$ will be trapped by ·NO to form $N_3O_3^-$, but at relatively low ·NO concentrations the rate of $N_2O_2^-$ trapping by ·NO is slow, and spontaneous decomposition of $N_2O_2^-$ to N_2O and HO· may compete. It should be noted that the generation of HO· from the decomposition of $N_2O_2^-$ has not been unequivocally demonstrated. It was postulated on the basis of kinetic studies performed at low ·NO concentrations that indicated the existence of a decomposition pathway for $N_2O_2^-$ competing with reaction

(44) (Seddon *et al.,* 1973). Thus, further chemical verification of reaction (45) is needed.

$$NO^- + \cdot NO \rightarrow N_2O_2^- \ (k = 1.7 \times 10^9\ M^{-1}\ s^{-1}) \quad (43)$$

$$N_2O_2^- + \cdot NO \rightarrow N_3O_3^- \ (k = 3.0 \times 10^6\ M^{-1}\ s^{-1}) \quad (44)$$

$$N_2O_2^- + H^+ \rightarrow HO\cdot + N_2O \ (k = 3.5 \times 10^2\ s^{-1}) \quad (45)$$

$$N_3O_3^- \rightarrow N_2O + NO_2^- \ (k = 235\ s^{-1}) \quad (46)$$

The reaction of ·NO with NO^- [reaction (43)] is particularly fast, as indicated by the large rate constant. Thus, NO^- is a good trap for ·NO and may attenuate or completely change its physiological actions.

As indicated by the negative reduction potential for the $NO/^1NO^-$ couple ($E° = -0.35$ V), singlet NO^- can act as a one-electron reducing agent. An example of this is represented by the ability of NO^- to reduce the cupric form of the enzyme, superoxide dismutase ($SODCu^{II}$), to the cuprous form ($SODCu^{I}$) (Murphy and Sies, 1991; Fukuto *et al.,* 1993; Hobbs *et al.,* 1994) [reaction (47)].

$$NO^- + SODCu^{II} \rightarrow \cdot NO + SODCu^{I} \quad (47)$$

In these studies, the source of NO^- dictated that its spin state be a singlet and, therefore, highly reducing. Thus, in the presence of a one-electron oxidizing species, NO^- can be easily converted to ·NO, resulting in an environment where both ·NO and NO^- exist. Due to the rate of reaction (43) and the postulated existence of reaction (45), mixtures of NO^- and ·NO may lead to the generation of the potent and indiscriminate oxidizing agent, HO·.

Nitroxyl can also react with oxidized hemoproteins such as methemoglobin or metmyoglobin to generate the reduced ferrous nitrosyl adducts ($HbFe^{II}-NO$) (Bazylinski and Hollocher, 1985b) [reaction (48)].

$$NO^- + HbFe^{III} \rightarrow HbFe^{II}-NO \quad Hb = \text{hemoglobin} \quad (48)$$

As indicated earlier in this chapter, the metal–nitrosyl bond can be especially strong; thus the Fe^{II}–NO complex can be extremely stable.

Nitroxyl is also electrophilic, as evidenced by its reaction with thiols to generate an *N*-hydroxysulfenamide [reaction (49)] (Doyle *et al.,* 1988).

$$HNO + RSH \rightarrow RS\text{-}NHOH \quad (49)$$
(*N*-hydroxysulfenamide)

This intermediate can react further with excess thiol to give hydroxylamine and the corresponding disulfide [reaction (50)].

$$RS-NHOH + RSH \rightarrow RSSR + NH_2OH \quad (50)$$

Alternatively, the *N*-hydroxysulfenamide can rearrange to generate a sulfinamide (Fig. 11) (Wong *et al.,* 1998).

Since the *N*-hydroxysulfenamide is a common intermediate in the generation of both the disulfide and sulfinamide, the predominance of one product over the other will undoubtedly be a function of the thiol:HNO ratio. That is, at a high thiol:HNO ratio, it may be expected that reaction (50), generation of the disulfide and NH_2OH, will predominate since a second thiol is required to react with the *N*-hydroxysulfenamide. However, at a low thiol:HNO ratio, sulfinamide formation would predominate. Significantly, disulfide formation is physiologically reversible since there are a variety of reductases capable of converting disulfides back to the corresponding thiols. Sulfinamide formation, however, may represent irreversible thiol modification since there are, to date, no reported physiological systems capable of reducing this functional group.

Another example of the electrophilic nature of HNO can be seen in its reaction with olefinic species. In the singlet spin state, HNO can undergo reactions comparable to that observed for singlet oxygen ($^1O_2$). For example, HNO reacts with 1,3-dienes by a 4 + 2 cycloaddition and can react with olefins in an ''ene-like'' reaction (Fig. 12) (Ensley and Mahadevan, 1989).

It should be noted that HNO has a possible isomer, NOH. This species has a triplet ground state and has never been observed directly. Moreover, calculations indicate that HNO is the thermodynamically favored isomer (Gallup, 1975). Thus, NOH generation is rare and, in fact, has only been reported to occur in the photolysis of the HNO donor molecule, Angeli's salt (Donald *et al.,* 1986) and in the thermal reaction of ·NO with NH_2OH (Bonner and Wang, 1986).

HNO/O_2 Chemistry

Very little is understood about the interaction of HNO and O_2. Most studies of HNO, using HNO donors, were carried out under strict anaerobic conditions precluding any $HNO-O_2$ chemistry. One previous report, however, indicated that HNO will react with O_2 since a decomposing solution of the HNO donor, Angeli's salt, was found to consume O_2 (Fukuto *et al.,* 1993). Moreover, it was observed that the reaction of HNO with O_2 results in the generation of H_2O_2, indicating that O_2 is capable of accepting an electron from HNO/NO^-. The reaction of NO^- with dioxygen is expected to be highly dependent on its spin state. Most of the reports on HNO chemistry involve HNO donor molecules

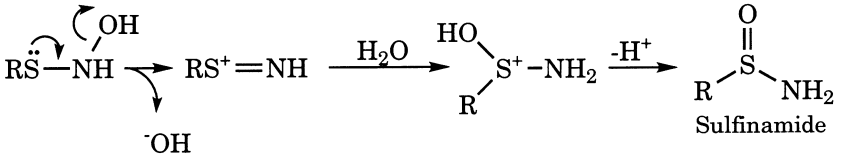


Figure 11 Mechanism of sulfinamide formation from the reaction of HNO with thiols.

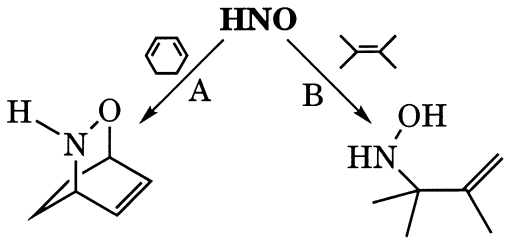


Figure 12 (A) Reaction of HNO with a diene by a 4 + 2 cycloaddition reaction and (B) HNO reaction with an olefin by an ene-type reaction.

that generate the singlet species. It has been reported that singlet NO^- generated *in situ* does *not* react at an appreciable rate with O_2 to form peroxynitrite (^-OONO) (Donald *et al.,* 1986) (although this certainly does not preclude reaction of HNO with O_2 to give other products). However, *in situ* generated triplet NO^- (via photolysis of the HNO donor molecule, Angeli's salt) has been reported to react with O_2 to give peroxynitrite (^-OONO) (Donald, *et al.,* 1986) [reaction (51).

$$^3NO^- + {}^3O_2 \rightarrow {}^-OONO \qquad (51)$$

The reaction of triplet NO^- with O_2 is reminiscent of and isoelectronic with the well-known reaction between ·NO and superoxide ($O_2^{\cdot-}$), which also generates ^-OONO [reaction (15)]. Thus, the likelihood of ^-OONO generation from NO^- in an aerobic environment will be a function of the spin state of NO^-.

HNO Donors

HNO is typically generated from precursor molecules due to its self-reactivity [reaction (42)]. Perhaps the best studied and most utilized HNO donor is sodium trioxodinitrate ($Na_2N_2O_3$), better known as Angeli's salt (Bonner and Hughes, 1988, and references cited therein). This inorganic salt is fairly stable under basic conditions but will spontaneously release HNO between pH 4 and 8 with a first order rate constant of $4.6 \times 10^4\ s^{-1}$ [reaction (52)] (Bonner and Ravid, 1975).

$$N_2O_3^{-2} + H^+ \rightarrow HN_2O_3^- \rightarrow HNO + NO_2^- \qquad (52)$$

The species released thermally from Angeli's salt is singlet HNO, as evidenced by its lack of reactivity with ground state O_2 (Donald *et al.,* 1986). A number of laboratories have used Angeli's salt in examining the chemistry of HNO; indeed, much of the HNO chemistry discussed above was delineated using Angeli's salt as the source of HNO.

Another possible way of generating HNO is via the decomposition of compounds with the *N*-hydroxysulfonamide functional group. The best known of these is *N*-hydroxybenzenesulfonamide or Piloty's acid [$C_6H_5S(O)_2NHOH$], which, under basic conditions, disproportionates to HNO and benzenesulfinic acid [$C_6H_5-S(O)O^-$] [reactions (53) and (54)].

$$C_5H_6S(O)_2NHOH + HO^- \rightarrow C_5H_6S(O)_2NHO^- + H_2O \qquad (53)$$

$$C_5H_6S(O)_2NHO^- \rightarrow C_5H_6S(O)O^- + HNO \qquad (54)$$

As is the case with Angeli's salt, the spin state of NO^- generated from Piloty's acid is singlet (Bonner and Ko, 1992).

Other Reduced ·NO Species; NH_2OH

Reduction of ·NO by three electrons (or two-electron reduction of HNO) results in the formation of hydroxylamine (NH_2OH), and further reduction by two electrons gives ammonia (NH_3) (Fig. 4). As previously indicated, HNO can be reduced by thiols to form NH_2OH via reactions (49) and (50). The physiologically relevant chemistry of NH_2OH includes its ability to serve as a reducing agent and nucleophile. The ability of NH_2OH to serve as a reducing agent is exemplified by its reactions with hemoproteins. For example, reaction of NH_2OH with oxyhemoglobin results in the initial formation of the hydronitroxide radical ($NH_2O\cdot$) and the corresponding peroxide intermediate ($HbFe^{III}-OOH$) [reaction (55)] (Stolze and Nohl, 1989; Stolze *et al.,* 1996).

$$[HbFe^{II}-O_2 \leftrightarrow HbFe^{III}-O_2^{\cdot-}] + NH_2OH \rightarrow HbFe^{III}-OOH + NH_2O\cdot \qquad (55)$$

The $NH_2O\cdot$ radical is unstable and may further react via a number of pathways depending on the conditions. For example, it may react with itself in a second order process to generate N_2 and H_2O (Stolze and Nohl, 1989) [reaction (56)].

$$2NH_2O\cdot \rightarrow N_2 + 2H_2O \qquad (56)$$

[The physiological relevance of reaction (56) may be precluded by the second order dependence on $NH_2O\cdot$, however.] The putative and fleeting ferric peroxide intermediate in reaction (55) has been proposed to either release H_2O_2 or decompose to generate a high valent oxo intermediate which can perform further oxidation chemistry (Stolze and Nohl, 1989, 1990; Stolze *et al.,* 1996). Regardless, it is apparent that NH_2OH is capable of acting as a reducing agent (or H-atom donor) for oxygen bound hemoproteins. Hydroxylamine is also able to reduce ferric hemes. For example, reaction of NH_2OH with methemoglobin under anaerobic conditions has been proposed to generate deoxyhemoglobin via reaction (57) (Bazylinski *et al.,* 1987).

$$2HbFe^{III} + 2NH_2OH \rightarrow 2\ HbFe^{II} + 2H_2O + N_2 + 2H^+ \qquad (57)$$

It has also been reported that NH_2OH can oxidize deoxyhemoglobin, under anaerobic conditions, to generate methemoglobin [reaction (58)] (Bazylinski *et al.,* 1987).

$$2\ HbFe^{II} + NH_2OH + H_2O \rightarrow 2HbFe^{III} + NH_3 + 2HO^- \qquad (58)$$

The combination of reactions (57) and (58) indicates that hemoglobin can act as a catalyst for the disproportionation of NH_2OH to N_2 and NH_3 [reaction (59)].

$$3NH_2OH \rightarrow NH_3 + N_2 + 3H_2O \qquad (59)$$

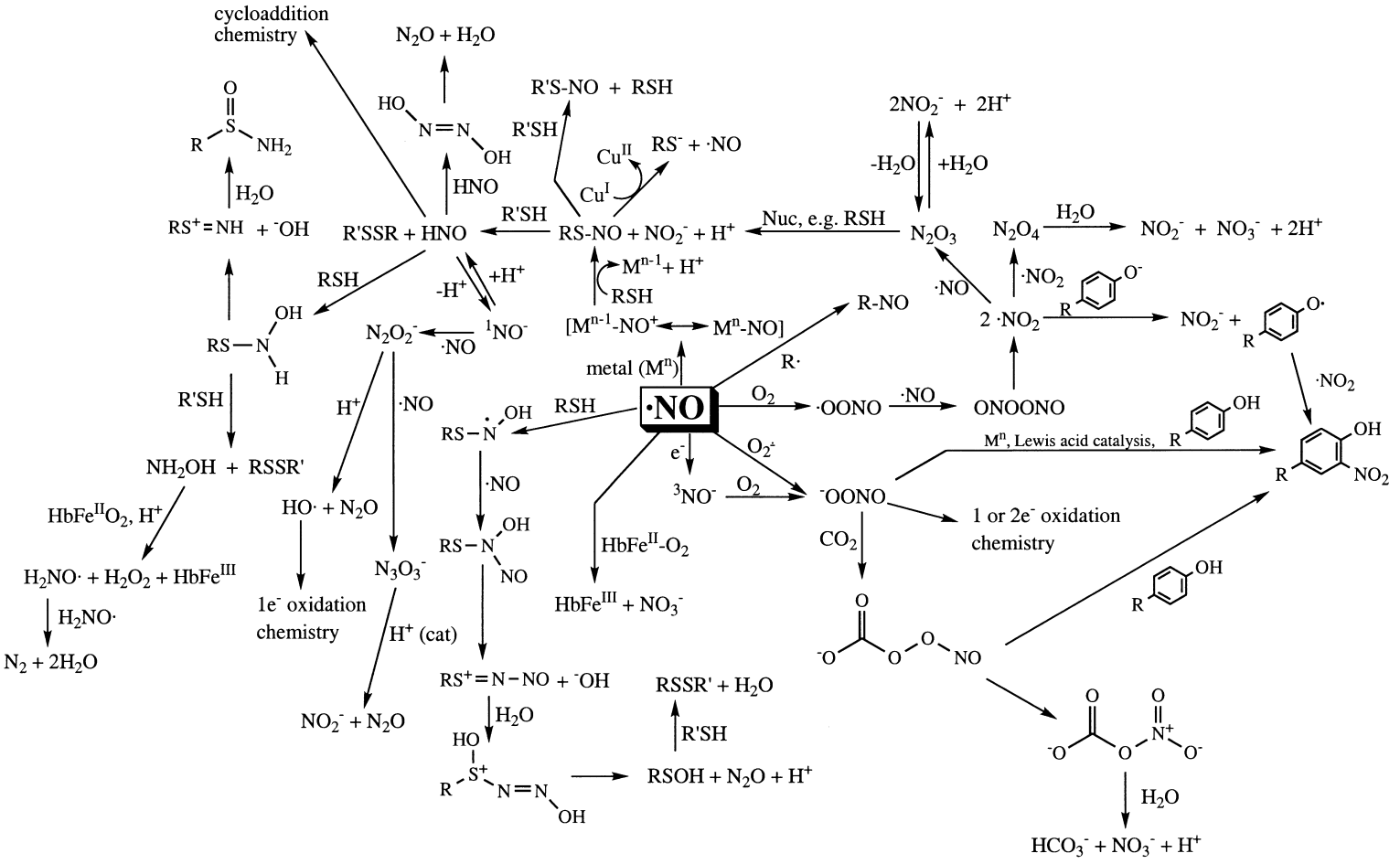


Figure 13 Schematic of some of the physiologically relevant reactions of ·NO and ·NO-derived species in aqueous, aerobic solution.

Along with its ability to serve as a reducing agent, due to a phenomenon known as the α effect, hydroxylamine is also a potent nucleophile (much better than, e.g., ammonia). This effect results from the juxtaposition of the nucleophilic lone pair of electrons on the nitrogen atom and the lone pairs on the adjacent oxygen atom. Thus, NH_2OH will react with a variety of electrophiles, such as aldehydes and ketones, which are converted to the corresponding oximes.

Summary

The chemistry of ·NO and ·NO-derived species consists of numerous interrelated and interdependent processes. This is best exemplified by the scheme shown in Fig. 13 where some of the more important and previously discussed aspects of the physiological chemistry of ·NO are depicted. As is evident from Fig. 13, the chemistry of ·NO and related nitrogen oxides will be a function of their concentration and environment. For example, many of the pathways depicted have greater than first order dependencies on ·NO concentration and, therefore, are not as likely to occur at low concentrations as those with a first order dependence on ·NO. Other reaction pathways involve participation of other chemical entities such as $O_2^{\cdot-}$ or thiols that may have widely varying physiological concentrations. To be sure, Fig. 13 is by no means intended to be a comprehensive depiction of physiological nitrogen oxide chemistry, and many of the processes have been simplified for the sake of convenience. Furthermore, it is not the intent of this chapter to indicate that all the intricacies of nitrogen oxide physiological chemistry are currently understood. On the contrary, this topic remains a vital and active field, and it is likely that numerous other important reactions and/or metabolic pathways for ·NO and related nitrogen oxides will be demonstrated in the future. What is clear, however, is that the chemistry and physiology of ·NO and related nitrogen oxides are intimately linked. The physiological importance of nitrogen oxides will undoubtedly lead to the discovery (or rediscovery) of new aspects of nitrogen oxide chemistry.

References

Arnelle, D. R., and Stamler, J. S. (1995). NO^+, NO and NO^- donation by *S*-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch. Biochem. Biophys.* **318,** 279–285.

Barnett, D. J., Rios, A., and Williams, D. L. H. (1994a). NO-group transfer (transnitrosation) between *S*-nitrosothiols and thiols. Part 2. *J. Chem. Soc., Perkin Trans. 2,* 1279–1282.

Barnett, D. J., McAninly, J., and Williams, D. L. H. (1994b). Transnitrosation between nitrosothiols and thiols. *J. Chem. Soc., Perkin Trans. 2,* 1131–1133.

Bartberger, M. D., Olson, L. P., and Houk, K. N. (1998). Mechanisms of peroxynitrite oxidations and rearrangements: The theoretical perspective. *Chem. Res. Toxicol.* **11,** 710–711.

Bartlett, D., Church, D. F., Bounds, P. L., and Koppenol, W. H. (1995). The kinetics of the oxidation of L-ascorbic acid by peroxynitrite. *Free Radicals Biol. Med.* **18,** 85–92.

Bazylinski, D. A., and Hollocher, T. C. (1985a). Evidence from the reaction between trioxodinitrate(II) and ^{15}NO that trioxodinitrate(II) decomposes into nitrosyl hydride and nitrite in neutral aqueous solution. *Inorg. Chem.* **24,** 4285–4288.

Bazylinski, D. A., and Hollocher, T. C. (1985b). Metmyoglobin and methemoglobin as efficient traps for nitrosyl hydride (nitroxyl) in neutral aqueous solution. *J. Am. Chem. Soc.* **107,** 7982–7986.

Bazylinski, D. A., Arkowitz, R. A., and Hollocher, T. C. (1987). Decomposition of hydroxylamine by hemoglobin. *Arch. Biochem. Biophys.* **259,** 520–526.

Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994). Oxidative chemistry of peroxynitrite. *Methods Enzymol.* **233,** 229–240.

Blough, N. V., and Zafiriou, O. C. (1985). Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline aqueous solution. *Inorg. Chem.* **24,** 3502–3504.

Bonner, F. T., and Hughes, M. N. (1988). The aqueous solution chemistry of nitrogen in low positive oxidation states. *Comments Inorg. Chem.* **7,** 215–234.

Bonner, F. T., and Ko, Y. (1992). Kinetic, isotopic and ^{15}N NMR study of *N*-hydroxybenzenesulfonamide decomposition: An HNO source. *Inorg. Chem.* **31,** 2514–2519.

Bonner, F. T., and Ravid, B. (1975). Thermal decomposition of oxohyponitrite (sodium trioxodinitrate(II)) in aqueous solution. *Inorg. Chem.* **14,** 558–563.

Bonner, F. T., and Stedman, G. (1996). The chemistry of nitric oxide and redox-related species. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. S. Stamler, eds.), pp. 3–18. Wiley, Chichester.

Bonner, F. T., and Wang, N. Y. (1986). Reduction of nitric oxide by hydroxylamine. 1. Kinetics and mechanism. *Inorg. Chem.* **25,** 1858–1862.

Casassa, M. P., Stephenson, J. C., and King, D. S. (1986). Vibrational predissociation of the nitric oxide dimer: Total energy distribution in the fragments. *J. Chem. Phys.* **85,** 2333–2334.

Casassa, M. P., Stephenson, J. C., and King, D. S. (1988). Time- and state-resolved measurements of nitric oxide dimer infrared photodissociation. *J. Chem. Phys.* **89,** 1966–1976.

Castro, C. E., and Bartnicki, E. W. (1994). The interconversion of nucleic acids bases by iron(III) porphyrins and nitric oxide. *J. Org. Chem.* **59,** 4051–4052.

Challis, B. C., and Kyrtopoulos, S. A. (1978). The chemistry of nitroso compounds. Part 12. The mechanism of nitrosation and nitration of aqueous piperidine by gaseous dinitrogen tetraoxide and dinitrogen trioxide in aqueous alkaline solutions. Evidence for the existence of molecular isomers of dinitrogen tetraoxide and dinitrogen trioxide. *J. Chem. Soc. Perkin Trans. 2,* 1296–1302.

Chien, J. C. W. (1969). Reactions of nitric oxide with methemoglobin. *J. Am. Chem. Soc.* **91,** 2166–2168.

Collman, J. P., Hegedus, L. S., Norton, J. R., and Finke, R. G. (1987). ''Principles and Applications of Organotransition Metal Chemistry,'' 2nd Ed. Univ. Science Books, Mill Valley, California.

Cotton, F. A., and Wilkinson, G. (1988). ''Advanced Inorganic Chemistry,'' 5th Ed. Wiley, New York.

DeMaster, E. G., Quast, B. J., Redfern, B., and Nagasawa, H. T. (1995). Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. *Biochemistry* **34,** 11494–11499.

Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* **333,** 49–58.

Dicks, A. P., Swift, H. R., Williams, D. L. H., Butler, A. R., Al-Sa'doni, H. H., and Cox, B. G. (1996). Identification of Cu^+ as the effective reagent in nitric oxide formation from *S*-nitrosothiols (RSNO). *J. Chem. Soc., Perkin Trans. 2,* 481–487.

Dkhissi, A., Soulard, P., Perrin, A., and Lacome, N. (1997). The NO dimer. *J. Mol. Spectrosc.* **183,** 12–17.

Donald, C. E., Hughes, M. N., Thompson, J. M., and Bonner, F. T. (1986). Photolysis of the N=N bond in trioxodinitrate: Reaction between triplet ^-NO and O_2 to form peroxynitrite. *Inorg. Chem.* **25,** 2676–2677.

Doyle, M. P., and Hoekstra, J. W. (1981). Oxidation of nitrogen oxides by bound dioxygen in hemeproteins. *J. Inorg. Biochem.* **14,** 351–358.

Doyle, M. P., Herman, J. G., and Dykstra, R. L. (1985). Autocatalytic oxidation of hemoglobin induced by nitrite: Activation and chemical inhibition. *J. Free Radicals Biol. Med.* **1,** 145–154.

Doyle, M. P., Mahapatro, S. N., Broene, R. D., and Guy, J. K. (1988). Oxidation and reduction of hemoproteins by trioxodinitrate(II). The role of nitrosyl hydride and nitrite. *J. Am. Chem. Soc.* **110,** 593–599.

Drapier, J. C. (1997). Interplay between NO and [Fe−S] clusters: Relevance to biological systems. *Methods (San Diego)* **11,** 319–329.

Duarte, H. A., Proynov, E., and Salahub, D. R. (1998). Density functional study of the NO dimer using GGA and LAP functionals. *J. Chem. Phys.* **109,** 26–35.

East, A. L. L. (1998). The 16 valence electronic states of nitric oxide dimer (NO)(2). *J. Chem. Phys.* **109,** 2185–2193.

East, A. L. L., McKellar, A. R. W., and Watson, J. K. G. (1998). The intermolecular vibrations of the NO dimer. *J. Chem. Phys.* **109,** 4378–4383.

Edwards, J. O., and Plumb, R. C. (1994). The chemistry of peroxonitrites. *Prog. Inorg. Chem.* **41,** 599–635.

Eisenberg, R., and Meyer, C. D. (1975). The coordination chemistry of nitric oxide. *Acc. Chem. Res.* **8,** 26–34.

Ensley, H. E., and Mahadevan, S. (1989). Diels-Alder and ene reaction of nitrosyl and nitrosoformaldehyde. *Tetrahedron Lett.* **30,** 3255–3258.

Farias-Eisner, R., Chaudhuri, G., Aeberhard, E., and Fukuto, J. M. (1996). The chemistry of tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. *J. Biol. Chem.* **271,** 6144–6151.

Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993). Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* **326,** 1–3.

Forte, E., and van den Bergh, H. (1978). The heat of formation of the nitric oxide dimer and its UV spectrum. *Chem. Phys.* **30,** 325–31.

Fukuto, J. M., Hobbs, A. J., and Ignarro, L. J. (1993). Conversion of nitroxyl (HNO) to nitric oxide in biological systems: The role of physiological oxidants and relevance to the biological activity of HNO. *Biochem. Biophys. Res. Commun.* **196,** 707–713.

Gallup, G. A. (1975). Self-consistent field calculation of nitrosyl hydride and nitrogen hydroxide. *Inorg. Chem.* **14,** 563–565.

Gilbert, J. R., and Thomas, J. H. (1972). Reaction of oxides of nitrogen. *In* ''Comprehensive Chemical Kinetics'' (C. H. Bamford and C. F. H. Tipper, eds.) Vol. 6, pp. 139–200. Elsevier, New York.

Goldstein, S., and Czapski, G. (1995). The reaction of ·NO with O_2^- and HO_2·: A pulse radiolysis study. *Free Radicals Biol. Med.* **19,** 505–510.

Gow, A., Duran, D., Thom, S. R., and Ischiropoulos, H. (1996). Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch. Biochem. Biophys.* **333,** 42–48.

Gratzel, V. M., Taniguchi, S., and Henglein, A. (1970). Pulsradiolytische untersuchung kurzlebiger zwischenprodukte der NO-reduktion in wassriger losung. *Ber. Bunsen-Ges. Phys. Chem.* **74,** 1003–1010.

Gwost, D., and Caulton, K. G. (1973). Reductive nitrosylation of group VIIIb compounds. *Inorg. Chem.* **12,** 2095–2099.

Ha, T.-K. (1981). *Ab initio* CI study of the nitric oxide dimer (N_2O_2). *Theor. Chim. Acta* **58,** 125–130.

Hetzler, J. R., Casassa, M. P., and King, D. S. (1991). Product energy correlations in the dissociation of overtone excited NO dimer. *J. Phys. Chem.* **95,** 8086–8095.

Hibbs, J. B. Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Comm.* **157,** 87–94.

Hobbs, A. J., Fukuto, J. M., and Ignarro, L. J. (1994). Formation of free nitric oxide from L-arginine by nitric oxide synthase: Direct enhancement of generation by superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 10992–10996.

Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., and Parthasarathy, S. (1993). Inhibition of low-density lipoprotein oxidation by nitric oxide. *FEBS Lett.* **334,** 170–174.

Hoshino, M., Ozawa, K., Seki, H., and Ford, P. C. (1993). Photochemistry of nitric oxide adducts of water soluble iron(III) porphyrins and ferrihemoproteins by nanosecond laser photolysis. *J. Am. Chem. Soc.* **115,** 9568–9575.

Houk, K. N., Condroski, K. R., and Pryor, W. A. (1996). Radical and concerted mechanisms in oxidations of amines by peroxynitrite, peroxynitrous acid and peroxynitrite-CO_2 adduct: Density functional theory transition structure and energetics. *J. Am. Chem. Soc.* **118,** 13002–13006.

Huber, K. P., and Herzberg, G. (1979). ''Molecular Spectra and Molecular Structure.'' Van Nostrand-Reinhold, New York.

Huie, R. E. (1994). The reaction kinetics of NO_2. *Toxicology* **89,** 193–216.

Huie, R. E., and Padmaja, S. (1993). The reaction of ·NO with superoxide. *Free Radical Res. Commun.* **18,** 195–199.

Ignarro, L. J. (1989). Heme-dependent activation of soluble guanylate cyclase by nitric oxide: Regulation of enzyme activity by porphyrins and metalloporphyrins. *Semin. Hematol.* **26,** 63–76.

Jorgensen, W. L., and Salem, L. (1973). ''The Organic Chemist's Book of Orbitals,'' p. 80. Academic Press, New York.

Jursic, B. S. (1995). Theoretical investigation of *cis*-nitric oxide dimer with hybrid density functional theory methods. *Chem. Phys. Lett.* **236,** 206–210.

Kanner, J. (1996). Nitric oxide and metal-catalyzed reactions. *Methods Enzymol.* **269,** 218–229.

Kanner, J., Harel, S., and Granit, R. (1991). Nitric oxide as an antioxidant. *Arch. Biochem. Biophys.* **289,** 130–136.

Kanner, J., Harel, S., and Granit, R. (1992). Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemoglobin. *Lipids* **27,** 46–49.

Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997). An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. *J. Biol. Chem.* **272,** 20340–20347.

Koppenol, W. H., and Traynham, J. G. (1996). Say NO to nitric oxide: Nomenclature for nitrogen- and oxygen-containing compounds. *Methods Enzymol.* **268,** 3–6.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ishiropoulos, H., and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5,** 834–842.

Kosaka, H., Imaizumi, K., and Tyuma, I. (1981). Mechanism of autocatalytic oxidation of oxyhemoglobin by nitrite: An intermediate detected by electron spin resonance. *Biochim. Biophys. Acta* **702,** 237–241.

Kukolich, S. G. (1982). Structure of the NO dimer. *J. Am. Chem. Soc.* **104,** 4715–4716.

Lee, T. J., Rendell, A. P., and Taylor, P. R. (1990). Comparison of the quadratic configuration interaction and coupled-cluster approaches to electron correlation including the effect of triple excitations. *J. Phys. Chem.* **94,** 5463–5468.

Lemercier, J. L., Squadrito, G. L., and Pryor, W. A. (1995). Spin trap studies on the decomposition of peroxynitrite. *Arch. Biochem. Biophys.* **321,** 31–39.

Lewis, R. S., and Deen, W. M. (1994). Kinetics of the reaction of nitric oxide with oxygen in aqueous solution. *Chem. Res. Toxicol.* **7,** 568–574.

Lissi, E. (1998). Autocatalytic oxidation of hemoglobin by nitrite: A possible mechanism. *Free Radicals Biol. Med.* **24,** 1535–1536.

Liu, X., Miller, M. J. S., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998). Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2175–2179.

Lymar, S. V., and Hurst, J. K. (1995). Rapid reaction between peroxonitrite ion and carbon dioxide: Implication for biological activity. *J. Am. Chem. Soc.* **117,** 8867–8868.

McCleverty, J. A. (1979). Reaction of nitric oxide coordinated to transition metals. *Chem. Rev.* **79,** 53–76.

McKellar, A. R. W., Watson, J. K. G., and Howard, B. J. (1995). The NO dimer: ^{15}N isotopic infrared spectra, line widths and force field. *Mol. Phys.* **86,** 273–286.

McMillen, D. F., and Golden, D. M. (1982). Hydrocarbon bond dissociation energies. *Annu. Rev. Phys. Chem.* **33,** 493–532.

Mahoney, L. R. (1970). Evidence for the formation of hydroxyl radical in

the isomerization of pernitrous acid to nitric acid in aqueous solution. *J. Am. Chem. Soc.* **92,** 5262–5263.

Merenyi, G., and Lind, J. (1997). Thermodynamics of peroxynitrite and its CO_2 adduct. *Chem. Res. Toxicol.* **10,** 1216–1220.

Merenyi, G., and Lind, J. (1998). Free radical formation in the peroxynitrous acid (ONOOH)/Peroxynitrite ($OONO^-$) system. *Chem. Res. Toxicol.* **11,** 243–246.

Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hansert, B., Wink, D. A., and Grisham, M. B. (1996). Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J. Biol. Chem.* **271,** 40–47.

Murphy, M. E., and Noack, E. (1994). Nitric oxide assay using hemoglobin method. *Methods Enzymol.* **233,** 240–250.

Murphy, M. E., and Sies, H. (1991). Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10860–10864.

Oae, S., Kim, Y. H., Fukushima, D., and Shinhama, K. (1978). New synthesis of thionitrites and their chemical reactivities. *J. Chem. Soc., Perkin Trans. 1,* 913–917.

Olbregts, J. (1985). Termolecular reaction of nitrogen monoxide and oxygen: A still unsolved problem. *Int. J. Chem. Kinet.* **17,** 835–848.

Perez Jigato, M., Termath, V., Gardner, P., Handy, N. C., King, D. A., Rassias, S., and Surman, M. (1995). Soft X-ray photoabsorption of the NO dimer. *Mol. Phys.* **85,** 619–633.

Pfeiffer, S., Gorren, A. C. F., Schmidt, K., Werner, E. R., Hansert, B., Bohle, D. S., and Mayer, B. (1997). Metabolic fate of peroxynitrite in aqueous solution. *J. Biol. Chem.* **272,** 3465–3470.

Pou, S., Nguyen, S. Y., Gladwell, T., and Rosen, G. M. (1995). Does peroxynitrite generate hydroxyl radical? *Biochim. Biophys. Acta* **1244,** 62–68.

Prutz, W. A., Monig, H., Butler, J., and Land, E. J. (1985). Reactions of nitrogen dioxide in aqueous model systems: Oxidation of tyrosine units in peptides and proteins. *Arch. Biochem. Biophys.* **243,** 125–134.

Pryor, W. A., and Squadrito, G. L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.* **268**(*Lung Cell Mol. Physiol.* **12**), L699–L722.

Pryor, W. A., Church, D. F., Govindan, C. K., and Crank, G. (1982). Oxidation of thiols by nitric oxide and nitrogen dioxide: Synthetic utility and toxicological implications. *J. Org. Chem.* **47,** 156–159.

Pryor, W. A., Jin, X., and Squadrito, G. L. (1994). One- and two-electron oxidations of methionine by peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 11173–11177.

Pryor, W. A., Jin, X., and Squadrito, G. L. (1996). Insensitivity of the rate of decomposition of peroxynitrite to changes in viscosity; evidence against free radical formation. *J. Am. Chem. Soc.* **118,** 3125–3128.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls. *J. Biol. Chem.* **266,** 4244–4250.

Ragsdale, R. O. (1973). Reaction of nitrogen(II) oxide. *In* ''Developments in Inorganic Chemistry'' (C. B. Colburn, ed.) Vol. 2, pp. 1–26. Elsevier, New York.

Richter-Addo, G. B., and Legzdins, P. (1992). ''Metal Nitrosyls,'' Oxford Univ. Press, New York.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. *J. Biol. Chem.* **269,** 26066–26075.

Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaraman, B., and Freeman, B. A. (1995). Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324,** 15–25.

Rubbo, H., Darley-Usmar, V., and Freeman, B. A. (1996). Nitric oxide regulation of tissue free radical injury. *Chem. Res. Toxicol.* **9,** 809–820.

Schmidt, H. H. H. W., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D., and Feelisch, M. (1996). No NO from nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14492–14497.

Schwartz, S. S., and White, W. H. (1983). Kinetics of reactive dissolution of nitrogen oxides in aqueous solution. *In* ''Trace Atmospheric Constituents, Properties, Transformation and Fates'' (S. E. Schwartz, ed.), pp. 1–116. Wiley, New York.

Seddon, W. A., Fletcher, J. W., and Soppchyshyn, F. C. (1973). Pulse radiolysis of nitric oxide in aqueous solution. *Can. J. Chem.* **51,** 1123–1130.

Shaw, A. W., and Vosper, A. J. (1977). Solubility of nitric oxide in aqueous and nonaqueous solvents. *J. Chem. Soc. Faraday Trans. 1,* **8,** 1239–1244.

Shi, X., Lenhart, A., and Mao, Y. (1994). ESR spin trapping investigation on peroxynitrite decomposition: No evidence for hydroxyl radical production. *Biochem. Biophys. Res. Commun.* **203,** 1515–1521.

Singh, S. P., Wishnok, J. S., Keshive, M., Deen, W. M., and Tannenbaum, S. R. (1996). The chemistry of the *S*-nitrosoglutathione/glutathione system. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14428–14433.

Skaarup, S., Skancke, P. N., and Boggs, J. E. (1976). Structure and force fields of the isomers of dinitrogen dioxide. *J. Am. Chem. Soc.* **98,** 6106–6109.

Squadrito, G. L., and Pryor, W. A. (1995). Stopped-flow kinetic study of the reaction of ascorbic acid with peroxynitrite. *Arch. Biochem. Biophys.* **322,** 53–59.

Stanbury, D. M. (1989). Reduction potentials involving inorganic free radicals in aqueous solution. *Adv. Inorg. Chem.* **33,** 69–138.

Stolze, K., and Nohl, H. (1989). Detection of free radicals as intermediates in the methemoglobin formation from oxyhemoglobin induced by hydroxylamine. *Biochem. Pharmacol.* **38,** 3055–3059.

Stolze, K., and Nohl, H. (1990). Free radical intermediates in the oxidation of *N*-methylhydroxylamine and *N,N*-dimethylhydroxylamine by oxyhemoglobin. *Free Radical Res. Commun.* **8,** 123–131.

Stolze, K., Dadak, A., Liu, Y., and Nohl, H. (1996). Hydroxylamine and phenol-induced formation of methemoglobin and free radical intermediates in erythrocytes. *Biochem. Pharmacol.* **52,** 1821–1829.

Struck, A. T., Hogg, N., Thomas, J. P., and Kalyanaraman, B. (1995). Nitric oxide donor compounds inhibit the toxicity of oxidized low-density lipoprotein to endothelial cells. *FEBS Lett.* **361,** 291–294.

Tonner, B. P., Kao, C. M., Plummer, E. W., Caves, T. C., Messmer, R. P., and Salaneck, W. R. (1983). Intermolecular screening in core-level photoemission from the nitric-oxide dimer. *Phys. Rev. Lett.* **51,** 1378–1381.

Tran, D., Skelton, B. W., White, A. H., Laverman, L. E., and Ford, P. C. (1998). Investigation of the nitric oxide reduction of the bis(2,9-dimethyl-1,10-phenanthroline) complex of copper (II) and the structure of $[Cu(dmp)_2(H_2O)](CF_3SO_3)_2$. *Inorg. Chem.* **37,** 2505–2511.

Traylor, T. G., and Sharma, V. S. (1992). Why NO? *Biochemistry* **31,** 2847–2849.

Traylor, T. G., Duprat, A. F., and Sharma, V. S. (1993). Nitric oxide-triggered heme-mediated hydrolysis: A possible model for biological reactions of NO. *J. Am. Chem. Soc.* **115,** 811–813.

Uppu, R. M., Squadrito, G. L., and Pryor, W. A. (1996). Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327,** 335–343.

van der Vliet, A., O'Neill, C. A., Halliwell, B., Cross, C. E., and Kaur, H. (1994). Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett.* **339,** 89–92.

Vosper, A. J. (1975). Nitrogen oxides and oxyacids. *In Int. Rev. Sci., Inorg. Chem. Ser. 2,* 123–165.

Wade, R. S., and Castro, C. E. (1990). Redox reactivity of iron(III) porphyrins and heme proteins with nitric oxide. Nitrosyl transfer to carbon, oxygen, nitrogen and sulfur. *Chem. Res. Toxicol.* **3,** 289–291.

Wallace, W. J., and Caughey, W. S. (1975). Mechanism for the oxidation of hemoglobin by phenols, nitrite and ''oxidant'' drugs. Peroxide formation by one electron donation to bound oxygen. *Biochem. Biophys. Res. Commun.* **62,** 561–567.

Warneck, P., and Wurzinger, C. (1988). Product quantum yields for the 305-nm photodecomposition of NO_3^- in aqueous solution. *J. Chem. Phys.* **92,** 6278–6283.

Wayland, B. B., and Olson, L. W. (1974). Spectroscopic studies and bonding model for nitric oxide complexes of iron porphyrins. *J. Am. Chem. Soc.* **96,** 6037–6041.

Williams, D. L. H. (1983). Nitrosation mechanisms. *Adv. Phys. Org. Chem.* **19,** 381–429.

Williams, D. L. H. (1996). Nitric oxide release from *S*-nitrosothiols (RSNO)—The role of copper ions. *Transition Met. Chem.* **21,** 189–191.

Williams, J. E., and Murrell, J. N. (1971). Structure and stability of the nitric oxide dimer. *J. Am. Chem. Soc.* **93,** 7149–52.

Wink, D. A., Hanbauer, I., Krishna, M. C., DeGraff, W., Gamson, J., and Mitchell, J. B. (1993). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9813–9817.

Winterbourn, C. C., and Metodiewa, D. (1995). The reaction of superoxide with glutathione and other thiols. *Methods Enzymol.* **251,** 81–86.

Wong, P. S. Y., Hyun, J., Fukuto, J. M., Shirota, F. N., DeMaster, E. G., Shoeman, D. W., and Nagasawa, H. T. (1998). Reaction between *S*-nitrosothiols and thiols: Generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry* **37,** 5362–5371.

Zhang, H., Squadrito, G. L., Uppu, R. M., Lemercier, J. L., Cueto, R., and Pryor, W. A. (1997). Inhibition of peroxynitrite-mediated oxidation of glutathione by carbon dioxide. *Arch. Biochem. Biophys.* **339,** 183–189.

CHAPTER 3

The Chemical Biology of Nitric Oxide

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THE MULTIPLE EFFECTS OF NO IN BIOLOGICAL SYSTEMS HAVE RESULTED IN INTENSE INVESTIGATION INTO THE MECHANISMS OF NO-MEDIATED EVENTS. THE CHEMISTRY OF NO IS THE PRIMARY DETERMINANT OF ITS BIOLOGICAL PROPERTIES. HOWEVER, NOT ALL THE REACTIONS OF NO THAT CAN BE PERFORMED IN THE TEST TUBE ARE PERTINENT *IN VIVO.* THIS CHAPTER PROVIDES A GUIDE THROUGH THE DIVERSE REACTIONS OF NO IN BIOLOGICAL SYSTEMS. THE SCHEME OF THE CHEMICAL BIOLOGY OF NO DIVIDES THE REACTIONS OF NO INTO THE TWO CATEGORIES OF DIRECT AND INDIRECT EFFECTS. DIRECT EFFECTS ARE DEFINED AS THOSE REACTIONS THAT ARE FAST ENOUGH TO OCCUR BETWEEN NO AND SPECIFIC BIOLOGICAL TARGETS. INDIRECT EFFECTS DO NOT INVOLVE NO BUT RATHER ARE MEDIATED BY REACTIVE NITROGEN OXIDE SPECIES FORMED FROM THE REACTION OF NO WITH EITHER OXYGEN OR SUPEROXIDE. THESE SPECIES CAN MEDIATE EITHER NITROSATIVE OR OXIDATIVE STRESS. THIS REPORT DISCUSSES ASPECTS OF THE CHEMICAL BIOLOGY OF NO RELATING TO BIOLOGICAL MOLECULES SUCH AS GUANYLATE CYCLASE, CYTOCHROME P-450, NITRIC OXIDE SYNTHASE, CATALASE, AND DNA AND EXPLORES THE POSSIBLE ROLES NO PERFORMS IN DIFFERENT BIOLOGICAL SITUATIONS.

Introduction

Nitric oxide (NO) is an endogenous mediator of numerous physiological processes that range from regulation of cardiovascular function to participation in memory (Dawson *et al.,* 1992; Feldman *et al.,* 1993; Ignarro, 1989; Moncada *et al.,* 1991). In the immune system this diatomic radical is involved in various antipathogenic and tumoricidal responses (Hibbs, 1991; MacMicking *et al.,* 1997). Paradoxically, NO has also been implicated as a promoter of the severity of different diseases including cancer and stroke (Gross and Wolin, 1995; Wink *et al.,* 1998a). In fact, both protective and deleterious properties have been assigned to NO even in the context of the same physiological event. Therefore, further mechanistic explanations are necessary to account for these contradictory effects.

Unlike most other biological mediators, the *in vivo* properties of NO are determined by its chemistry. Nitric oxide can undergo numerous reactions, and these often result in the formation of additional reactive nitrogen oxide species (RNOS). In an attempt to determine the pertinence of these diverse reactions to biological systems, we have developed the concept of the *chemical biology of nitric oxide* (Espey *et al.,* 2000; Wink and Mitchell, 1998; Wink *et al.,* 1996a,b, 1999). This scheme classifies the chemical reactions of NO into the two basic categories of direct and indirect effects (Fig. 1).

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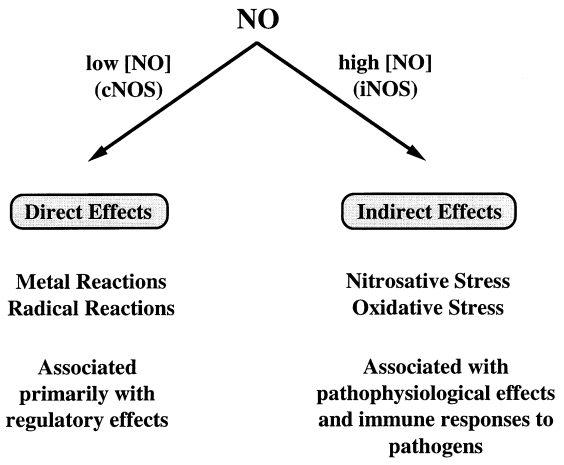


Figure 1 Chemical biology of nitric oxide.

Direct chemical reactions are those in which NO interacts directly with biological targets. The most common reactions of this type are between NO and heme-containing proteins. These reactions are generally rapid and are the genesis of the majority of the *in vivo* effects of NO. Conversely, indirect effects involve other RNOS, usually derived from the reactions between NO and O_2 or superoxide (O_2^-) rather than NO itself. Indirect effects require much higher concentrations of NO than direct reactions. Therefore, NO produced at low concentrations for short periods of time will primarily mediate direct effects, while indirect effects will occur in regions where higher local NO concentrations are sustained for prolonged time periods.

Direct and indirect effects correlate with the different isozymes of nitric oxide synthase (NOS) (Griffith and Stuehr, 1995; Nathan and Xie, 1994). A variety of cells such as endothelia (eNOS) and neurons (nNOS) express NOS constitutively. These isozymes can briefly generate submicromolar NO concentrations at the cellular level. Conversely, inducible NOS (iNOS) can produce NO for extended periods of time, with concentrations as high as 10 μM in the vicinity of the cell (Lewis *et al.,* 1995). In general, the chemistry and the ultimate biological outcome of NO synthesis will be dictated by the isozyme type. Additional consideration must be given to the proximity of the biological target to the NO source. Both direct and indirect effects would be expected to occur adjacent to cells such as activated macrophages, which produce high levels of NO, while more distant locations would primarily experience direct effects.

As the reactive intermediates responsible for the indirect effects of NO can also undergo a variety of reactions, indirect effects can be further subdivided into the categories of nitrosative and oxidative stress (Wink and Mitchell, 1998). Nitrosation mediated by RNOS appears to occur *in vivo* primarily through reaction with N_2O_3, whereas oxidation is a consequence of peroxynitrite ($ONOO^-$) or nitroxyl (NO^-) ion formation (Wink *et al.,* 1999). Several studies indicate that nitrosative stress is orthogonal to oxidative stress (Wink and Mitchell, 1998; Wink *et al.,* 1997). It also appears that biological effects such as cytotoxicity vary under nitrosative and oxidative stress (Wink and Mitchell, 1998). This suggests that there is a balance between these two types of stress and that a shift in this balance may determine the functional outcome in responses ranging from cell death to signal transduction. In this chapter, we explore the pertinent chemical reactions in the chemical biology of NO and discuss them in the context of oxidative and nitrosative stress.

Direct Effects

The primary criterion for the significance of direct reactions *in vivo* is reaction rate. The reactions in which NO combines with biological substrates at sufficiently rapid rates to be of consequence involve either metals or radicals (Fig. 2). Direct reactions between NO and thiols, for example, are far too slow to occur to any considerable extent.

The two major types of reactions between NO and biological metals are direct reactions with metal centers and redox reactions with metal dioxygen complexes or high valence metal–oxo complexes. These reactions are rapid, often nearing the diffusion controlled limit, and so are relevant under both physiological and pathophysiological conditions.

Reaction between NO and Metal Complexes

Nitric oxide reacts with a variety of metals to form nitrosyl complexes. The vast majority of these reactions *in vivo* are with metalloproteins containing iron. Although copper compounds will form nitrosyl complexes, the rates are too slow to be of major significance in the biology of NO (Cotton and Wilkinson, 1988). Other physiologically important transition metals such as zinc do not react with NO under biological conditions.

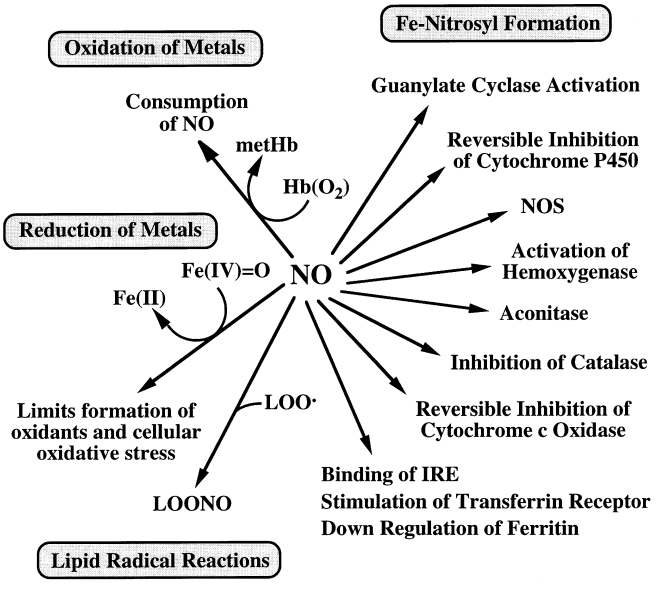


Figure 2 Direct effects of nitric oxide.

Nitrosylation of hemes is particularly facile and should be of primary consideration in any mechanism involving NO. Many basic regulatory functions involve reaction of NO with heme complexes such as guanylate cyclase, cytochrome P-450, NOS, and hemoglobin. The facility of these reactions is such that the NO source can be in another cell or even in different tissue than the target protein. On the contrary, reaction with the Fe–S cluster protein, aconitase, requires higher NO concentrations and thus a closer proximity to the NO source (Bouton *et al.,* 1996).

The most notable heme protein that forms an Fe−NO adduct *in vivo* is soluble guanylate cyclase (Murad, 1994). On NO binding, the position of the iron within the porphyrin ring is shifted such that the distal histidine is decoupled in favor of the five-coordinate nitrosyl complex (Stone and Marletta, 1994; Yu *et al.,* 1994). This alteration in protein configuration activates the enzyme at relatively low NO concentrations (EC_{50} of 100 n*M;* Forstermann and Ishii, 1996) and leads to conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in the GTPase domain. Production of cGMP has ramifications in a variety of tissues, but particularly in vascular smooth muscle where NO mediates vasodilation and has profound effects on platelet function as well as other intercellular interactions (Murad, 1994). Activation of guanylate cyclase by NO has also been suggested to prevent some apoptotic processes (Estevez *et al.,* 1998).

In contrast to guanylate cyclase, binding of NO to monooxygenases such as cytochrome P-450 results in potent competitive inhibition of O_2 binding to the heme site (Fig. 3) (Khatsenko *et al.,* 1993; Stadler *et al.,* 1994; Wink *et al.,* 1993a). Cytochrome P-450 enzymes require O_2 to facilitate oxidative chemistry via the production of iron-peroxo and high valence iron-oxo species. Inhibition of P-450 activity by NO has been postulated to regulate hormone metabolism such as testosterone synthesis (Adams *et al.,* 1992).

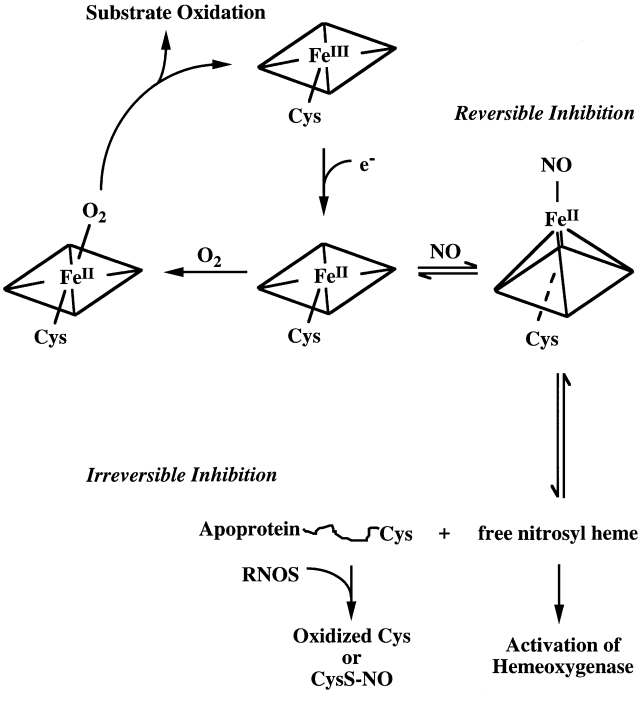


Figure 3 Mechanisms for NO-mediated inhibition of cytochrome P-450.

Nitric oxide-mediated inhibition of cytochrome P-450 has some important pathophysiological sequelae as well. The copious amounts of NO produced during chronic infection or septic shock can lead to inhibition of liver cytochrome P-450s (Stadler *et al.,* 1994; Wink *et al.,* 1993a) and subsequent repression of metabolism of clinically administered drugs (Khatsenko *et al.,* 1993). In contrast, binding of NO to the heme domain of cytochrome P-450 may serve as a protective mechanism against a variety of pathophysiological conditions by releasing free heme and activating hemeoxygenase in hepatocytes (Kim *et al.,* 1995a; Choi and Alam, 1996; Stocker, 1990). Thus, the interaction of NO with P-450s can have regulatory functions as well as deleterious or protective pathophysiological roles.

In the NOS protein, L-arginine is oxidized to L-citrulline and NO at the heme domain by a mechanism similar to substrate oxidation by cytochrome P-450 (Griffith and Stuehr, 1995). Nitric oxide can regulate its own synthesis through nitrosylation of this heme. Enzymatic oxidation is restricted by a negative feedback mechanism in which Fe–NO complex formation prevents O_2 binding at the active site (Abu-Soud *et al.,* 1995; Griscavage *et al.,* 1994, 1995; Hurshman and Marletta, 1995). Even under conditions of hyper intracellular calcium concentrations, this inhibition prevents production of significant amounts of RNOS from nNOS and eNOS. Compared to the constitutive NOS isozymes, the resultant Fe–NO complex in iNOS has a relative lower stability and thus a reduced susceptibility to inhibition by NO (Griscavage *et al.,* 1995). These data show that the predominant source of indirect effects *in vivo* may be from iNOS.

Competitive inhibition of NOS activity by NO can influence direct effects. In recent studies, NOS in lung alveoli was shown to produce NO in response to different concentrations of O_2 (Dweik *et al.,* 1998). Since NO and O_2 compete for the NOS heme binding site, the relative stability of the nitrosyl versus the dioxygen adduct determines the level of NO produced. Formation of the Fe–NO complex in NOS makes the K_m for O_2 binding linear in the physiological range suggesting that NOS may serve as both an O_2 sensor and a regulator of O_2 supply to tissue (Abu-Soud *et al.,* 1996). This mechanism may play a crucial role in regulating blood flow through different tissue in an O_2 tension-dependent manner.

Biological complexes containing metals other than iron are also affected by NO binding. As an example, nitrosylation of the aquo form of the vitamin B_{12} derivative cobalamin results in a diminished ability for this complex to serve as a cofactor for methionine synthase (Brouwer *et al.,* 1996). However, consideration of the consequences of nitrosylation must be extended from the effect on a particular compound to the impact on metabolism in general. For instance, an *in vivo* study showed that scavenging of NO by cobalamin reduced the loss in mean arterial blood pressure induced by

lipopolysaccharide (LPS) activated NOS (Greenberg *et al.,* 1995). Furthermore, the nitrosyl cobalamin complex can also perturb biological systems by nitrosating glutathione-(GSH) or thiol-containing proteins, as discussed later (Brouwer *et al.,* 1996).

Interaction of NO with Metal–Oxygen and Metal–Oxo Complexes

The reactivity of NO with metals is not limited simply to covalent interactions. The rapid reaction between NO and oxyhemoglobin to produce methemoglobin and nitrate ($k = 5 \times 10^7\ M^{-1}\ s^{-1}$; Eich *et al.,* 1996) is the primary endogenous mechanism by which NO diffusion and concentration are controlled (Doyle and Hoekstra, 1981; Feelisch, 1991; Lancaster, 1994).

$$\text{oxyHb } (Fe^{II}-O_2) + NO \rightarrow \text{metHb } (Fe^{III}) + NO_3^- \quad (1)$$

Nitric oxide also reacts rapidly with metal–oxo and metal–peroxo species (discussed in Wink and Mitchell, 1998; Wink *et al.,* 1999). These highly reactive metal complexes are formed from oxidation by agents such as hydrogen peroxide (H_2O_2) and can result in cellular damage through mechanisms such as lipid peroxidation (Puppo and Halliwell, 1988).

$$Fe^{II} + H_2O_2 \rightarrow Fe^{IV}{=}O + H_2O \quad (2)$$

The oxidative chemistry of these hypervalent metal complexes can be abated via reduction by NO to a normal valence (Gorbunov *et al.,* 1995; Kanner *et al.,* 1991; Wink *et al.,* 1994a).

$$Fe^{IV}{=}O + NO \rightarrow Fe^{II} + NO_2^- \quad (3)$$

Consumption of H_2O_2 by catalase is inhibited by cytokine stimulated hepatocytes and by synthetic NO donors (Kim *et al.,* 1995b; Wink *et al.,* 1996c). This reaction likely plays a role in the tumoricidal activity of stimulated macrophages (Farias-Eisner *et al.,* 1996). It is also of interest in this discussion, as catalase inhibition can occur through either metal nitrosyl formation or reaction with a metal–oxo species.

Nitric oxide binds to the catalase heme moiety to form a ferric nitrosyl complex with a rate constant of $3 \times 10^7\ M^{-1}\ s^{-1}$ and a K_{dis} of $1 \times 10^5\ M^{-1}$ (Hoshino *et al.,* 1993). In analogy to inhibition of P-450 and NOS (Fig. 3; see above), the Fe–NO adduct prevents binding of H_2O_2 to the metal ion by occupying the coordination site. It is estimated that 10–15 μM NO inhibits H_2O_2 consumption by 80% by this mechanism (Farias-Eisner *et al.,* 1996). Since cells that express iNOS have reduced catalase activity, local NO concentrations may be this high for prolonged periods of time.

In the enzymatic mechanism (Fig. 4), H_2O_2 first reacts with catalase to form complex I ($Fe^V{=}O$) and H_2O in a manner similar to reaction (2). The enzyme is then regenerated by further reaction with H_2O_2 to produce O_2 and H_2O. However, NO can also rapidly react with complex I to form complex II and NO_2^-. Complex II then reacts with an additional

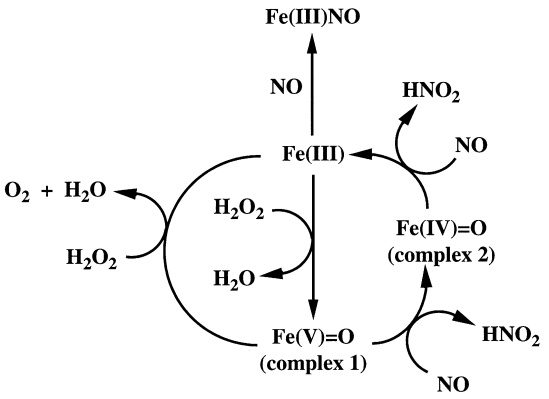


Figure 4 Interactions of nitric oxide with catalase.

NO to yield Fe^{3+} and NO_2^-. Thus, NO consumption retards H_2O_2 depletion with a K_i for NO of 0.18 μM (Brown, 1995a). This mechanism indicates that NO may inhibit H_2O_2 catabolism but, more importantly, that catalase may regulate NO concentration (Li *et al.,* 1992).

Reaction of NO with Radical Species

Lipid and carbon-centered radicals are formed as a result of both oxidative stress and normal metabolism. Nitric oxide can abate the effects these radicals have on biological systems. For instance, the catalytic turnover of ribonucleotide reductase produces a tyrosyl radical. Depletion of this radical by reaction with NO inhibits the enzyme (Kwon *et al.,* 1991; Lepoivre *et al.,* 1990, 1991, 1992). The resulting suppression of DNA synthesis is one of the deleterious (to the cell in question) functions of NO.

The interaction of NO with radicals can also have protective effects. Nitric oxide can react with oxyradicals formed during lipid peroxidation, which is an important component of the inflammatory process and cell death (Halliwell, 1991; Hogg *et al.,* 1993; Rubbo *et al.,* 1995). These oxyradicals convert lipids into a variety of lipid–oxy and lipid–peroxy adducts, which ultimately leads to cell membrane compromise through perpetuation of lipid oxidation (Fig. 5). Nitric oxide protects against reactive oxygen species (ROS) cytotoxicity by terminating lipid peroxidation (Gupta *et al.,* 1997; Padmaja and Huie, 1993; Wink *et al.,* 1994a, 1995).

$$LOO\cdot + NO \rightarrow LOONO \quad (4)$$

In addition, lipid peroxidation induced by oxidants formed as a result of exposure to copper, xanthine oxidase, or azobisamidinopropane is terminated by NO (Hogg *et al.,* 1995; Rubbo *et al.,* 1995). Chain termination can prevent oxidation of low density lipoprotein in both endothelial (Struck *et al.,* 1995) and macrophage cells (Hogg *et al.,* 1995). Reduction of oxidized cholesterol levels is thought to impede initiation of atherosclerosis mediated by activated foamy macrophages.

Other processes in inflammation, such as production of leukotrienes from arachodonic acid, are also affected by NO.

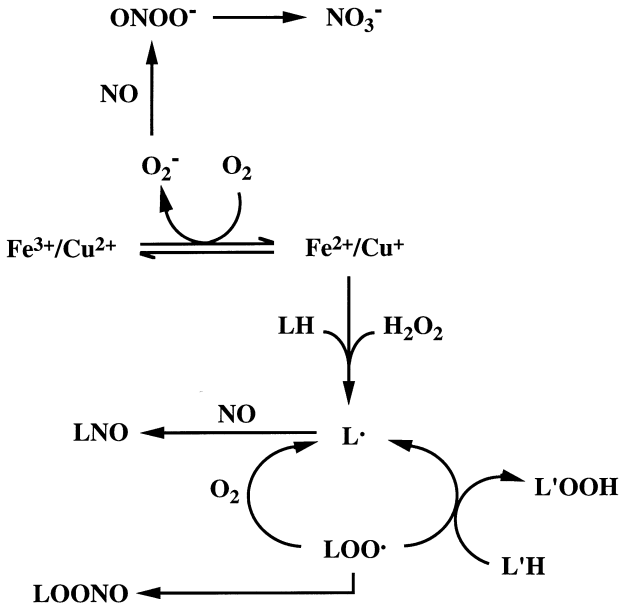


Figure 5 Chemistry of NO-mediated inhibition of lipid peroxidation.

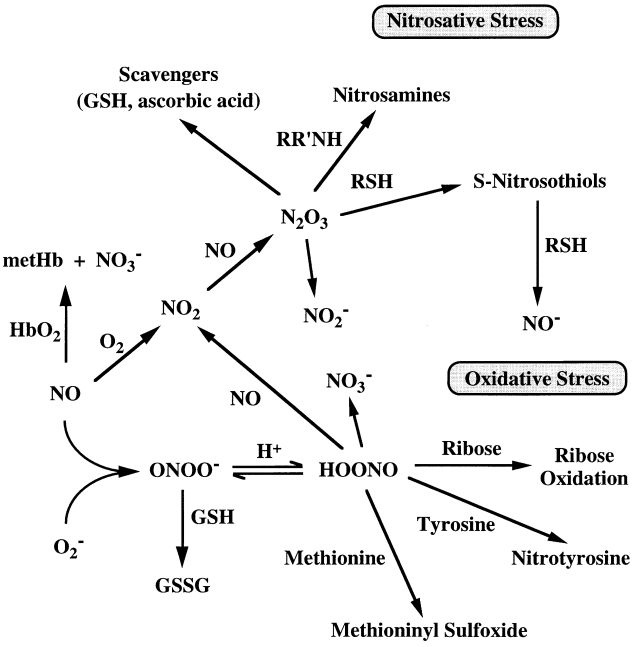


Figure 6 Chemistry of indirect effects.

Lipoxygenase, which mediates lipid oxidation, is inhibited by NO. At low concentrations, NO reacts with the lipid radicals directly, while at higher concentrations NO forms metal nitrosyl complexes that inhibit enzymatic activity. Therefore, arachodonic acid metabolism is influenced in a NO concentration-dependent manner.

Indirect Effects

The indirect effects of NO can be subdivided into the categories of nitrosation and oxidation. Nitrosation reactions primarily affect amines and thiols. Oxidation chemistry can result in the modification of a variety of macromolecules ranging from mild reducing agents such as catacholamines and metal centers to those with higher oxidation potentials such as DNA, proteins, and lipids. In biological systems, oxidation and nitrosation chemistry is a normal, constitutive component of cellular metabolism. However, these processes can also result in chemical intermediates that can stress the cell or produce toxic effects. In this instance the chemical reactions involved are referred to as nitrosative and oxidative stress. Under conditions of chemical stress, proteins and DNA can be irreversibly damaged. Thus, the indirect effects of NO are often associated with pathophysiological conditions, and higher nitrogen oxides are thought to be the chemical species responsible for the etiology of numerous diseases.

The biological properties of these species, principally N_2O_3, $ONOO^-$, NO^-, and NO_2, are chemically driven much the same as NO itself (Fig. 6). For instance, N_2O_3 is a relatively mild oxidant that modifies substrates with potentials of less than +0.7 V and thus does not affect biomolecules such as DNA. However, N_2O_3 will readily nitrosate nucleophiles and may be the principal nitrosating species *in vivo* (Wink *et al.,* 1996a). Conversely, the peroxynitrite and nitroxyl ions do not nitrosate substrates but can mediate oxidation of macromolecules (Miles *et al.,* 1996; Wink *et al.,* 1997; Wong *et al.,* 1998). A comparison of the resultant thiol products in the presence of various RNOS can illustrate this point. Aerobic NO solutions produce N_2O_3 and, when exposed to solutions of GSH, form the nitrosative product *S*-nitrosoglutathione (GSNO) nearly quantitatively (Wink *et al.,* 1994b). However, if GSH is exposed to $ONOO^-$, NO^-, or NO_2, the major products are oxidized, not nitrosated, thiols (Doyle *et al.,* 1988; Pryor *et al.,* 1982; Radi *et al.,* 1991; Wong *et al.,* 1998).

To further complicate the issue, NO can react with $ONOO^-$, NO^-, and NO_2 (Pryor *et al.,* 1982; Wink *et al.,* 1997). For instance, the reaction between NO and $ONOO^-$ produces N_2O_3 (Beckman *et al.,* 1994; Koppenol *et al.,* 1992; Miles *et al.,* 1996).

$$ONOO^- + 2\,NO \rightarrow N_2O_3 + NO_2^- \qquad (5)$$

This reaction facilitates thiol nitrosation at the expense of thiol oxidation and provides a means of balancing oxidative and nitrosative stress. In addition to these interactions with RNOS, NO can interact with ROS such as O_2^- to further abate oxidative chemistry. Therefore, it is not sufficient to consider a single reaction; one must contemplate many potential reactants and reactions. With this in mind, in the following sections we discuss the sources and conditions that might lead to nitrosative or oxidative stress, the chemical reactions that are responsible for both types of stress, and the likely biological targets.

Nitrosative Stress

The study of nitrosation chemistry dates back to the turn of the twentieth century (reviewed in Williams, 1988). In the mid 1970s nitrosation of amines derived from NO_2^- metabolism in the gastrointestinal tract became a concern as a potential source of carcinogens (Bartsch *et al.,* 1990). A decade later, the observations that nitrosamines and NO_2^- are produced as a result of infection or by activated leukocytes were critical links in the discovery of endogenous NO (Green *et al.,* 1981; Hibbs, 1991; Marletta, 1988; Stuehr and Marletta, 1985). *S*-Nitrosothiol (RSNO) formation has also been demonstrated to be physiologically relevant in processes ranging from cardiovascular function to cancer (Stamler, 1994; Wink *et al.,* 1998a).

Nitrosation is defined as the donation of a nitrosonium ion (NO^+) to a nucleophile, whereas nitrosylation is the formation of a nitrosyl adduct ($\cdot NO$) such as those between NO and metals as described earlier for direct effects. Both metals and RNOS mediate nitrosation, although to different extents. Transnitrosation between thiols may also affect protein function.

Metal-Mediated Nitrosation

Chemically, it is possible that metal-mediated nitrosation can occur. For example, iron containing compounds ranging from sodium nitroprusside to hemes can catalyze thiol and amine nitrosation. Generally, these compounds must be in the ferric state to transfer a nitrosonium ion such that substrates are reductively nitrosylated.

$$Fe^{III} \overset{NO}{\rightleftharpoons} Fe^{III}\text{–}NO \overset{RSH}{\rightleftharpoons} Fe^{II} + RSNO + H^+ \qquad (6)$$

Cobalamin, which forms a stable Co^{III}–NO complex under physiological conditions, has also been shown to nitrosate thiols (Brouwer *et al.,* 1996).

Under anaerobic conditions, ferrous nitrosyl hemes are quite stable. However, the corresponding ferric complexes require high NO concentrations for stability, as NO easily disassociates from the ferric ion. Additionally, heme- and iron-mediated nitrosation of amines requires an atmosphere of NO for days (Wade and Castro, 1990). These are conditions unlikely to be encountered *in vivo.* Metal-induced nitrosation also does not result in deamination of DNA or nitrosation of lysine groups.

Physiological transport of NO and the formation of *S*-nitrosothiols may occur through nonheme iron sulfur nitrosyl complexes such as those observed in activated macrophages. Under high fluxes of NO, formation of these complexes has been indicated by electron paramagnetic resonance (EPR) spectroscopy (Lancaster and Hibbs, 1990). Ferritin, aconitase, and even metallothionein are responsible for the EPR signal observed in activated macrophages. These observations imply that *S*-nitrosothiol formation may occur via these nonheme nitrosyl complexes.

Studies have been conducted to mimic the chemistry of these complexes. Nitrosation of thiols can be catalyzed by ferrous iron under anaerobic conditions. As this

$$Fe^{II} \overset{RSH}{\rightleftharpoons} Fe^{II}(RS)_2 \overset{NO}{\rightleftharpoons} Fe^{II}(RS)_2(NO)_2 \overset{NO}{\rightleftharpoons} Fe^{II} + 2\ RSNO \qquad (7)$$

chemistry is readily reversible, it is not likely to account for inactivation of enzymes in cells exposed to high NO concentrations but may produce a small amount of *S*-nitrosothiol. However, studies using different NO donors showed that enzymes such as DNA-repair enzymes are not inhibited by GSNO (Laval and Wink, 1994; Wink and Laval, 1994), and this chemistry [Eq. (7)] **cannot** be a mechanism for nitrosative stress. As a result, metal-mediated nitrosation has a minor role at best *in vivo.*

RNOS-Mediated Nitrosation

Generally, nitrosation *in vivo,* particularly of amines, is a result of RNOS formation. As described below, there are three potential sources for these RNOS (Fig. 7): the autoxidation of NO, the dehydration of acidic NO_2^-, and the NO/O_2^- reaction under an excess of NO. With the exception of the gastric regions (pH $<$ 1.5) where NO^+ can be formed from acidic NO_2^-, the primary nitrosating intermediates are isomers of N_2O_3.

The chemical reaction most noted for the formation of N_2O_3 is the autoxidation, or reaction with O_2, of NO. This reaction has been studied for decades due to its importance in nitrogen oxide chemistry in the atmosphere (Schwartz and White, 1983). In aqueous, hydrophobic, and gas phases, the autoxidation of NO has a third order rate equation with a second order dependence on NO.

$$2\ NO + O_2 \rightarrow 2\ NO_2 \quad -d[NO]/dt = k_{NO}[NO]^2[O_2] \qquad (8)$$

Due to the instability of NO in the presence of O_2 as well as its role in the formation of toxic chemical species such as NO_2 and N_2O_3, it was difficult to envision why nature would choose NO as a physiological mediator. However, the lifetime of NO, which is inversely proportional to its concentration, is dictated by the second order dependence of the autoxidation reaction [Eq. (8)] (Ford *et al.,* 1993; Wink *et al.,* 1993b). Therefore, as NO diffuses away from the cellular

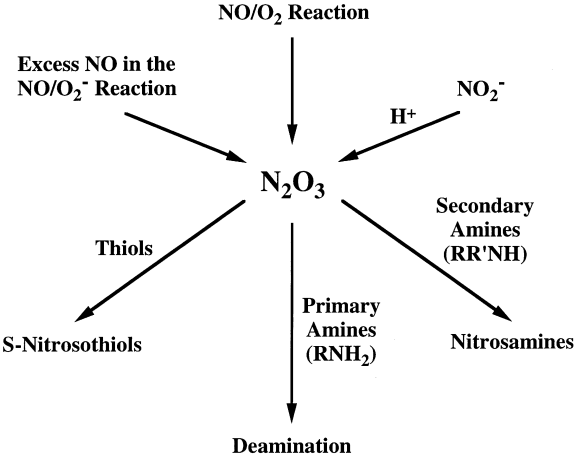


Figure 7 Nitrosative stress.

source and is thereby diluted, its lifetime increases sufficiently to allow it to react with other biological targets such as guanylate cyclase. It is only in local regions of high NO output where intermediates associated with NO autoxidation will be formed to a significant extent *in vivo*.

The rate constants for the autoxidation of NO in hydrophobic and aqueous solutions are similar ($k \approx 8 \times 10^6\ M^{-2}\ s^{-1}$; Ford *et al.,* 1993), whereas the concentrations of NO and O_2 are 10–50 times higher in lipid membranes than in aqueous solution (Denicola *et al.,* 1996). Consequently, the rate of autoxidation is dependent on the solubility of both NO and O_2 in a particular medium. Exposure to a NO flux from either a chemical donor or an *in vivo* source results in NO levels at least 10 times higher in membranes than in the surrounding medium and a corresponding elevation in the rate of autoxidation. In micelles this rate was demonstrated to increase 1500 times as compared to that in aqueous solution (Liu *et al.,* 1998). For this reason, nitrosation reactions mediated via the autoxidation reaction are likely to predominate in the membrane where proteins that are functionally and structurally dependent on thiols or amines would be most affected by nitrosative stress. However, thiol nitrosation is also an important pathway in membrane-associated protein chemistry.

PEPTIDE-MEDIATED NITROSATION

Small thiol peptides such as glutathione and cysteine can also be affected by nitrosative stress. In turn, these RSNO species can influence cellular metabolism and cardiovascular function by transnitrosating (transfer of NO^+) other reduced thiols (Stamler, 1994). This has been proposed as a mechanism by which NO can be transported through biological systems. The interaction of *S*-nitrosothiol peptides and reduced thiols can also result in reductive elimination of NO^- (Arnelle and Stamler, 1995). In addition, GSNO can react with various iron (Vanin, 1995) and copper complexes (Gorren *et al.,* 1996; Williams, 1996) and with O_2^- (Aleryani *et al.,* 1998; Jourd'heuil *et al.,* 1998; Trujillo *et al.,* 1998).

Low-molecular-weight thiols for the most part are less stable than proteins. For instance, CysNO in biological solutions has a shorter half-life than GSNO, which in turn has a shorter lifetime than *S*-nitrosothiol proteins (Feelisch and Stamler, 1996). Therefore, CysNO will typically transfer NO^+ to higher molecular weight thiols more quickly than GSNO. Zinc finger proteins are exceptions, as they do not react with RSNOs (Wink and Laval, 1994). However, as transnitrosation is often an equilibrium reaction in which the products are thermodynamically controlled, proteins such as alkyltransferases are not irreversibly inhibited by GSNO and CysNO (Laval and Wink, 1994). Inactivation of these proteins requires RNOS mediated nitrosation.

MECHANISM OF N_2O_3 FORMATION

The above sections demonstrate that nitrosative stress is primarily driven by N_2O_3. The mechanisms by which N_2O_3 is formed are crucial to the chemistry of nitrosative stress. The autoxidation of NO in either the gas phase or in hydrophobic solvents initially produces NO_2 [Eq. (8)]. Further reaction between NO and NO_2 results in an equilibrium with N_2O_3.

$$NO + NO_2 \rightleftharpoons N_2O_3 \tag{9}$$

In aqueous solution, competition reactions showed that NO_2 could not be trapped during the autoxidation of NO (Wink and Ford, 1995; Wink *et al.,* 1993b). These data suggest that dimerization to N_2O_4 occurs before NO_2 can escape the solvent cage. Under these conditions, formation of N_2O_3 takes place by Eq. (10) rather than Eq. (9).

$$2\ NO + N_2O_4 \rightleftharpoons 2\ N_2O_3 \tag{10}$$

Subsequently, N_2O_3 rapidly converts to nitrite in the absence of substrate.

$$N_2O_3 + H_2O \rightarrow 2\ H^+ + 2\ NO_2^- \tag{11}$$

Whether autoxidation takes place in hydrophobic/gas phase or in aqueous media affects the subsequent RNOS chemistry. For example, exposure of tyrosine to RNOS synthesized in the gas phase or from acidified nitrite solutions resulted exclusively in nitrotyrosine formation (Wink *et al.,* 1994b). However, this product was not observed as a result of the autoxidation reaction in H_2O. These findings suggest that nitrotyrosine formation by NO autoxidation is more likely to occur in hydrophobic environments such as membranes than in the cytosol.

Since N_2O_3 is hydrolyzed to NO_2^- with a half-life of 1 ms in aqueous media, only substrates that are present in high enough concentrations and have sufficient affinity will react with N_2O_3 (Wink *et al.,* 1996d). Thiol-containing peptides have an affinity for N_2O_3 that is 1000 times greater than any other amino acid at neutral pH. The primary products of the NO/O_2 reaction in aqueous solutions or biological media will likely then be *S*-nitrosothiols and nitrite (Wink *et al.,* 1994b, 1996d).

$$N_2O_3 + RSH \rightarrow RSNO + H^+ + NO_2^- \tag{12}$$

Oxidative Stress

Oxidation, or the removal of electrons from a substrate, occurs as part of normal metabolism. However, there is a significant difference between this cellular redox chemistry and that associated with oxidative stress. Under conditions of oxidative stress, powerful oxidizing agents abnormally modify biomolecules affecting their function and severely impacting the health status of cells or tissue. For example, oxidation of DNA can lead to strand breaks (Halliwell and Gutteridge, 1984). Unabated oxidation of lipids and proteins is associated with the onset of numerous pathophysiological conditions, and chronic oxidative stress is involved in the etiology of many disease states.

RNOS-mediated oxidative stress is induced primarily by NO_2, $ONOO^-$, and NO^-. The autoxidation of NO and the dehydration of acidic NO_2^- can form NO_2, while both NO_2 and $ONOO^-$ can be produced by the reaction between NO

and O_2^-. Interestingly, these are the same pathways that can lead to nitrosative stress. This unequivocally demonstrates that a dynamic equilibrium exists between oxidative and nitrosative reactions during periods of cellular stress.

NO_2-Mediated Oxidative Stress

The reactivity of NO_2 illustrates that this balance is controlled by the local NO concentration. The autoxidation of NO [Eq. (8)] is one mechanism by which NO_2 is produced. However as discussed earlier, this reaction occurs to a significant extent *in vivo* only in regions of high NO output. Under these conditions, NO_2, formed by any mechanism, will react with excess NO to yield N_2O_3 [Eq. (9)], thus stimulating nitrosative rather than oxidative stress. Conversely, the acidification of NO_2^- does not require free NO (Williams, 1988).

$$NO_2^- + H^+ \rightleftharpoons HNO_2 \quad (pK_a\, HNO_2 = 3.15) \qquad (13)$$

$$HNO_2 + H^+ \rightarrow H_2ONO^+ \qquad (14)$$

$$H_2ONO^+ + HNO_2 \rightarrow N_2O_3 + H_3O^+ \qquad (15)$$

At sufficiently low NO concentrations, the equilibrium in Eq. (9) will favor N_2O_3 disproportionation into NO_2 and NO. Although NO_2 does not appear to alter DNA (Routledge *et al.,* 1994a,b), this reaction can induce lipid peroxidation (Pryor, 1982).

Excess NO [Eq. (5)] or O_2^- [Eq. (16)] can react with $ONOO^-$ to form NO_2 (Beckman *et al.,* 1994; Koppenol *et al.,* 1992; Miles *et al.,* 1996).

$$ONOO^- + O_2^- + 2\,H^+ \rightarrow NO_2 + H_2O + O_2 \qquad (16)$$

This imparts a further balance between oxidative and nitrosative stress. However, the oxidative chemistry mediated by NO_2 *in vivo* is probably limited to regions of low pH or high (relative to NO) O_2^- concentrations.

NO/O_2^- Chemistry

The reaction between NO and O_2^- produces the powerful oxidant, $ONOO^-$ (Huie and Padmaja, 1993; Pryor and Squadrito, 1995).

$$NO + O_2^- \rightarrow ONOO^- \quad k = 6 \times 10^9\, M^{-1}\, s^{-1} \qquad (17)$$

The observation that superoxide dismutase (SOD) enhanced the effect of endothelial-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980) was followed by a large literature on its potential importance in biological systems (Beckman *et al.,* 1990). Although the rate constant for $ONOO^-$ formation approaches diffusion control, the relative pseudo first order rate constant is of equal importance in determining whether a reaction occurs *in vivo.* In other words, the impact a particular reaction will have *in vivo* is governed by the concentration of the reactants as much as by the rate constant (for a summary of evaluating RNOS, see Wink *et al.,* 1996d).

The estimated cellular concentrations of O_2^- and NO under normal conditions are 1 p*M* and 0.1–1 μ*M,* respectively (Tyler, 1975). Thus, the location and amount of $ONOO^-$ formation from reaction between these two radicals are likely to be controlled by O_2^- production. Since O_2^- reacts with SOD and NO with similar rate constants, $ONOO^-$ formation will also be dependent on the competing reaction of O_2^- with SOD. The intracellular concentration of SOD is thought to be between 4 and 10 μ*M,* while the mitochondrial enzyme, MnSOD, content is probably as high as 50 μ*M* (Nakano *et al.,* 1990). Therefore, in order for 10% of the available O_2^- to be converted to $ONOO^-$, the NO concentration would have to be 0.4–5 μ*M*. In addition, other reaction partners of O_2^- such as aconitase ($3 \times 10^7\, M^{-1}\, s^{-1}$) and ferricytochrome *c* ($5 \times 10^6\, M^{-1}\, s^{-1}$) could also abate $ONOO^-$ production, although to a lesser extent. Therefore, the reaction of O_2^- with NO will be constrained to specific sites (Fig. 8).

In neutral solution, $ONOO^-$ has been shown to be a potent modifier of biological compounds through oxidation of thiols, initiation of lipid peroxidation, nitration of tyrosine, cleavage of DNA, and nitration or oxidation of guanosine and methionine. The modifying species is an excited state of peroxynitrous acid (HOONO*) (Koppenol *et al.,* 1992). In the absence of adequate substrate, HOONO* simply rearranges to NO_3^- (Fig. 9). This isomerization can be considered as a detoxification pathway. However, in high enough concentrations, substrates such as tyrosine can react with HOONO* (1 m*M* to yield 50% nitrotyrosine).

The direct interactions between metals and $ONOO^-$ can catalyze substrate modifications. For example, the metals in SOD and FeEDTA enhance nitration reactions (Beckman *et al.,* 1992). Heme containing enzymes such as myeloperoxidase ($k = 6 \times 10^6\, M^{-1}\, s^{-1}$) and lactoperoxidase ($k = 3.3 \times 10^5\, M^{-1}\, s^{-1}$) also react with $ONOO^-$ (Floris *et al.,* 1993) such that complex II is formed (Fig. 4). In contrast, horseradish peroxidase ($k = 3.2 \times 10^6\, M^{-1}\, s^{-1}$) is converted to complex I by $ONOO^-$. Floris *et al.* proposed an interesting mechanism by which complex I is initially formed and then

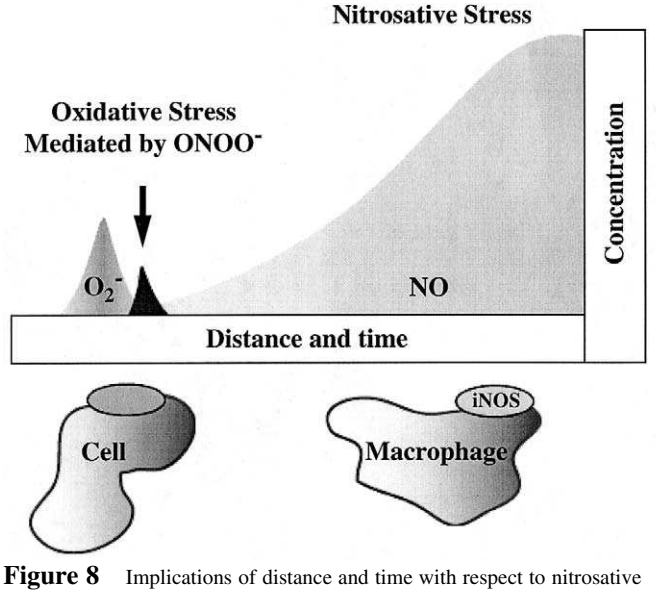


Figure 8 Implications of distance and time with respect to nitrosative and oxidative stress in the NO/O^{2-} reaction.

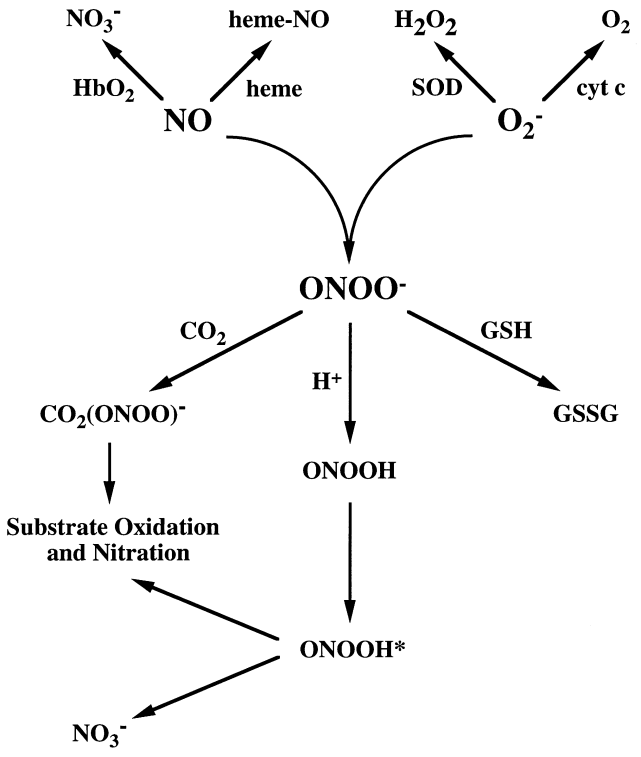


Figure 9 Chemistry of formation and reactivity of peroxynitrite.

rapidly oxidizes NO_2^- to NO_2. In the presence of NO, a number of nitrosation reactions would subsequently be facilitated via N_2O_3 [Eq. (9)]

As $ONOO^-$ reacts with excess NO and O_2^- [Eqs. (5) and (16)], the effects of variance in the reactant fluxes on the balance between nitrosative and oxidative chemistry were examined using NO donors and xanthine oxidase (XO) (Jourd'heuil *et al.,* 1999; Miles *et al.,* 1996; Wink *et al.,* 1997). Xanthine oxidase is considered to be a model for oxidative stress and generates the ROS, O_2^- and H_2O_2. The oxidation of hypoxanthine and the production of H_2O_2 from XO were unaffected by the presence of the NO releasing agent DEA/NO [$(C_2H_5)_2N[N(O)NO]^-Na^+$](Wink *et al.,* 1996c). However, the amount of O_2^- formed was dramatically reduced due to reaction with NO to form $ONOO^-$, which then isomerizes to NO_3^- (Fig. 9) (Clancy *et al.,* 1992; Miles *et al.,* 1995; Rubbo *et al.,* 1994; Wink *et al.,* 1993c). Addition of dihydrohodamine (DHR) to the reaction mixture resulted in an increase in substrate oxidation to rhodamine (Miles *et al.,* 1996). The hypothesis that $ONOO^-$ is a RNOS generated from the interaction of NO with O_2^- generated from XO is further supported by these data (Kooy *et al.,* 1994; Jourd'heuil *et al.,* 1999).

A crucial finding of this study is that maximal oxidation was only achieved at a 1:1 ratio of NO to O_2^-. When either radical was in excess, DHR oxidation was quenched due to conversion of $ONOO^-$ to NO_2 [Eqs. (5) and (16)] (Beckman *et al.,* 1994; Koppenol *et al.,* 1992; Miles *et al.,* 1996). Under conditions of excess NO, NO_2 is converted to the nitrosating species, N_2O_3 [Eq. (9)], illustrating an additional mechanism by which oxidative stress can be transformed to nitrosative stress by the action of NO (Fig. 8). This places further restrictions on the chemistry mediated directly by $ONOO^-$. We emphasize that the predominant factor is relative concentrations of the reactants involved in nitrogen oxide chemistry.

NO^--Mediated Oxidative Stress

The nitroxyl anion is a species with emerging importance in the understanding of nitrogen oxide biology. Nitroxyl can be formed as a result of several different processes. A primary source of NO^- is the nucleophilic attack of reduced thiols by RNOS to form RSNO, which can subsequently form NO^- and disulfide.

$$N_2O_3 + RSH \rightarrow RSNO + HNO_2 \quad (18)$$

$$RSNO + RSH \rightarrow RSSR + NO^- + H^+ \quad (19)$$

Decomposition of dithiothreitol-*S*-nitrosthiol to oxidized thiol can release NO^- by a similar mechanism (Arnelle and Stamler, 1995; Wink and Feelisch, 1996).

$$DTT{-}SNO \rightarrow DTT + NO^- \quad (20)$$

Nitroxyl has also been suggested to be formed from the decomposition of iron dinitrosyl complexes similar to those observed in tumor cells exposed to activated macrophages (Bonner and Pearsall, 1982). Last, NO^- may be derived from NOS (Hobbs *et al.,* 1994; Schmidt *et al.,* 1996). The mechanism may entail oxidation of hydroxyarginine, which is an intermediate in NOS activity (Pufahl *et al.,* 1995).

The synthesis of several substances that release NO^- has provided a method to study the effects of NO^- in biology (Feelisch and Stamler, 1996). Angeli's salt (AS; $Na_2N_2O_3$) releases NO^- and NO_2^- at neutral pH. Nitroxyl is isoelectric to O_2 and most frequently exists in either the triplet ground state or a singlet excited state. As the reactivity of a compound is in part controlled by its state, defining the state of AS is fundamental to understanding its chemistry. Nitroxyl is released from AS in the singlet state (Bonner and Stedman, 1996) and reacts with substrates, generally other singlets, more quickly than intersystem crossing (or state changes) to the ground state occurs.

In biological systems, NO^- can react by either an oxygen-independent or an oxygen-dependent pathway. Our preliminary results indicate that amines (e.g., hydroxylamine) and thiols rapidly react directly with NO^-. In addition, NO^- can react directly with NO, NADPH, and metalloproteins (e.g., SOD, myoglobin, and catalase) (Murphy and Sies, 1991; Wink *et al.,* 1998b; Miranda and Wink, 1999, personal observations). The NO^- reactions that require O_2 include hydroxylation and oxidation (e.g., methionine modification) and may figure prominently in NO^--mediated cytoxicity (Wink *et al.,* 1998b).

The toxicity of AS toward Chinese hamster V79 fibroblasts is two orders of magnitude greater than that of NO released from SIN-1 (3-morpholinosydnonimine) and the NONOate, DEA/NO, and comparable to that of H_2O_2 and

alkyhydroperoxide. Hypoxia abates NO^- toxicity, which demonstrates a requirement for reaction between NO^- and O_2. The lack of effect of metal chelators indicates that ROS produced via Fenton type reactions are not involved. A unique consequence of NO^--mediated cytotoxicity is the formation of DNA double strand breaks. This characteristic is not observed with either H_2O_2 or $ONOO^-$.

Although the reaction between NO^- and O_2 might be expected to give the same product as the reaction between NO and O_2^-, a preliminary analysis using several oxidative probes showed that $ONOO^-$ is not involved (Wink *et al.,* 1998b). Similar selectivity for two-electron oxidation of DHR to the fluorescent compound rhodamine was observed for NO^- from AS and for $ONOO^-$. However, the oxidative yield from AS was twice that for $ONOO^-$. Oxidation of DHR by AS was not quenched by azide, which indicates that N_2O_3 is not a component of NO^- chemistry. Peroxynitrite is very effective at nitrating and oxidizing (by one electron) hydroxyphenylacetic acid (HPA) but is a poor hydroxylating agent for benzoic acid. In contrast, AS is an efficient hydroxylating agent but fails to oxidize or nitrate HPA. Therefore, NO^- and O_2 produce an intermediate that is distinct from $ONOO^-$.

Characterization of this intermediate was undertaken. The stoichiometry of the NO^-/O_2 reaction was determined to be one to one (Wink *et al.,* 1998b). Singlet NO^- from AS decomposition in aqueous solution is proposed to react with H_2O (singlet ground state) to form an equilibrium with the hydrated species, H_2ONHO. This hydrated HNO adduct then could react with O_2 to form $(OH)_2$ NOO^- (Fig. 10). The intermediate decomposes to form NO_3^- and NO_2^- approximately equally. In the presence of HEPES, up to 25% H_2O_2 is also generated (Miranda and Wink, unpublished data, 1999). The intermediate, $(OH)_2NOO^-$, may be the species responsible for the oxidative chemistry and the cytotoxicity under aerobic conditions in cells. The inability of metal chelators to abate toxicity eliminates H_2O_2 as the toxic agent.

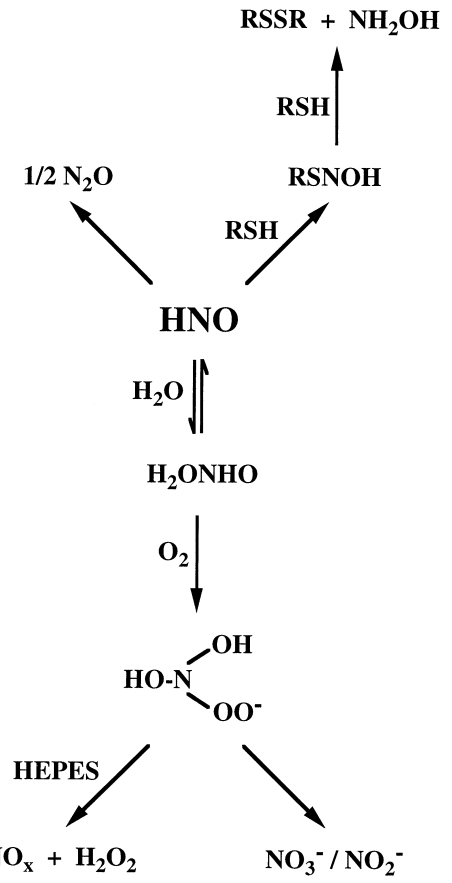


Figure 10 Possible mechanism for the oxidative chemistry of nitroxyl in the presence of oxygen.

Mixed Direct and Indirect Effects

When elucidating mechanisms under conditions that produce indirect effects, it is important to consider direct effects as well. One may envision direct effects occurring within the NO-generating cell, as well as within neighboring or distant cells, concurrently with indirect effects depending on the redox environment.

NO Inhibition of Mitochondrial Respiration

A primary cellular target for the cytotoxic action of NO is the mitochondrion (Hibbs *et al.,* 1987; Lancaster and Hibbs, 1990; Moncada *et al.,* 1991). Dinitrosyl adducts of aconitase were observed in activated macrophages by EPR (Lancaster and Hibbs, 1990), showing that iNOS catalyzed chemistry results in mitochondrial inhibition within the source cells and potentially causes cytostasis or cytotoxicity in target cells. However, the effects of NO can also be regulatory rather than deleterious.

Nitric oxide can repress oxidative phosphorylation in a reversible manner through regulation of intracellular calcium levels (Laffranchi *et al.,* 1995; Schweizer and Richter, 1994). Additionally, nitrosylation of cytochrome *c* oxidase reversibly inhibits respiration (Brown *et al.,* 1995; Brown, 1995b; Cassina and Radi, 1996; Cleeter *et al.,* 1994; Poderoso *et al.,* 1996; Lizasoain *et al.,* 1996; Rousseau *et al.,* 1988). The enzyme reverts to the active state when the bound NO is reduced to nitrogenous products by electrons from the respiratory chain (Borutaite and Brown, 1996; Clarkson *et al.,* 1995). Under aerobic conditions, O_2^- is formed when the electrons are diverted from the Fe–NO adduct to the O_2 binding site (Fig. 11) (Poderoso *et al.,* 1996). This O_2-dependent partitioning between the reduction of NO and O_2 results in respiration modulation by low levels of NO. The interesting possibility of intraorganelle regulation of tissue O_2 levels is raised by demonstration of an eNOS-like isoform in mitochondria (Bates *et al.,* 1996).

Similar to the mechanism described earlier for cytochrome P-450 (Fig. 3), inhibition of mitochondrial respiration appears to have a reversible component mediated by direct effects and an irreversible component mediated by indirect effects. Deciphering which oxidative and nitrosative

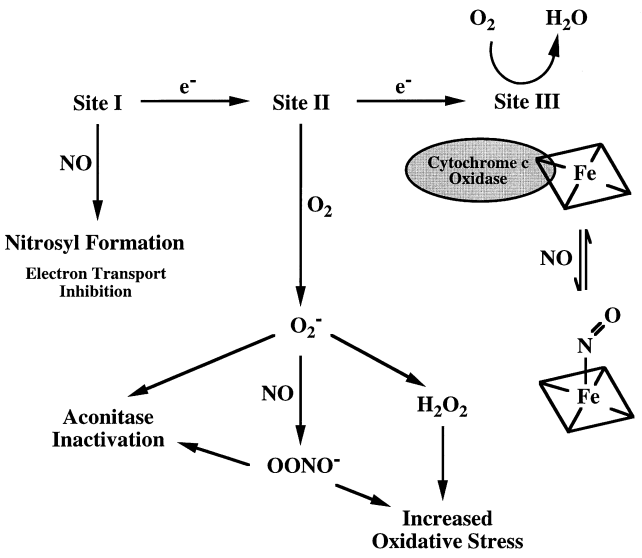


Figure 11 Nitric oxide and mitochondrial function.

mediators are involved in the pathogenic inhibition of mitochondrial functions is key to understanding disease mechanisms. RNOS may play a role in irreversible inhibition of complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) (Cassina and Radi, 1996) under inflammatory conditions in which cells can experience prolonged exposure to high levels of NO. As NO concentrations and time of exposure increase, there are corresponding increases in RNOS formed in the mitochondria (Fig. 8). Under aerobic conditions, O_2^- is a product of respiration and can react with NO to form $ONOO^-$. A significant role for $ONOO^-$ in mitochondria has been suggested. Both GSH and glucose prevented inhibition of mitochondrial respiration by $ONOO^-$, which suggests that this species is unlikely to directly inhibit respiration in cells unless it is formed within the mitochondria (Lizasoain *et al.,* 1996). However, the high concentration of mitochondrial MnSOD limits $ONOO^-$ formation. The amount of NO required to form $ONOO^-$ results in higher NO fluxes than O_2^- fluxes, favoring the conversion of $ONOO^-$ to N_2O_3 [Eqs. (5) and (9)]. These conditions indicate that the oxidative chemistry mediated by $ONOO^-$ probably does not play a significant role in mitochondrial function or dysfunction. Rather other RNOS such as NO_2 and N_2O_3 are likely responsible for irreversible inhibition of cellular respiration, and nitrosative, not oxidative, chemistry would be the predominant indirect effect in mitochondria under high NO fluxes.

The body may have several protective mechanisms to limit the indirect effects of RNOS on mitochondria. Inhibition of respiration was not observed in cells isolated from sites of experimentally induced inflammation *in vivo* (Fisch *et al.,* 1996; Stadler *et al.,* 1991). This may suggest that oxyhemoglobin and diffusion of NO away from NOS containing cells play important roles in the extent of mitochondrial inhibition where RNOS formation is limited and reversible inhibition is only transient.

Metal Homeostatics

Nitric oxide can regulate intracellular iron status under either physiological or pathophysiological conditions (see reviews in Drapier and Bouton, 1996; Hentze and Kuhn, 1996). Ferrochelatase is the enzyme that incorporates iron into the porphyrin center. Nitric oxide can substantially inhibit this process by direct interaction with the Fe–S complex while increasing the activity of the heme catabolic enzyme, heme oxygenase (Kim *et al.,* 1995a). These actions reduce the overall heme availability including that for the heme domain of NOS, which may serve as a negative feedback mechanism for NO formation.

Nitric oxide interacts with iron responsive-binding proteins (IRB) to regulate the uptake and storage of cellular iron. As their names imply, IRBs regulate the translation of proteins containing iron responsive element (IRE) motifs in their mRNA such as transferrin receptor and ferritin (Drapier and Bouton, 1996; Hentze and Kuhn, 1996; Klausner *et al.,* 1993). Interestingly, the mitochondrial enzyme aconitase acts as an IRB in the cytoplasm. The iron sulfur cluster within this IRB has two forms, apoprotein (Fe_3S_4) and holoprotein (Fe_4S_4), with the fourth iron in the apical position. The loss of iron at this site signals an overall cellular requirement to increase iron levels. Binding of IRB apoproteins to their respective IREs results in upregulation of transferrin receptor and downregulation of ferritin production (Klausner *et al.,* 1993). Conversely, the intact Fe–S cluster in IRBs leads to a reduction of iron uptake.

Nitrosylation of the apical iron results in Fe−NO expulsion to give the Fe_3S_4 cluster. This direct effect leads to inhibition of aconitase activity and to IRB binding to the IRE (Fig. 12). Although $ONOO^-$ and O_2^- also inhibit aconitase activity, they decrease the ability of IRB to bind to the IRE (Castro *et al.,* 1994; Hausladen and Fridovich, 1994). Oxidative inactivation of aconitase by either $ONOO^-$ or O_2^- may limit iron availability as a *protective* mechanism against Haber–Weiss chemistry. Iron is released from ferritin by an O_2-mediated mechanism involving NADPH oxidase. Through direct inhibition of NADPH oxidase assembly (Clancy *et al.,* 1992), NO can decrease the availability of ferrous ion. In addition, the indirect effect of O_2^- scavenging by NO inhibits the reduction of ferritin bound iron. These examples illustrate the complex checks and balances that have evolved in the regulation of basal metabolism and in protection against oxidative stress by NO.

Conclusion

The chemical biology of NO outlined in this chapter is meant to provide a guide for investigation of the cellular and molecular aspects of NO. The importance of concentration and timing with ROS cannot be overstated. Direct effects predominate in normal physiology, however, indirect effects can govern the characteristics of NO during pathology. With stoichiometric, temporal, and spatial considerations, the po-

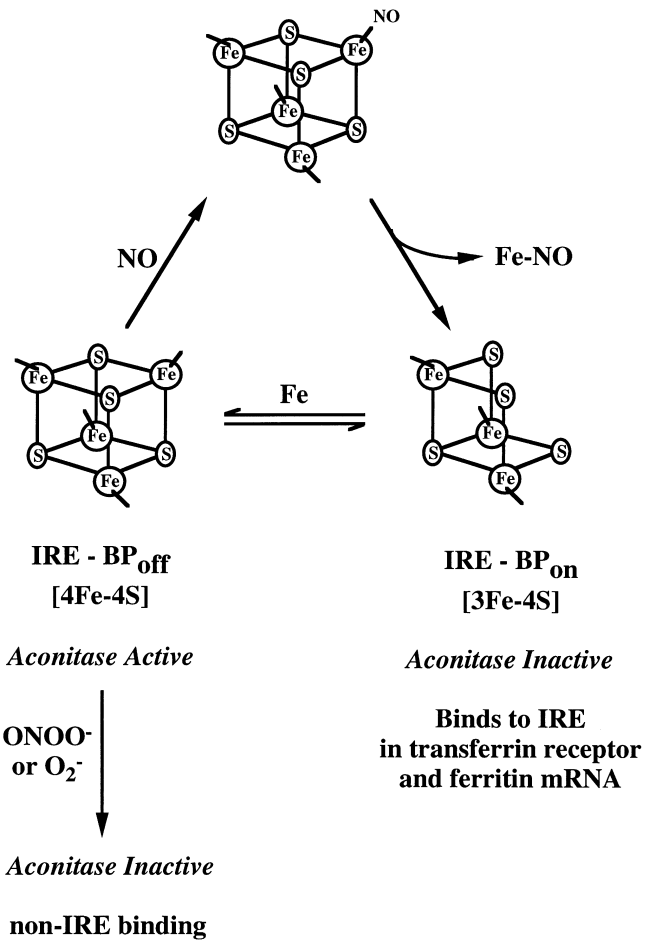


Figure 12 Nitric oxide and aconitase.

tential reactions of NO can be placed in proper context for biological systems.

References

Abu-Soud, H. M., Wang, J., Rousseau, D. L., Fukuto, J. M., Ignarro, L. J., and Stuehr, D. J. (1995). Neuronal nitric oxide synthase self-inactivates by forming a ferrous–nitrosyl complex during aerobic catalysis. *J. Biol. Chem.* **270,** 22997–23006.

Abu-Soud, H. M., Rousseau, D. L., and Stuehr, D. J. (1996). Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. *J. Biol. Chem.* **271,** 32515–32518.

Adams, M. L., Nock, B., Truong, R., and Cicero, T. J. (1992). Nitric oxide control of steroidogenesis: endocrine effects of N^G-nitro-L-arginine and comparisons to alcohol. *Life Sci.* **50,** PL35–PL40.

Aleryani, S, Milo, E., Rose, Y., and Kostka, P. (1998). Superoxide-mediated decomposition of biological *S*-nitrosothiols. *J. Biol. Chem.* **273,** 6041–6045.

Arnelle, D. R., and Stamler, J. S. (1995). NO^+, NO, and NO^- donation by *S*-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch. Biochem. Biophys.* **318,** 279–285.

Bartsch, H., Ohshima, H., Shuker, D. E. G., Pignatelli, B., and Calmels, S. (1990). Exposure of humans to endogenous *N*-nitroso compounds: Implications in cancer etiology. *Mutat. Res.* **238,** 255–267.

Bates, T. E., Loesch, A., Burnstock, G., and Clark, J. B. (1996). Mitochondrial nitric oxide synthase: A ubiquitous regulator of oxidative phosphorylation? *Biochem. Biophys. Res. Commun.* **218,** 40–44.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992). Kinetics of superoxide dismutase and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch. Biochem. Biophys.* **298,** 438–445.

Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994). Oxidative chemistry of peroxynitrite. *Methods Enzymol.* **233,** 229–240.

Bonner, F. T., and Pearsall, K. A. (1982). Aqueous nitrosyliron(II) chemistry. 1. Reduction of nitrite and nitric oxide by iron(II) and (trioxodinitrato)iron(II) in acetate buffer. Intermediacy of nitrosyl hydride. *Inorg. Chem.* **21,** 1973–1978.

Bonner, F. T., and Stedman, G. (1996). The chemistry of nitric oxide and redox-related species. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. Stamler, eds.), pp. 3–18. Wiley, New York.

Borutaite, V., and Brown, G. C. (1996). Rapid reduction of nitric oxide by mitochondria and reversible inhibition of mitochondrial respiration by nitric oxide. *Biochem. J.* **315,** 295–299.

Bouton, C., Raveau, M., and Drapier, J.-C. (1996). Modulation of iron regulatory protein functions. Further roles of nitrogen and oxygen-derived reactive species. *J. Biol. Chem.* **271,** 2300–2306.

Brouwer, M., Chamulitrat, W., Ferruzzi, G., Sauls, D. L., and Weinberg, J. B. (1996). Nitric oxide interactions with cobalamins: Biochemical and functional consequences. *Blood* **88,** 1857–1864.

Brown, G. C. (1995a). Reversible binding and inhibition of catalase by nitric oxide. *Eur. J. Biochem.* **232,** 188–191.

Brown, G. C. (1995b). Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett.* **369,** 136–139.

Brown, G. C., Bolanos, J. P., Heales, S. J. R., and Clark, J. B. (1995). Nitric oxide produced by activated astrocytes rapidly and reversibly inhibits cellular respiration. *Neurosci. Lett.* **193,** 201–204.

Cassina, A., and Radi, R. (1996). Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* **328,** 309–316.

Castro, L., Rodriguez, M., and Radi, R. (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.* **269,** 29409–29415.

Choi, A. M., and Alam, J. (1996). Heme oxygenase-1: Function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Respir. Cell. Mol. Biol.* **15,** 9–19.

Clancy, R. M. K., Leszczynska-Piziak, J., and Abramson, S. B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.* **90,** 1116–1121.

Clarkson, R. B., Norby, S. W., Smirnov, A., Vahdi, N., Boyer, S., Nims, R. W., and Wink, D. A. (1995). Direct measurement of the accumulation and mitochondrial conversion of nitric oxide within Chinese hamster ovary cells using an intracellular electron paramagnetic resonance technique. *Biochim. Biophys. Acta* **1243,** 496–502.

Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett.* **345,** 50–54.

Cotton, F. A., and Wilkinson, G. (1988). ''Advanced Inorganic Chemistry.'' Wiley, New York.

Dawson, T. M., Dawson, V. L., and Synder, S. H. (1992). A novel neuronal messenger molecule in brain: The free radical, nitric oxide. *Ann. Neurol.* **32,** 297–311.

Denicola, A., Souza, J. M., Radi, R., and Lissi, E. (1996). Nitric oxide diffusion in membranes determined by fluorescence quenching. *Arch. Biochem. Biophy.* **328,** 208–212.

Doyle, M. P., and Hoekstra, J. W. (1981). Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Biochem.* **14,** 351–358.

Doyle, M. P., Mahapatro, S. N., Broene, R. D., and Guy, J. K. (1988).

Oxidation and reduction of hemoproteins by trioxodinitrate(II). The role of nitrosyl hydride and nitrite. *J. Am. Chem. Soc.* **110,** 593–599.

Drapier, J.-C., and Bouton, C. (1996). Modulation by nitric oxide of metalloprotein regulatory activities. *BioEssays* **18,** 549–556.

Dweik, R. A., Laskowski, D., Abu-Soud, H. M., Kaneko, F. T., Hutte, R., Stuehr, D. J., and Erzurum, S. C. (1998). Nitric oxide synthesis in the lung. Regulation by oxygen through a kinetic mechanism. *J. Clin. Invest.* **101,** 660–666.

Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N. Jr., and Olson J. S. (1996). Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* **35,** 6976–6983.

Espey, M. G., Miranda, K. M., Feelisch, M., Fukuto, J., Grisham, M. B., Vitek, M. P., and Wink, D. A. (2000). Mechanisms of cell death governed by the balance between nitrosative and oxidative stress. *NY Acad. Sci* in press.

Estevez, A. G., Spear, N., Thompson, J. A., Cornwell, T. L.,Radi, R., Barbeito, L., and Beckman, J. S. (1998). Nitric oxide-dependent production of cGMP supports the survival of rat embryonic motor neurons cultured with brain-derived neurotrophic factor. *J. Neurosci.* **18,** 3708–3714.

Farias-Eisner, R., Chaudhuri, G., Aeberhard, E., and Fukuto, J. M. (1996). The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. *J. Biol. Chem.* **271,** 6144–6151.

Feelisch, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: Appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.* **17,** S25–S33.

Feelisch, M., and Stamler, J. S. (1996). Donors of nitrogen. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. Stamler, eds.), pp. 71–115. Wiley, New York.

Feldman, P. L., Griffith, O. W., and Stuehr, D. J. (1993). The surprising life of nitric oxide. *Chem. Eng. News* **Dec. 20,** 26–38.

Fisch, C., Robin, M.-A., Letteron, P., Fromenty, B., Berson, A., Renault, S., Chachaty, C., and Pessayre, D. (1996). Cell-generated nitric oxide inactivates rat hepatocyte mitochondria in vitro but reacts with hemoglobin in vivo. *Gastroenterology* **110,** 210–220.

Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993). Interaction of myeloperoxidase with peroxynitrite. A comparison with lactoperoxidase, horseradish preoxidase and catalase. *Eur. J. Biochem.* **215,** 767–775.

Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993). Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* **326,** 1–3.

Forstermann, U., and Ishii, K. (1996). Measurement of cyclic GMP as an indicator of nitric oxide production. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. Stamler, eds.), pp. 555–566. Wiley, New York.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Gorbunov, N. V., Osipov, A. N., Day, B. W., Zayas-Rivera, B., Kagan, V. E., and Elsayed, N. M. (1995). Reduction of ferrylmyoglobin and ferrylhemoglobin by nitric oxide: A protective mechanism against ferryl hemoprotein-induced oxidations. *Biochemistry* **34,** 6689–6699.

Gorren, A. C. F., Schrammel, A., Schmidt, K., and Mayer, B. (1996). Decomposition of *S*-nitrosoglutathione in the presence of copper ions and glutathione. *Arch. Biochem. Biophys.* **330,** 219–228.

Green, L. C., Tannenbaum, S. R., and Goldman, P. (1981). Nitrate synthesis in the germfree and conventional rat. *Science* **212,** 56–58.

Greenberg, S. S., Xie, J., Zatarain, J. M., Kapusta, D. R., and Miller, M. J. S. (1995). Hydroxocobalamin (vitamin B12a) prevents and reverses endotoxin-induced hypotension and mortality in rodents: Role of nitric oxide. *J. Pharmacol. Exp. Ther.* **273,** 257–265.

Griffith, O. W., and Stuehr, D. J. (1995). Nitric oxide synthases: Properties and catalytic mechanism. *Annu. Rev. Physiol.* **57,** 707–736.

Griscavage, J. M., Fukuto, J. M., Komori, Y., and Ignarro, L. J. (1994). Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. *J. Biol. Chem.* **269,** 21644–21649.

Griscavage, J. M., Hobbs, A. J., and Ignarro, L. J. (1995). Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. *Adv. Pharmacol.* **34,** 215–234.

Gross, S. S., and Wolin, M. S. (1995). Nitric oxide: Pathophysiological mechanisms. *Annu. Rev. Physiol.* **57,** 737–769.

Gupta, M. P., Evanoff, V., and Hart, C. M. (1997). Nitric oxide attenuates hydrogen peroxide-mediated injury to porcine pulmonary artery endothelial cells. *Am. J. Physiol.* **272,** L1133–L1141.

Halliwell, B. (1991). Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. *Am. J. Med.* **91,** 3C-14S–22S.

Halliwell, B., and Gutteridge, J. M. C. (1984). Oxygen toxicity, oxygen radicals, transition metals, and disease. *Biochem. J.* **219,** 1–14.

Hausladen, A., and Fridovich, I. (1994). Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.* **269,** 29405–29408.

Hentze, M. W., and Kuhn, L. C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8175–8182.

Hibbs, J. B., Jr.(1991). Synthesis of nitric oxide from L-arginine: A recently discovered pathway induced by cytokines with antitumour and antimicrobial activity. *Res. Immunol.* **142,** 565–569.

Hibbs, J. B., Jr., Vavrin, Z., and Taintor, R. R. (1987). L-arginine is required for the expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* **138,** 550–565.

Hobbs, A. J., Fukuto, J. M., and Ignarro, L. J. (1994). Formation of free nitric oxide from L-arginine by nitric oxide synthase: Direct enhancement of generation by superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 10992–10996.

Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., and Parthasarathy, S. (1993). Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett.* **334,** 170–174.

Hogg, N., Struck, A., Goss, S. P. A., Santanam, N., Joseph, J., Parthasarathy, S., and Kalyanaraman, B. (1995). Inhibition of macrophage-dependent low density lipoprotein oxidation by nitric-oxide donors. *J. Lipid Res.* **36,** 1756–1762.

Hoshino, M., Ozawa, K., Seki, H., and Ford, P. C. (1993). Photochemistry of nitric oxide adducts of water-soluble iron(III) porphyrin and ferrihemoproteins studied by nanosecond laser photolysis. *J. Am. Chem. Soc.* **115,** 9568–9575.

Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radical Res. Comms.* **18,** 195–199.

Hurshman, A. R., and Marletta, M. A. (1995). Nitric oxide complexes of inducible nitric oxide synthase: Spectral characterization and effect on catalytic activity. *Biochemistry* **34,** 5627–5634.

Ignarro, L. J. (1989). Endothelium-derived nitric oxide: Pharmacology and relationship to the actions of organic nitrate esters. *Pharm. Res.* **6,** 651–659.

Jourd'heuil, D., Mai, C. T., Laroux, F. S., Wink, D. A., and Grisham, M. B. (1998). The reaction of *S*-nitrosoglutathione with superoxide. *Biochem. Biophys. Res. Commun.* **244,** 525–30 .

Jourd'heuil, D., Miranda, K. M., Kim, S. M., Espey, M. G., Vodovotz, Y., Laroux, S., Mai, C. T., Miles, A. M., Grisham, M. B., and Wink, D. A. (1999). The oxidative and nitrosative chemistry of the nitric oxide/superoxide reaction in the presence of bicarbonate. *Arch. Biochem. Biophys.* **365,** 92–100.

Kanner, J., Harel, S., and Granit, R. (1991). Nitric oxide as an antioxidant. *Arch. Biochem. Biophys.* **289,** 130–136.

Khatsenko, O. G., Gross, S. S., Rifkind, A. B., and Vane, J. R. (1993). Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11147–11151.

Kim, Y.-M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D., and Lancaster, J. R., Jr. (1995a). Loss and degradation of enzyme-bound

heme induced by cellular nitroxide synthesis. *J. Biol. Chem.* **270,** 5710–5713.

Kim, Y.-M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D., and Lancaster, J. R. (1995b). Nitric oxide and intracellular heme. *Adv. Pharmacol.* **34,** 277–291.

Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993). Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell* **72,** 19–28.

Kooy, N. W., Royall, J. A., Ischiropoulos, H., and Beckman, J. S. (1994). Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radicals Biol. Med.* **16,** 149–156.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulus, H., and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5,** 834–842.

Kwon, N. S., Stuehr, D. J., and Nathan, C. F. (1991). Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.* **174,** 761–767.

Laffranchi, R., Gogvadze, V., Richter, C., and Spinas, G. A. (1995). Nitric oxide (nitrogen monoxide, NO) stimulates insulin secretion by inducing calcium release from mitochondria. *Biochem. Biophys. Res. Commun.* **217,** 584–591.

Lancaster, J. R., Jr. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8137–8141.

Lancaster, J. R., Jr., and Hibbs, J. B., Jr. (1990). EPR demonstration of iron–nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1223–1227.

Laval, F., and Wink, D. A. (1994). Inhibition by nitric oxide of the repair protein O^6-methylguanine-DNA-methyltransferase. *Carcinogenesis* **15,** 443–447.

Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelander, L., and Tenu, J.-P. (1990). Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. *J. Biol. Chem.* **265,** 14143–14149.

Lepoivre, M., Fieschi, F., Coves, J., Thelander, L., and Fontecave, M. (1991). Inactivation of ribonucleotide reductase by nitric oxide. *Biochem. Biophys. Res. Commun.* **179,** 442–448.

Lepoivre, M., Flaman, J.-M., and Henry, Y. (1992). Early loss of the tyrosyl radical in ribonucleotide reductase of adenocarcinoma cells producing nitric oxide. *J. Biol. Chem.* **267,** 22994–23000.

Lewis, R. S., Tamir, S., Tannenbaum, S. R., and Deen, W. H. (1995). Kinetic analysis of the fate of nitric oxide synthesized by macrophages in vitro. *J. Biol. Chem.* **270,** 29350–29355.

Li, Y., Severn, A., Rogers, M. V., Palmer, R. M. J., Moncada, S., and Liew, F. Y. (1992). Catalase inhibits nitric oxide synthesis and the killing of intracellular *Leishmania major* in murine macrophages. *Eur. J. Immunol.* **22,** 441–446.

Liu, X., Miller, M. J. S., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998). Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2175–2179.

Lizasoain, I., Moro, M. A., Knowles, R. G., Darley-Usmar, V., and Moncada, S. (1996). Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *Biochem. J.* **314,** 877–880.

MacMicking, J., Xie, Q., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.

Marletta, M. A. (1988). Mammalian synthesis of nitrite, nitrate, nitric oxide and N-nitrosating agents. *Chem. Res. Toxicol.* **1,** 249–257.

Miles, A. M., Gibson, M. F., Krishna, M., Cook, J. C., Pacelli, R., Wink, D., and Grisham, M. B. (1995). Effects of superoxide on nitric oxide-dependent N-nitrosation reactions. *Free Radical Res.* **23,** 379–390.

Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hansert, B., Wink, D. A., and Grisham, M. B. (1996). Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J. Biol. Chem.* **271,** 40–47.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Murad, F. (1994). The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. *Rec. Prog. in Horm. Res.* **49,** 239–248.

Murphy, M. E., and Sies, H. (1991). Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10860–10864.

Nakano, M., Kimura, H., Hara, M., Kuroiwa, M., Kato, M., Totsune, K., and Yoshikawa, T. (1990). A highly sensitive method for determining both Mn- and Cu-Zn superoxide dismutase activities in tissue and blood cells. *Anal. Biochem.* **187,** 277–280.

Nathan, C., and Xie, Q. (1994). Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* **269,** 13725–13728.

Padmaja, S., and Huie, R. E. (1993). The reaction of nitric oxide with organic peroxyl radicals. *Biochem. Biophys. Res. Commun.* **195,** 539–544.

Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996). Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch. Biochem. Biophys.* **328,** 85–92.

Pryor, W. A. (1982). ''Lipid Peroxides in Biology and Medicine'' (K. Yagi, ed.), pp. 1–22. Academic Press, New York.

Pryor, W. A., and Squadrito, G. L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Phys.* **268,** L699–L722.

Pryor, W. A., Church, D. F., Govindan, C. K., and Crank, G. (1982). Oxidation of thiols by nitric oxide and nitrogen dioxide: Synthetic utility and toxicological implications. *J. Org. Chem.* **47,** 156–159.

Pufahl, R. A., Wishnok, J. S., and Marletta, M. A. (1995). Hydrogen peroxide-supported oxidation of N^G-hydroxy-L-arginine by nitric oxide synthase. *Biochemistry* **34,** 1930–1941.

Puppo, A., and Halliwell, B. (1988). Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron: Is haemoglobin a biological Fenton reagent? *Biochem. J.* **249,** 185–190.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Rousseau, D. L., Singh, S., Ching, Y.-C., and Sassaroli, M. (1988). Nitrosyl cytochrome c oxidase. Formation and properties of mixed valence enzyme. *J. Biol. Chem.* **263,** 5681–5685.

Routledge, M. N., Mirsky, F. J., Wink, D. A., Keefer, L. K., and Dipple, A. (1994a). Nitrite-induced mutations in a forward mutation assay: Influence of nitrite concentration and pH. *Mutat. Res.* **322,** 341–346.

Routledge, M. N., Wink, D. A., Keefer, L. K., and Dipple, A. (1994b). DNA sequence changes induced by two nitric oxide donor drugs in the supF assay. *Chem. Res. Toxicol.* **7,** 628–632.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation: Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **269,** 26066–26075.

Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaraman, B., and Freeman, B. A. (1995). Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324,** 15–25.

Schmidt, H. H. H., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D., and Feelisch, M. (1996). No·NO from NO synthase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14492–14497.

Schwartz, S. E., and White, W. H. (1983). Kinetics of reactive dissolutions of nitrogen oxides into aqueous solution. *In* ''Trace Atmospheric Constituents, Properties, Transformation and Fates,'' Wiley, New York. (S. E. Schuartz, ed.), pp. 1–117.

Schweizer, M., and Richter, C. (1994). Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem. Biophys. Res. Commun.* **204,** 169–175.

Stadler, J., Billiar, T. R., Curran, R. D., Stuehr, D. J., Ochoa, J. B., and Simmons, R. L. (1991). Effect of exogenous and endogenous nitric

oxide on mitochondrial respiration of rat hepatocytes. *Am. J. Physiol.* **260,** C910–C916.

Stadler, J., Trockfeld, J., Shmalix, W. A., Brill, T., Siewert, J. R., Greim, H., and Doehmer, J. (1994). Inhibition of cytochrome P4501A by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 3559–3563.

Stamler, J. S. (1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78,** 931–936.

Stocker, R. (1990). Induction of haem oxygenase as a defense against oxidative stress. *Free Radicals Res. Commun.* **9,** 101–112.

Stone, J. R., and Marletta, M. A. (1994). Soluble guanylate cyclase from bovine lung: Activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric state. *Biochemistry* **33,** 5636–5640.

Struck, A. T., Hogg, N., Thomas, J. P., and Kalyanaraman, B. (1995). Nitric oxide donor compounds inhibit the toxicity of oxidized low-density lipoprotein to endothelial cells. *FEBS Lett.* **361,** 291–294.

Stuehr, D. J., and Marletta, M. A. (1985). Mammalian nitrate biosynthesis: Mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7738–7742.

Trujillo, M., Alvarez, M. N., Peluffo, G., Freeman, B. A., and Radi, R. (1998). Xanthine oxidase-mediated decomposition of *S*-nitrosothiols. *J. Biol. Chem.* **273,** 7828–7834.

Tyler, D. D. (1975). Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochem. J.* **147,** 493–504.

Vanin, A. F. (1995). Roles of iron ions and cysteine in formation and decomposition of *S*-nitrosocysteine and *S*-nitrosoglutathione. *Biochemistry (Moscow)* **80,** 441–447.

Wade, R. S., and Castro, C. E. (1990). Redox reactivity of iron(III) porphyrins and heme proteins with nitric oxide. Nitrosyl transfer to carbon, oxygen, nitrogen, and sulfur. *Chem. Res. Toxicol.* **3,** 289–291.

Williams, D. L. H. (1996). *S*-nitrosothiols and role of metal ions in decomposition to nitric oxide. *Methods Enzymol.* **268,** 299–308.

Williams, D. L. H. (1988). *In* ''Nitrosation.'' Cambridge Univ. Press, Oxford.

Wink, D. A., and Feelisch, M. (1996). Formation and detection of nitroxyl and nitrous oxide. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. Stamler, eds.), pp. 403–412. Wiley, New York.

Wink, D. A., and Ford, P. C. (1995). Nitric oxide reactions important to biological systems: A survey of some kinetics investigations. *Methods (San Diego)* **7,** 14–20.

Wink, D. A., and Laval, J. (1994). The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. *Carcinogenesis* **15,** 2125–2129.

Wink, D. A., Osawa, Y., Darbyshire, J. F., Jones, C. R., Eshenaur, S. C., and Nims, R. W. (1993a). Inhibition of cytochromes P450 by nitric oxide and a nitric oxide-releasing agent. *Arch. Biochem. Biophys.* **300,** 115–123.

Wink, D. A., and Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic and cytoprotective mechanisms of nitric oxide. *Free Radicals Biol. Med.* **25,** 434–456.

Wink, D. A., Darbyshire, J. F., Nims, R. W., Saveedra, J. E., and Ford, P. C. (1993b). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: Determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol.* **6,** 23–27.

Wink, D. A., Hanbauer, I., Krishna, M. C., DeGraff, W., Gamson, J., and Mitchell, J. B. (1993c). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9813–9817.

Wink, D. A., Hanbauer, I., Laval, F., Cook, J. A., Krishna, M. C., and Mitchell, J. B. (1994a). Nitric oxide protects against the cytotoxic effects of reactive oxygen species. *Ann. N.Y. Acad. Sci.* **738,** 265–278.

Wink, D. A., Nims, R. W., Darbyshire, J. F., Christodoulou, D., Hanbauer, I., Cox, G. W., Laval, F., Laval, J., Cook, J. A., Krishna, M. C., DeGraff, W., and Mitchell, J. B. (1994b). Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol.* **7,** 519–525.

Wink, D. A., Cook, J. A., Krishna, M. C., Hanbauer, I., DeGraff, W., Gamson, J., and Mitchell, J. B. (1995). Nitric oxide protects against alkyl peroxide-mediated cytotoxicty: Further insights into the role nitric oxide plays in oxidative stress. *Arch. Biochem. Biophys.* **319,** 402–407.

Wink, D. A., Grisham, M. B., Mitchell, J. B., and Ford, P. C. (1996a). Direct and indirect effects of nitric oxide in chemical reactions relevant to biology. *Methods Enzynol.* **268,** 12–31.

Wink, D. A., Hanbauer, I., Grisham, M. B., Laval, F., Nims, R. W., Laval, J., Cook, J., Pacelli, R., Liebmann, J., Krishna, M., Ford, P. C., and Mitchell, J. B. (1996b). Chemical biology of nitric oxide. Regulation and protective and toxic mechanisms. *Curr. Top. Cell. Regul.* **34,** 159–187.

Wink, D. A., Cook, J. A., Pacelli, R., DeGraff, W., Gamson, J., Liebmann, J., Krishna, M. C., and Mitchell, J. B. (1996c). Effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity. A direct correlation between nitric oxide formation and protection. *Arch. Biochem. Biophys.* **331,** 241–248.

Wink, D. A., Grisham, M. B., Miles, A. M., Nims, R. W., Krishna, M. C., Pacelli, R., Teague, D., Poore, C. M. B., Cook, J. A., and Ford, P. C. (1996d). Determination of selectivity of reactive nitrogen oxide species for various substrates. *Methods Enzynol.* **268,** 120–130.

Wink, D. A., Feelisch, M., Vodovotz, Y., Fukuto, J., and Grisham, M. B. (1999). The chemical biology of nitric oxide. *In* ''Reactive Oxygen Species in Biological Systems.'' (D. L. Gilbert and C. A. Colton, eds.), pp. 245–291. Kluwer Academic/Plenum Publishers, New York.

Wink, D. A., Cook, J. A., Kim, S. Y., Vodovotz, Y., Pacelli, R., Krishna, M. C., Russo, A., Mitchell, J. B. Jourd'heuil, D., Miles, A. M., and Grisham, M. B. (1997). Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide-derived reactive intermediates. *J. Biol. Chem.* **272,** 11147–11151.

Wink, D. A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M. W., and Mitchell, J. B. (1998a). The multifaceted roles of nitric oxide in cancer. *Carcinogenesis* **19,** 711–721.

Wink, D. A., Feelisch, M., Fukuto, J., Chistodoulou, D., Jourd'heuil, D., Grisham, M. B., Vodovotz, Y., Cook, J. A., Krishna, M., DeGraff, W. G., Kim, S., Gamson, J., and Mitchell, J. B. (1998b). The cytotoxicity of nitroxyl: Possible implications for the pathophysiological role of NO. *Arch. Biochem. Biophys.* **351,** 66–74.

Wong, P. S., Hyun, J., Fukuto, J. M., Shirota, F. N., DeMaster, E. G., Shoeman, D. W., and Nagasawa, H. T. (1998). Reaction between *S*-nitrosothiols and thiols: Generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry* **37,** 5362–5371.

Yu, A. E., Hu, S., Spiro, T. G., and Burstyn, J. N. (1994). Resonance Raman spectroscopy of soluble guanylyl cyclase reveals displacement of distal and proximal heme ligand by NO. *J. Am. Chem. Soc.* **116,** 4117–4118.

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CHAPTER 4

The Biological Chemistry of Peroxynitrite

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PEROXYNITRITE ANION ($ONOO^-$) IS FORMED *IN VIVO* AS A RESULT OF THE DIFFUSION-CONTROLLED REACTION BETWEEN NITRIC OXIDE ($\cdot NO$) AND SUPEROXIDE ANION ($O_2^{\cdot-}$) RADICALS. PEROXYNITRITE ANION AND ITS CONJUGATED ACID, PEROXYNITROUS ACID (ONOOH, $pK_a = 6.8$), ARE STRONG OXIDANT SPECIES THAT CAUSE MOLECULAR DAMAGE IN A VARIETY OF PATHOPHYSIOLOGICAL CONDITIONS. PEROXYNITRITE REACTS FAST ($k \simeq 10^3$–$10^6\ M^{-1}\ s^{-1}$) WITH A NUMBER OF BIOLOGICAL TARGETS, INCLUDING THIOLS, METALLOPROTEINS, AND CARBON DIOXIDE, OR MORE SLOWLY DECOMPOSES TO HYDROXYL ($\cdot OH$) AND NITROGEN DIOXIDE ($\cdot NO_2$) RADICALS BY PROTON-CATALYZED HOMOLYSIS ($k = 0.9\ s^{-1}$ AT PH 7.4 AND 37°C). CARBON DIOXIDE ACCOUNTS FOR A SIGNIFICANT FRACTION OF PEROXYNITRITE CONSUMPTION AND LEADS TO THE SECONDARY FORMATION OF CARBONATE ($CO_3^{\cdot-}$) AND NITROGEN DIOXIDE ($\cdot NO_2$) RADICALS. AT THE MOLECULAR LEVEL, THE PREDOMINANT OUTCOME OF PEROXYNITRITE REACTIONS *IN VIVO* IS ONE- OR TWO-ELECTRON OXIDATIONS AND NITRATIONS. PROTEIN TYROSINE NITRATION REPRESENTS A KEY FOOTPRINT OF THE BIOLOGICAL FORMATION AND REACTIONS OF PEROXYNITRITE, AND CAN ALSO DIRECTLY CONTRIBUTE TO PEROXYNITRITE-MEDIATED TOXICITY. WHEREAS TYROSINE NITRATION BY PEROXYNITRITE IS A RELATIVELY LOW-YIELD PROCESS (~6%), YIELDS ARE SIGNIFICANTLY ENHANCED BY TRANSITION METALS AND CARBON DIOXIDE. PEROXYNITRITE CAN DIFFUSE THROUGH TISSUE COMPARTMENTS, BEING ABLE TO CROSS BIOMEMBRANES BY BOTH PASSIVE DIFFUSION AND ANION CHANNELS. THUS, ALTHOUGH THE BIOLOGICAL HALF-LIFE OF PEROXYNITRITE IS SHORT (<20 MS), IT IS SUFFICIENT FOR PEROXYNITRITE TO DIFFUSE A COUPLE OF CELL DIAMETERS AND CAUSE BIOLOGICAL EFFECTS DISTANT FROM ITS SITE OF PRODUCTION. CELLULAR DYSFUNCTION BY PEROXYNITRITE RELIES ON A COMBINATION OF MECHANISMS, MOST NOTABLY MITROCHONDRIAL DAMAGE, WHICH MAY CONTRIBUTE TO THE INITIATION OF PEROXYNITRITE-MEDIATED APOPTOSIS. AT THE EXTRACELLULAR LEVEL, PEROXYNITRITE IS IMPLICATED IN THE ATHEROGENIC PROCESS VIA LOW-DENSITY LIPOPROTEIN OXIDATION OF CRITICAL AMINO ACID RESIDUES IN APO-B-100 AND BY INITIATING LIPID OXIDATION. PHARMACOLOGICAL DEVELOPMENT OF PEROXYNITRITE SCAVENGERS IS BASED ON OUR CURRENT KNOWLEDGE OF ITS REACTION CHEMISTRY AND BIOLOGY. HEREIN, WE PROVIDE A COMPREHENSIVE OVERVIEW OF THE PHYSICAL AND BIOLOGICAL CHEMISTRY OF PEROXYNITRITE. THE AIM IS TO PROVIDE A FOUNDATION TO RATIONALIZE THE BIOLOGICAL FATE AND ACTIONS OF PEROXYNITRITE AND THE STRATEGIES FOR PREVENTING PEROXYNITRITE-DEPENDENT BIOLOGICAL DAMAGE AND PATHOLOGY.

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Introduction

Excess production of the free radicals nitric oxide (·NO) superoxide ($O_2^{\cdot-}$) is related to cell and tissue pathology (Beckman and Koppenol, 1996; Freeman and Crapo, 1982; White *et al.,* 1994). Unraveling the mechanisms by which these moderately reactive radicals disrupt biomolecular structure and function has been challenging due to both their transient nature and the potential multiplicity of cellular and extracellular target molecules. Substantial progress was made when a hypothesis was elaborated in the early 1990s, proposing that the pathways of ·NO and $O_2^{\cdot-}$-dependent molecular damage can merge into a common route involving the formation of peroxynitrite anion ($ONOO^-$) (Beckman *et al.,* 1990; Radi *et al.,* 1991a,b).

After the identification of ·NO as a cellular messenger, the interaction of ·NO with $O_2^{\cdot-}$ became evident (Ignarro *et al.,* 1987; Palmer *et al.,* 1987). Early experiments showed that superoxide dismutase (SOD) prolonged the half-life and biological effects of the endothelium-derived relaxing factor (EDRF) (Gryglewski *et al.,* 1986; Rubanyi and Vanhoutte, 1986). Furthermore, a number of compounds described as inhibitors of EDRF were shown to act by generating $O_2^{\cdot-}$ in solution as a result of their redox properties, and SOD attenuated their actions on EDRF (Moncada *et al.,* 1986). Initially thought to represent a "scavenging" mechanism for ·NO leading to the formation of non-toxic nitrate, the reaction of $O_2^{\cdot-}$ with ·NO was later recognized to yield a reactive intermediate, $ONOO^-$, which could exert profound biological effects (Beckman, 1990; Beckman *et al.,* 1993; Radi *et al.,* 1991a).

The biological formation of peroxynitrite[1] has been demonstrated at cell and tissue levels under different pathophysiological conditions. Both $ONOO^-$ and its protonated form, peroxynitrous acid (ONOOH), are strong biological oxidants that can cause a variety of alterations on cell and tissue homeostasis. The interactions of peroxynitrite with cell components result in severe dysfunction and can ultimately lead to cell death (Denicola *et al.,* 1993; Estevez *et al.,* 1995; Lin *et al.,* 1995; Zhu *et al.,* 1992).

In this chapter we analyze the chemical and biological properties of peroxynitrite that make this molecule pathologically relevant. In addition, we aim to provide a sound biochemical background for the development of pharmacological interventions against the toxic actions of peroxynitrite *in vivo.*

Peroxynitrite Formation Pathways

Reaction of Nitric Oxide with Superoxide

The radical species ·NO and $O_2^{\cdot-}$ can be formed endogenously and can readily interact with each other to yield peroxynitrite.

$O_2^{\cdot-}$ is the one-electron reduction product of dioxygen. It can be formed through enzymes such as NADPH oxidase and xanthine oxidase, through the redox cycling of xenobiotics, via electron leakage in the mitochondrial respiratory chain, and at the endoplasmic reticulum (Freeman and Crapo, 1982; Turrens and Boveris, 1980). The main route of $O_2^{\cdot-}$ consumption in biological systems is the superoxide dismutase (SOD)-catalyzed dismutation to hydrogen peroxide (H_2O_2) and dioxygen. $O_2^{\cdot-}$ can also react with other targets such as iron sulfur clusters, transition metal centers, and sulfhydryls, but the rate constants of these reactions are $10^6\ M^{-1}\ s^{-1}$ or lower. Thus, due mainly to the preferential reaction with SOD, the steady state concentrations of $O_2^{\cdot-}$ are kept low, in the nanomolar to picomolar range (Chance *et al.,* 1979). However, the intracellular fluxes of $O_2^{\cdot-}$ are significant (exceeding micromolar per second) due to the high respiration rates of most cells, and it is estimated that around 1% of the dioxygen respired evolves to $O_2^{\cdot-}$. This value can increase severalfold under conditions of altered cellular homeostasis and inflammatory processes.

·NO in biological systems has a half-life of several seconds (Ignarro, 1990; Moncada *et al.,* 1991). Since its reaction with dioxygen is relatively slow, the consumption of ·NO is determined by its reactions with other targets such as transition metals, organic radicals, and $O_2^{\cdot-}$. ·NO will react with heme proteins such as the enzyme guanylate cyclase, responsible for the signaling effects of ·NO, and with oxyhemoglobin. The rate constants of these reactions are approximately $10^7\ M^{-1}\ s^{-1}$. ·NO may also react with organic radicals such as tyrosyl or peroxyl radicals with rate constants of $10^9\ M^{-1}\ s^{-1}$ (Eiserich *et al.,* 1995; Padmaja and Huie, 1993).

The reaction rate between ·NO and $O_2^{\cdot-}$ to form peroxynitrite is near to the diffusion-controlled limit [Eq. (1)].

$$\cdot NO + O_2^{\cdot-} \rightarrow ONOO^- \qquad v = k\,[\cdot NO]\,[O_2^{\cdot-}] \qquad (1)$$

The rate constant for reaction (1) has been reported as $6.7 \times 10^9\ M^{-1}\ s^{-1}$ (Huie and Padmaja, 1993) and $4.3 \times 10^9\ M^{-1}\ s^{-1}$ (Goldstein and Czapski, 1995a). A more recent redetermination with less potentially interfering secondary reactions reported a rate constant of $1.9 \times 10^{10}\ M^{-1}\ s^{-1}$ (Kissner *et al.,* 1997). Thus, it is safe to assume a k value of $10^{10}\ M^{-1}\ s^{-1}$ for this reaction, a value five times higher than that of Cu,Zn-SOD ($2 \times 10^9\ M^{-1}\ s^{-1}$). In fact, ·NO is the only biomolecule known to react fast enough and to be produced at sufficient concentrations to outcompete SOD for its reaction with $O_2^{\cdot-}$. It can be calculated that at physiological concentrations of SOD ($\sim 10\ \mu M$), a concentration of $2\ \mu M$ ·NO will react with about half of the $O_2^{\cdot-}$ formed, yielding peroxynitrite. Whereas the steady-state concentration of ·NO needed for exerting its messenger role is in the nanomolar range, in inflammatory sites ·NO can reach micromolar concentrations (Moncada *et al.,* 1991), allowing for peroxynitrite formation to be kinetically feasible. It is useful to recall that for every 10-fold increase in ·NO and $O_2^{\cdot-}$ concentration, a 100-fold increase in the rate of peroxynitrite formation should occur (Beckman, 1996).

[1] Throughout the text, the name peroxynitrite is used to refer to both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH). IUPAC recommends the names nitrogen monoxide for ·NO (nitric oxide), oxoperoxonitrate(1−) for $ONOO^-$ (peroxynitrite), and hydrogen oxoperoxonitrate for its conjugated acid, ONOOH (peroxynitrous acid).

Other Routes

NITRIC OXIDE SYNTHASE

The enzyme nitric oxide synthase produces ·NO. However, under arginine depletion, $O_2^{\cdot-}$ is concomitantly formed, promoting peroxynitrite formation, through the uncoupling of NADPH consumption from arginine oxidation. This process has been shown to occur both in isolated [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)] as well as in iNOS-containing macrophages and in nNOS transfected kidney cells (Vasquez-Vivar *et al.,* 1998; Xia *et al.,* 1996, 1998a,b; Xia and Zweier, 1997).

REACTION OF NITROXYL ANION WITH DIOXYGEN

Nitroxyl anion [NO^-, oxonitrate(1-)] can be formed through the one-electron reduction of ·NO [$E^{\circ\prime}(\cdot NO/NO^-) = 0.39$ V]. In turn, NO- can react with O_2, yielding peroxynitrite (Donald *et al.,* 1986).

$$NO^- + O_2 \rightarrow ONOO^- \qquad (2)$$

Since NO^- could be formed biologically (Hogg *et al.,* 1996; Wong *et al.,* 1998; Wink *et al.,* 1998), its reaction with dioxygen may represent a secondary route of peroxynitrite formation *in vivo.* Indeed, peroxynitrite formation was suggested after reduction of ·NO by mitochondrial ferrocytochrome *c* (Sharpe and Cooper, 1998a).

REACTIONS OF OXYMYOGLOBIN AND OXYHEMOGLOBIN WITH ·NO

The reaction of ·NO with oxymyoglobin or oxyhemoglobin [$k = 3 \times 10^7\ M^{-1}\ s^{-1}$ (Doyle and Hoekstra, 1981)] leads to nitrate. It has been proposed that this occurs through the formation of an intermediate peroxynitrite (Herold, 1999; Ignarro, 1990; Wade and Castro, 1996).

$$\cdot NO + [Hb^{2+}{-}O_2 \leftrightarrow Hb^{3+}{-}O_2^{\cdot-}] \rightarrow Hb^{3+}{-}OONO^- \rightarrow Hb^{3+} + NO_3^- \qquad (3)$$

Physicochemical Properties

Thermodynamic Properties

Thermochemical calculations on peroxynitrite are based on two experimentally determined values. First, the enthalpy of formation of peroxynitrite was determined as -10 ± 1 kcal mol^{-1} by calorimetric measurements of its decomposition to nitrate (-39 ± 1 kcal mol^{-1}) (Bohle *et al.,* 1994; Manuszak and Koppenol, 1996). Second, the enthalpy of peroxynitrous acid ionization was considered close to zero (Koppenol *et al.,* 1992) and recently was redetermined as 4 ± 2 kcal mol^{-1} (Koppenol and Kissner, 1998). In order to estimate the Gibbs energy of formation, the absolute entropy of peroxynitrite must be evaluated. Different authors have made discrepant estimations, leading to controversy regarding the possible pathways of peroxynitrite decomposition (Koppenol and Kissner, 1998; Koppenol *et al.,* 1992; Merenyi and Lind, 1997). Nevertheless, there is agreement that peroxynitrite is an oxidizing molecule, with estimated one- and two-electron reduction potentials of $E^{\circ\prime}(ONOOH, H^+/\cdot NO_2, H_2O) = 1.6–1.7$ V and $E^{\circ\prime}(ONOOH, H^+/NO_2^-, H_2O) = 1.3–1.37$ V (Koppenol and Kissner, 1998; Merenyi and Lind, 1997).

The activation parameters of peroxynitrite decomposition to nitrate were initially reported as 18 ± 1 kcal mol^{-1} for the activation enthalpy, 3 ± 2 cal $mol^{-1}\ K^{-1}$ for the activation entropy (Alvarez *et al.,* 1995; Koppenol *et al.,* 1992), and 1.7 ± 1.0 $cm^3\ mol^{-1}$ for the activation volume (Kissner *et al.,* 1997). More recently, values of 21.2 kcal/mol^{-1}, + 13 cal $mol^{-1}K^{-1}$, and +10 $cm^3\ mol^{-1}$, respectively, were reported (Goldstein *et al.,* 1999; Merenyi, *et al.,* 1999). These values have been discussed in terms of the mechanisms of peroxynitrite decomposition (Pryor and Squadrito, 1995).

Conformation

The possible conformations of peroxynitrite have been proposed based on quantum calculations and spectral data. According to the former, the bond between the nitrogen and the peroxidic oxygen has a partial double bond character. Around this bond, a *cis* and a *trans* conformer can be defined.

For peroxynitrite anion, the *cis* conformer would be more stable than the *trans* by 3–4 kcal mol^{-1}. For peroxynitrous acid, the *cis* would also be more stable, by 1–2 kcal mol^{-1} (Tsai *et al.,* 1996; Tsai *et al.,* 1994). There is no agreement on whether the rate of interconversion between the conformers is fast or slow, and the importance of these conformers for peroxynitrite chemistry is under debate (Pryor and Squadrito, 1995). Since the calculated barrier for interconversion between the *cis* and the *trans* anions would be 21–24 kcal mol^{-1}, and 10–12 kcal mol^{-1} lower for the acids, it has been proposed that $ONOO^-$ would predominate as the *cis* conformer; hydronation would enable it to switch to the more unstable *trans* and decompose (Tsai *et al.,* 1996; Tsai *et al.,* 1994). X-ray analysis suggests that peroxynitrite crystallized in the *cis* form (Worle *et al.,* 1999).

Spectral Properties

ULTRAVIOLET–VISIBLE SPECTROSCOPY

The absorption spectrum of $ONOO^-$ in the ultraviolet (UV) region consists of a single rather broad band centered at 302 nm in aqueous alkaline solution (Hughes and Nicklin, 1968). The maximum at 302 nm has been universally used to quantify peroxynitrite using the ϵ value reported by Hughes and Nicklin of $1670 \pm 50\ M^{-1}\ cm^{-1}$. More recently, Bohle *et al.*(1994) reported a more accurate value of $1700 \pm 10\ M^{-1}\ cm^{-1}$. Nevertheless, the former value is still the most widely used.

The UV absorption of $ONOO^-$ has been studied in detail in nonaqueous media such as solid argon matrixes at 12 K (Lo *et al.,* 1995), dichloromethane, or liquid ammonia (Bohle *et al.,* 1996). In these experiments it has been shown

that in non-hydrogen-bonding solvents and in solid $ONOO^-$ absorption is greatly red-shifted, indicating that peroxynitrite is greatly stabilized in aqueous solution through solvent interactions.

VIBRATIONAL SPECTRA

Infrared and Raman spectra of ONOOH and $ONOO^-$ have been obtained Cheng, 1991; Bohle *et al.,* 1994; Tsai *et al.,* 1994, #1053]. The bands are wide, suggesting interaction with water. However, the interpretation of vibrational spectra is not straightforward and the results do not clarify the issue of peroxynitrite conformers.

NUCLEAR MAGNETIC RESONANCE

^{15}N NMR experiments with peroxynitrite in basic aqueous solutions show the presence of only one peak at 191.4 ppm from nitrate (Bohle *et al.,* 1994; Tsai *et al.,* 1996). This was interpreted as indicative of only one conformer being present in solution or of rapid interconversion between the *cis* and *trans* conformers.

Acid–Base Properties

Peroxynitrous is a weak acid. Since it decays relatively fast, its ionization constant (K_a) cannot be measured directly. Thus, most reports on the pK_a of peroxynitrous acid are based on kinetic measurements of peroxynitrite decomposition in phosphate buffer (see following). There is agreement in the literature on a pK_a value of 6.8.

$$ONOOH \rightleftharpoons ONOO^- + H^+ \quad K_a = 1.58 \times 10^{-7}\, M^{-1} \quad (4)$$

At the physiological pH of 7.4, both $ONOO^-$ and ONOOH will be present, in proportions of 80 and 20%, respectively.

The decomposition of peroxynitrite can be affected by the concentration and the nature of the buffer. At low phosphate concentrations the pK_a can decrease from 6.8 to 6.5 (Kissner *et al.,* 1997), and in the presence of HEPES, CAPS, CAPSO, formate, borate, or ammonia the apparent pK_a increases to values near 8 (Beckman, 1996; Koppenol, 1998). Furthermore, reaction of peroxynitrite with the buffers can occur in some cases (Gadelha *et al.,* 1997).

In addition to the proton, other Lewis acids catalyze peroxynitrite decomposition, such as transition metals (Beckman *et al.,* 1992; Ferrer-Sueta *et al.,* 1997), aldehydes (Uppu *et al.,* 1997), and carbon dioxide (Denicola *et al.,* 1996; Lymar and Hurst, 1995). In the process, reactive intermediates are formed.

Biochemical Reactivity

Peroxynitrite formed in a biological system can have different possible fates. In fact, its high reduction potential indicates that it could oxidize a wide variety of biomolecules. Thus, in order to understand the effects of peroxynitrite formation in biological systems, it is important to characterize the kinetics of peroxynitrite reactions with biomolecules.

In contrast to extremely reactive biological oxidants such as the hydroxyl radical (·OH), whose high rate of reaction with most molecules ($>10^9\ M^{-1}\ s^{-1}$) determines that it will react nonspecifically and close to its site of formation, peroxynitrite reactions are relatively slow. Thus, peroxynitrite will be more selective in its target molecules and will be able to react relatively distant to its site of formation.

Approach to the Study of Peroxynitrite Reactivity

DIRECT STUDIES

Since peroxynitrite decomposition at neutral pH and 37°C occurs in less than 10 s, in order to study the kinetics of peroxynitrite reactions special techniques need to be used. The stopped-flow spectrophotometer has a fast mixing device that allows it to study reactions that occur in a time scale of milliseconds. Peroxynitrite absorption at 302 nm can be used to follow the reactions, and both integral or initial rate approaches can be designed (Alvarez *et al.,* 1999; Floris *et al.,* 1993; Radi *et al.,* 1991a).

COMPETITION STUDIES

When parallel reactions yield different products, it can be shown that the ratio of these products will be proportional to the relative rate of the reactions [Eqs. (5)–(7)].

$$ONOO^- + A_1 \xrightarrow{k_1} P_1 \quad (5)$$

$$ONOO^- + A_2 \xrightarrow{k_2} P_2 \quad (6)$$

$$\frac{[P_1]}{[P_2]} = \frac{k_1\,[A_1]}{k_2\,[A_2]} \quad (7)$$

Thus, the ratio of the rate constants can be deduced from the ratio of products formed. This approach has been used to determine the kinetics of peroxynitrite reaction with one target by measuring the amount of product formed in the presence of competing scavengers of known reactivity (Castro *et al.,* 1994; Souza and Radi, 1998).

For instance, to measure the rate constant of the reaction of an enzyme (E) with peroxynitrite, it is possible to determine the activity of the enzyme alone and exposed to peroxynitrite in the presence of increasing concentrations of a peroxynitrite scavenger. Defining F_I as the fraction of protection:

$$F_1 = \frac{A_S - A_P}{A_0 - A_P} \quad (8)$$

where A_0 is the enzymatic activity in the absence of peroxynitrite, A_P is in its presence, and A_S is the activity when a concentration [S] of scavenger was added, it can be shown that (Winterbourn, 1987)

$$\frac{F_1}{1 - F_1} = \frac{k_S[S]}{k_E[E]} \quad (9)$$

where k_S and k_E are the rate constants with the scavenger and with the enzyme, respectively. Rearranging the equation, plots of $[F_1/(1-F_1)](1/k_S)[E]$ versus [S] can be drawn. The slope of such plots represents $1/k_E$.

However, the competition approach should be used with caution. The scavengers present in excess may reduce a target molecule that was previously oxidized by peroxynitrite. Besides, secondary scavenger-derived reactive species may be formed and further react with the target. Finally, it is important to point out that at low target concentrations three processes will actually be competing. In addition to reacting with the target and with the scavenger, a significant fraction of peroxynitrite will decompose via the proton-catalyzed decomposition to nitrate, yielding reactive intermediates as described later.

Peroxynitrite Decomposition in the Absence of Targets

Peroxynitrite is relatively stable in alkaline solution. However, at neutral or acidic pH it decomposes fast. The absorbance at 302 nm then decreases following an exponential function (Radi *et al.,* 1991a). The rate constant of peroxynitrite decomposition at pH 7.4 is 0.26 s^{-1} at 25°C and 0.9 s^{-1} at 37°C. The rate of peroxynitrite decomposition increases at acidic pH with limiting rate constants of 1.3 and 4.5 at 25° and 37°C, respectively (Alvarez *et al.,* 1998; Koppenol *et al.,* 1992). From the half-maximum values, the equilibrium constant for peroxynitrous acid ionization was determined to be $pK_a = 6.8$ (Radi *et al.,* 1991a).

Keith and Powell (1969) proposed the following reaction scheme for the acid-catalyzed decomposition of peroxynitrite:

$$ONOOH \overset{K_a}{\rightleftharpoons} ONOO^- + H^+ \quad (10)$$

$$ONOOH \overset{k_{HA}}{\rightarrow} NO_3^- + H^+ \quad (11)$$

According to this scheme, the apparent rate constant of peroxynitrite decomposition (k) would follow the equation:

$$k = \frac{k_{HA}[H^+]}{K_a + [H^+]} \quad (12)$$

where k_{HA} is the pH-independent rate constant.

The final product of the spontaneous decomposition of peroxynitrite at acidic pH is nitrate (Anbar and Taube, 1954; Bohle and Hansert, 1997). However, at alkaline pH peroxynitrite also decomposes to nitrite with about 30% yield at pH 7.5, with the concomitant production of dioxygen (Pfeiffer *et al.,* 1997; Radi *et al.,* 1991b).

$$2\ ONOO^- \rightarrow 2\ NO_2^- + O_2 \quad (13)$$

Mechanism of Peroxynitrite Isomerization to Nitrate

There are two mechanistically feasible pathways by which peroxynitrous acid can isomerize to nitrate (Koppenol *et al.,* 1992). The first is homolysis to $\cdot NO_2$ and $\cdot OH$ followed by reaction of the radicals to form nitrate; the second is internal rearrangement of *trans*-peroxynitrous acid to nitric acid.

Homolysis of peroxynitrous acid has been the subject of debate. The proposal of potential formation of $\cdot OH$ from peroxynitrite (Beckman *et al.,* 1990; Radi *et al.,* 1991b) was received with interest because it provided a mechanism that was independent of transition metals for the formation of this extremely potent oxidant [$E^{\circ\prime}(\cdot OH, H^+/H_2O) = 2.3$ V] (Koppenol, 1989), whereas the mechanisms previously proposed depended on the interaction of hydrogen peroxide with free or protein-bound transition metals.

Homolysis would imply the initial formation of the radicals in a solvent cage (Pryor and Squadrito, 1995). The radicals would recombine inside the cage or diffuse. This proposal was raised after the fact that it was possible to trap only a fraction of peroxynitrite as $\cdot OH$ became known (caged radicals are untrappable by definition). Alternatively, it has been proposed that ONOOH rearranges into nitrate via the formation of a reactive intermediate, a vibrationally distorted *trans* peroxynitrous acid or a hydrogen-bonded radical pair ($OH^{\cdots}ONO$) (Houk *et al.,* 1996; Koppenol *et al.,* 1992).

Different approaches have been used to address the question of the pathway of peroxynitrite isomerization: thermochemical calculations (Koppenol and Kissner, 1998; Koppenol *et al.,* 1992; Merenyi and Lind, 1997), values of activation parameters for the isomerization reaction (Kissner *et al.,* 1997; Koppenol *et al.,* 1992; Goldstein *et al.,* 1999; Merenyi *et al.,* 1999), experiments varying the viscosity of the solvent (Pryor *et al.,* 1996), experiments with water isotopically labeled in the hydrogen or in the oxygen (Anbar and Taube, 1954; Bohle and Hansert, 1997; Koppenol, 1998), electronic paramagnetic resonance spectroscopy (Augusto *et al.,* 1994; Gatti *et al.,* 1998; Lemercier *et al.,* 1995; Pou *et al.,* 1995), competition and product analysis in the presence of hydroxyl radical scavengers (Beckman *et al.,* 1990) (Alvarez *et al.,* 1995; Augusto *et al.,* 1994; Beckman *et al.,* 1990; Goldstein and Czapski, 1995b; Lymar and Hurst, 1998a; Gerasimov and Lymar, 1999; Coddington *et al.,* 1999; Richeson *et al.,* 1998), and quantum-mechanical calculations (Houk *et al.,* 1996; Jursic *et al.,* 1997).

Overall, the results favor the hypothesis of homolysis. In this sense, electronic paramagnetic resonance (EPR) spin-trapping experiments have shown that in the presence of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), the DMPO–hydroxyl radical adduct is formed (Augusto *et al.,* 1994; Gatti *et al.,* 1998). The formation of the adduct is inhibited by formate and ethanol, as expected from their rate constants with $^{\cdot}OH$, with concomitant formation of the predicted trapped products. Experiments with ^{17}O-labeled water confirmed that the $^{\cdot}OH$ trapped by DMPO came from the peroxynitrite molecule, not the solvent.

Thus, in the pathway of peroxynitrite isomerization to nitrate, highly reactive $\cdot OH$ and $\cdot NO_2$ radicals are formed. We will refer to it throughout the text as the ''hydroxyl radical pathway.''

Peroxynitrite Reaction with Target Molecules

Molecules that are present during peroxynitrite decomposition can react with it through two main pathways

that have been proposed on the basis of kinetic studies (Fig. 1).

In one pathway (Fig. 1, I and II), $ONOO^-$ or ONOOH reacts directly with the target in processes that are second order overall: first order in peroxynitrite and first order in the target, so that the apparent rate constant of peroxynitrite decomposition increases linearly with target concentration (Fig. 2). In the other pathway (Fig. 1, III), ONOOH first rearranges into $\cdot OH$ and $\cdot NO_2$, which are the ultimate oxidants. These processes are first order in peroxynitrite and zero order in target (Fig. 2), since the formation of the radicals would be the rate-limiting step. However, in some cases, the plots of the apparent rate constant of peroxynitrite that decay as a function of target concentration are more complex (Alvarez *et al.,* 1999; Gatti *et al.,* 1998; Pryor *et al.,* 1994).

Peroxynitrite can perform both two- and one-electron oxidations to target molecules. The latter process leads to the formation of free radicals (Bartlett *et al.,* 1995; Gatti *et al.,* 1994; Pietraforte and Minetti, 1997a,b), which can in turn initiate radical chain reactions. In some cases, the formation

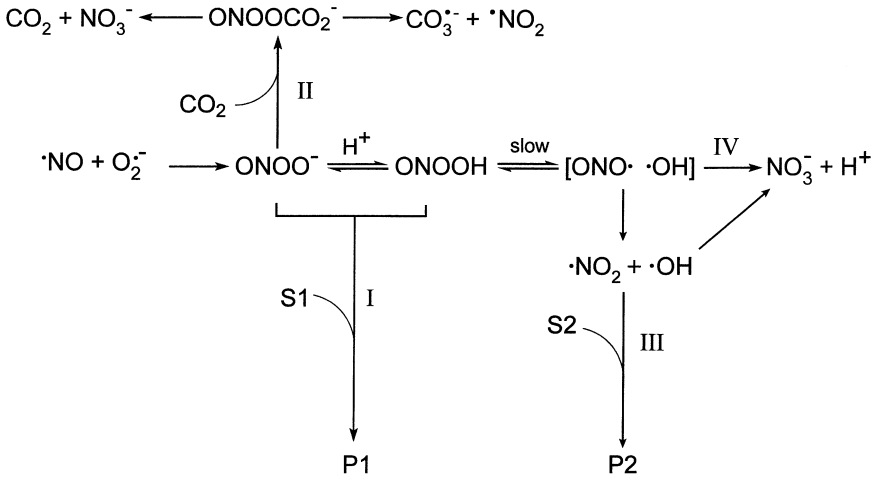


Figure 1 Pathways of peroxynitrite decomposition and reduction. Peroxynitrite can cause the oxidation of target molecules (S1) via direct reactions (I) or it can react with CO_2 to form $ONOOCO_2^-$ (II). In the absence of direct targets, peroxynitrite slowly evolves to hydroxyl radical and nitrogen dioxide which can oxidize/nitrate other targets (S2) (III) or isomerize to nitrate (IV).

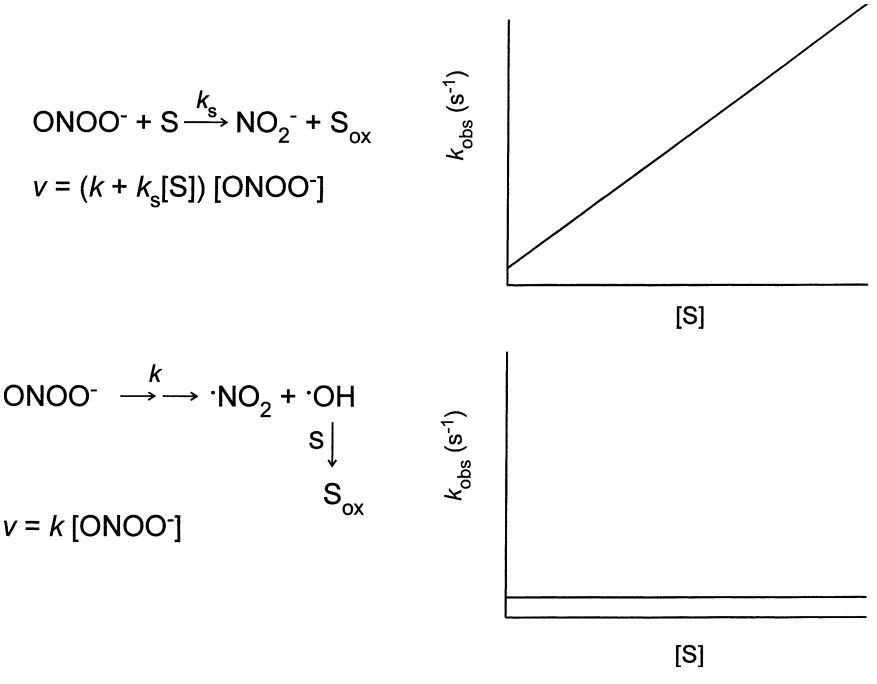


Figure 2 Peroxynitrite reaction with target molecules. In one pathway peroxynitrite reacts directly with the target molecule in an overall second-order process (top). In the other pathway peroxynitrite first decomposes into hydroxyl radical and nitrogen dioxide, which are the ultimate oxidants in a process that is zero order in the target (bottom).

of the one-electron oxidation product has been related to the hydroxyl radical pathway (Padmaja *et al.,* 1996a; Pryor *et al.,* 1994; Quijano *et al.,* 1997).

Direct Reactions

The second-order rate constants of peroxynitrite reaction with a number of molecules have been determined and are collected in Table I. Both $ONOO^-$ and ONOOH can undergo second-order reactions and in most cases the apparent rate constants at pH 7.4 are reported in Table I. Those reactions that are most important from a biochemical point of view will be described in detail following.

THIOLS

The reaction with the thiol groups of cysteine and albumin was the first reaction of peroxynitrite with a biomolecule studied by stopped-flow (Radi *et al.,* 1991a). Since then a number of reports on other low-molecular-weight sulfhydryls have been produced, all of them with rate constants in

Table I Second-Order Rate Constants of Peroxynitrite Reaction with Biologically and Pharmacologically Relevant Target Molecules

Target	k (M^{-1} s^{-1})	T (°C)	pH	Reference
Hydroxyl radical	4.8×10^9	21–24	12	Kissner *et al.* (1997); Goldstein *et al.* (1998)
Myeloperoxidase	6.2×10^6	12	7.2	Floris *et al.* (1993)
GPx (reduced)[a]	8×10^6	25	7.4	Briviba *et al.* (1998a)
GPx (oxidized)[a]	0.7×10^6	25	7.4	Briviba *et al.* (1998a)
Mn(III)T*M*-2-PyP[b]	1.85×10^7	37	7.4	Ferrer-Sueta *et al.* (1999)
Mn(III)TM-4-PyP[b]	1.8×10^6	22–24	7.4	Lee *et al.* (1997)
Fe(III)TM-4-PyP[b]	2.2×10^6	37	7.4	Stern *et al.* (1996)
Lactoperoxidase	3.3×10^5	12	7.4	Floris *et al.* (1993)
Horseradish peroxidase	3.2×10^6	25	ind[c]	Floris *et al.* (1993)
Ebselen[d]	2×10^6	25	>8	Masumoto *et al.* (1996)
Alcohol dehydrogenase	2.6×10^5	23	7.4	Crow *et al.* (1995)
Aconitase	1.4×10^5	25	7.6	Castro *et al.* (1994)
Tryptophan hydroxylase	3.4×10^4	25	7.4	Kuhn and Geddes (1999)
Cytochrome c^{2+}	2.5×10^4	37	7.4	Thomson *et al.* (1995)
Oxyhemoglobin (monomer)	1.04×10^4	25	7.4	Denicola *et al.* (1998)
Carbon dioxide	3×10^4	24	ind[c]	Lymar and Hurst (1995)
Carbon dioxide	4.6×10^4	37	7.4	Denicola *et al.* (1996)
Cu,Zn-SOD	1×10^3–10^5	37	8	Beckman *et al.* (1992)
Selenomethionine	6.1×10^3	25	6.8	Padmaja *et al.* (1997)
Cysteine	5.9×10^3	37	7.4	Radi *et al.* (1991a)
Mercaptoethyl guanidine	1.9×10^3	37	ind[c]	Szabo *et al.* (1997a)
Glutathione	1.35×10^3	37	7.4	Koppenol *et al.* (1992)
Creatine kinase	8.85×10^5	NR[e]	6.9	Konorev *et al.* (1998)
GAPDH[f]	2.5×10^5	25	7.4	Souza and Radi (1998)
Albumin thiol	3.8×10^3	37	7.4	Alvarez *et al.* (1999)
Human serum albumin	9.7×10^3	37	7.4	Alvarez *et al.* (1999)
Bovine serum albumin	7.5×10^3	37	7.4	Alvarez *et al.* (1999)
Acetaldehyde	8.3×10^2	25	7.0	Uppu *et al.* (1997)
Urate monoanion	4.8×10^2	37	7.3	Santos *et al.* (1999)
Methionine	1.8×10^2	25	7.4	Pryor *et al.* (1994)
Pyruvate	88	37	7.4	Vasquez-Vivar *et al.* (1997)
Ascorbic acid	47	25	7.4	Bartlett *et al.* (1995)
Tryptophan	37	37	7.4	Alvarez *et al.* (1996)

[a] GPx, Glutathione peroxidase, tetramer.
[b] TMPy*P,* 5,10,15,20-tetrakis(*N*-methyl-pyridyl)porphyrin.
[c] ind, pH-independent rate constant.
[d] Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one.
[e] NR, not reported.
[f] GAPDH, Glyceraldehyde-3-phosphate dehydrogenase, tetramer.

the vicinity of $10^3\ M^{-1}\ s^{-1}$ (Koppenol *et al.,* 1992; Quijano *et al.,* 1997; Szabo *et al.,* 1997a). On the other hand, protein thiol groups have reactivities that depend greatly on the protein environment. Whereas the albumin single thiol reacts much like a low-molecular-weight thiol (Alvarez *et al.,* 1999; Radi *et al.,* 1991a), the rate constant of peroxynitrite with the active site thiol of GAPDH is one order of magnitude higher (Souza and Radi, 1998).

The pH dependence of the rate constant of thiol oxidation by peroxynitrite has a bell-shape profile (Radi *et al.,* 1991a; Szabo *et al.,* 1997a). This profile indicates that the acid form of one reagent is reacting with the basic form of the other, for example, RSH with $ONOO^-$ (Radi *et al.,* 1991a), but does not reveal the identity of the actual reactants.

The products of peroxynitrite-mediated oxidation of thiols are dependent on a number of reaction conditions such as relative concentration of reagents, CO_2 concentration, pH, and the nature of the thiol involved (Quijano *et al.,* 1997). Two main pathways have been identified (Fig. 3), namely, a two-electron oxidation yielding the corresponding disulfide and nitrite (possibly via a sulfenic acid intermediate (pathway *a*), and a one-electron oxidation by the $\cdot OH$ or $\cdot NO_2$ yielding thiyl radical (pathway *b*) and starting an oxygen-dependent chain reaction (Quijano *et al.,* 1997).

Reactions involving nitro- and nitrosothiols as intermediates and/or final products have also been invoked (pathways *c* and *d*) (Balazy *et al.,* 1998; Pryor and Squadrito, 1995; van der Vliet *et al.,* 1998) but they involve a small percentage of the initial reactants and appear to be of limited biochemical significance.

Selenium Compounds

Selenium compounds, particularly 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one (ebselen), selenomethionine, and selenocysteine, and selenium-containing proteins such as glutathione peroxidase react faster than their sulfur analogs and were found to act as very efficient peroxynitrite scavengers (Briviba *et al.,* 1996, 1998b; Masumoto *et al.,* 1996; Masumoto and Sies, 1996a,b; Padmaja *et al.,* 1997). In particular, ebselen and glutathione peroxidase can be recycled by glutathione (Sies and Masumoto, 1997; Sies *et al.,* 1997).

Amino Acids

In addition to cysteine, two other amino acids, methionine (Alvarez *et al.,* 1999; Pryor *et al.,* 1994) and tryptophan (Alvarez *et al.,* 1996; Padmaja *et al.,* 1996b), have significant second-order reactions with peroxynitrite. The rest of the protein-forming amino acids have been studied and minimal or no bimolecular reactivity was observed. For instance, histidine, phenylalanine, leucine, and tyrosine did not change the rate of peroxynitrite decay (Alvarez *et al.,* 1999) (Fig. 4).

The amino acids that do not significantly affect the rate of peroxynitrite consumption can still be modified via the hydroxyl radical pathway (e.g., tyrosine and phenylalanine, see following). Furthermore, in metalloproteins, the metal center can alter the mechanism and products of amino acid modifications mediated by peroxynitrite (Ischiropoulos *et al.,* 1992a; Roberts *et al.,* 1998; Smith *et al.,* 1992; Yamakura *et al.,* 1998).

Metal Centers

Some transition metal centers are oxidized very fast with peroxynitrite, particularly metal porphyrin systems, as in heme proteins (Alayash *et al.,* 1998; Denicola *et al.,* 1998; Exner and Herold, 2000; Floris *et al.,* 1993; Ischiropoulos *et al.,* 1996; Pearce *et al.,* 1999; Thomson *et al.,* 1995), and synthetic metal porphyrin complexes (Balavoine *et al.,* 1997; Hunt *et al.,* 1997; Misko *et al.,* 1998; Salvemini *et al.,* 1998). In some cases the oxidized metal ion can be reduced back to the original oxidation state if a suitable reductant is present. For instance, myeloperoxidase, lactoperoxidase, and

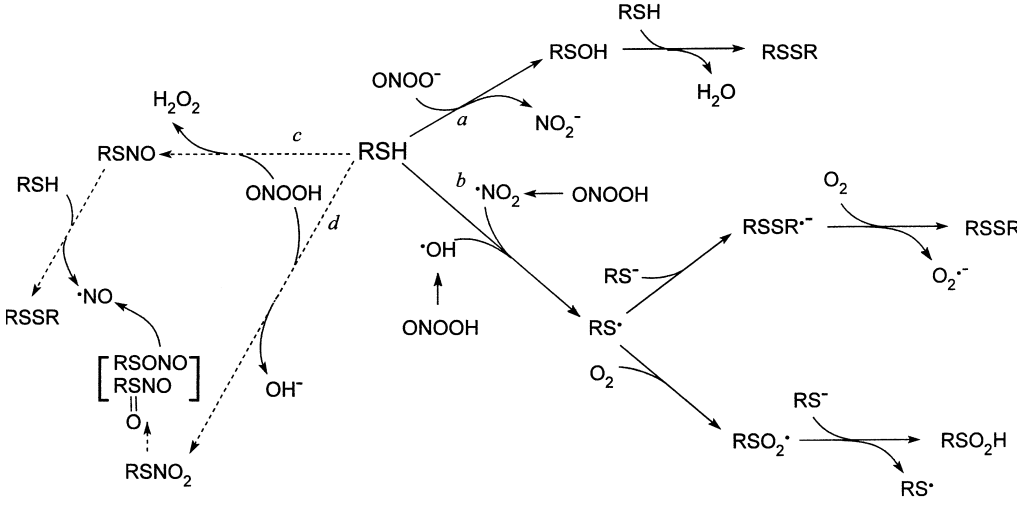


Figure 3 Pathways of thiol oxidation by peroxynitrite. (*a*) Two-electron pathway leading to disulfide formation. (*b*) One-electron oxidation associated with oxygen-dependent chain reaction may lead to higher oxidation states. (*c* and *d*) Proposed pathways for the formation of nitro and nitroso derivatives.

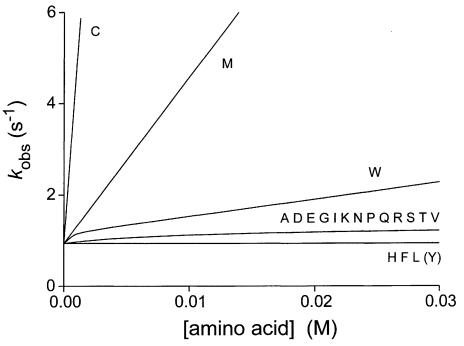


Figure 4 Kinetics of peroxynitrite reaction with amino acids. Apparent rate of peroxynitrite decomposition in the presence of increasing concentrations of the free L-amino acids.

horseradish peroxidase can use peroxynitrite as a substrate and then be reduced by endogenous reductants.

The iron–sulfur cluster in aconitase (Castro *et al.,* 1994; Hausladen and Fridovich, 1994) and the Zn–S active site of yeast alcohol dehydrogenase (Crow *et al.,* 1995) are also oxidized by peroxynitrite, but in this case the oxidation causes the disruption of the active site and the loss of enzymatic activity.

THE PEROXYNITRITE–CO_2 REACTION

Reaction Chemistry Early work pointed out the instability of peroxynitrite in carbonated buffers (Keith and Powell, 1969). Recent studies have shown that the bicarbonate–carbon dioxide pair has strong influence on peroxynitrite reactivity and kinetics (Denicola *et al.,* 1996; Goldstein and Czapski, 1997; Gow *et al.,* 1996; Lemercier *et al.,* 1997; Lymar and Hurst, 1995, 1996, 1998b; Lymar *et al.,* 1996; Pryor *et al.,* 1997; Radi *et al.,* 1993; Uppu *et al.,* 1996a; van der Vliet *et al.,* 1994; Zhang *et al.,* 1997).

Peroxynitrite decomposition in the presence of bicarbonate–carbon dioxide follows second-order kinetics (Denicola *et al.,* 1996; Lymar and Hurst, 1995; Uppu *et al.,* 1996a). The pH dependency of the reaction rate shows a bell-shape profile with a maximum at 6.5, indicating that ionization of both peroxynitrite and bicarbonate (apparent pK_a at 37°C is 6.1) contributes to the pH profile. pH jump (Lymar and Hurst, 1995) and carbonic anhydrase experiments (Uppu *et al.,* 1996a) have shown that the reacting species are $ONOO^-$ and CO_2. The pH-independent second-order rate constant for the peroxynitrite–CO_2 reaction is $5.8 \times 10^4\ M^{-1}\ s^{-1}$ at 37°C (Denicola *et al.,* 1996). Considering the relatively high concentration of CO_2 both in intra- and extracellular compartments (1–2 m*M*), its reaction with $ONOO^-$ becomes one of the fastest for peroxynitrite in biological systems.

Reactivity of the $ONOOCO_2^-$ Adduct The product of the peroxynitrite–CO_2 reaction is the adduct $ONOOCO_2^-$ (1-carboxylate-2-nitrosodioxidane), which rapidly decomposes to CO_2 and nitrate. The short half-life of $ONOOCO_2^-$ (< 1 μs) is attributable to the presence of a weak O−O bond (Merenyi and Lind, 1997).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow CO_2 + NO_3^- \quad (14)$$

In the absence of target molecules, CO_2 catalyzes the decomposition of peroxynitrite to nitrate at a higher rate than the proton-catalyzed isomerization (i.e., 46 versus 0.9 s^{-1} at 1 m*M* CO_2 and pH 7.4). However, CO_2 can not be considered a ''scavenger'' of peroxynitrite because the $ONOOCO_2^-$ adduct is not an inert species. In the presence of substrates, the adduct can perform one-electron oxidations as well as nitrations but with a reactivity different than peroxynitrite itself. In general the $ONOOCO_2^-$ adduct is a less potent oxidant but a more powerful nitrating agent than peroxynitrite itself (Denicola *et al.,* 1996; Goldstein and Czapski, 1997; Lemercier *et al.,* 1997; Lymar and Hurst, 1995, 1996, 1998b; Pryor *et al.,* 1997; Radi *et al.,* 1999; Uppu *et al.,* 1996a). This is due to the homolytic decomposition to yield carbonate radical ($CO_3^{\cdot-}$) and nitrogen dioxide ($NO_2^{\cdot}$) as the main pathway (Fig. 5). The formation of $CO_3^{\cdot-}$ has been indirectly detected by photochemical (Denicola *et al.,* 1996) and product distribution studies (Lymar and Hurst, 1998b) and more recently directly by EPR (Bonini *et al.,* 1999). Carbonate radical is a reactive species able to oxidize biomolecules with rate constants in the range of 10^5–$10^8\ M^{-1}\ s^{-1}$ (Chen and Hoffman, 1973; Chen and Hoffman, 1974) (e.g., $5 \times 10^7\ M^{-1}\ s^{-1}$ with tyrosine).

Maximum oxidation or nitration yields by the adduct are on the order of 20–35% (Goldstein and Czapski, 1997; Lymar and Hurst, 1998b) on line with the initial formation of $CO_3^{\cdot-}$ and $\cdot NO_2$ as a caged radical pair (Fig. 5).

The differential chemical reactivity of $ONOOCO_2^-$ and its significantly shorter half-life compared to $ONOO^-$ (∼15 versus <1 μs at pH 7.4) determine that (a) the presence of carbon dioxide redirects peroxynitrite reactivity and (b) the adduct can diffuse a significantly shorter distance than peroxynitrite in biological milieu.

Reactions via the Hydroxyl Radical Pathway

Among relevant targets that have been reported to react with the ·OH arising from peroxynitrite homolysis are phenolics such as 4-hydroxyphenylacetic acid (Beckman *et al.,* 1992), tyrosine, phenol, and salicylate (Ramezanian *et al.,* 1996); phenylalanine (van der Vliet *et al.,* 1994); histidine (Alvarez *et al.,* 1999); hydrogen peroxide (Alvarez *et al.,* 1995); deoxyribose (Beckman *et al.,* 1990); mannitol; ethanol (Alvarez *et al.,* 1998); ubiquinol (Schöpfer *et al.,* 2000); and lipids (Radi *et al.,* 1991b; Shustov *et al.,* 2000).

The oxidation yield of these processes was found to be less than 30% of the peroxynitrite added, even when the values were extrapolated to infinite substrate concentration (Beckman *et al.,* 1990).

When the one-electron oxidation product of the target leads to $O_2^{\cdot-}$ formation, such as in the case of hydrogen peroxide and ethanol, peroxynitrate (O_2NOO^-) may be formed

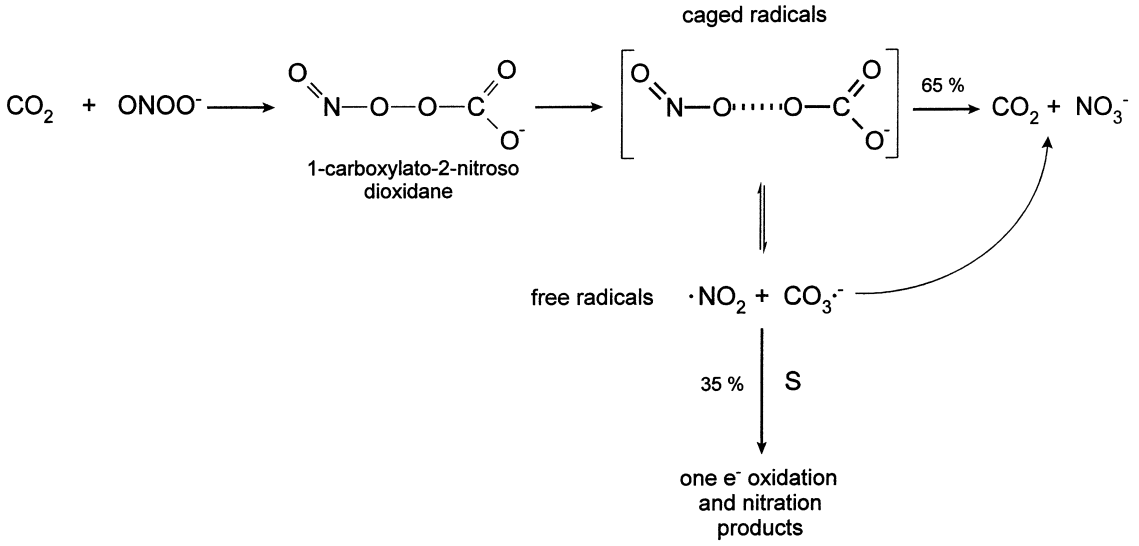


Figure 5 Formation of carbonate radical from $ONO_2CO_2^-$ adduct decomposition.

from the reaction of $O_2^{\cdot-}$ and $\cdot NO_2$ (Alvarez *et al.,* 1995; Goldstein and Czapski, 1998; Hodges and Ingold, 1999).

NITRATION

Peroxynitrite is a good nitrating agent for aromatic compounds (e.g., tyrosine, tryptophan). At 25°C and neutral pH peroxynitrite-mediated tyrosine nitration in phosphate has a maximum yield of 6–8% nitrotyrosine formed with respect to peroxynitrite (Ischiropoulos *et al.,* 1992a). Tyrosine is nitrated without affecting the rate of peroxynitrite decomposition, indicating that $\cdot OH$ and $\cdot NO_2$ are responsible for this reaction (Ramezanian *et al.,* 1996). The formation of bityrosine as a side product during the nitration reaction is a clear indication of a radical mechanism (van der Vliet *et al.,* 1995). The reaction is complex and involves many competing reactions:

$$ONOOH \rightarrow [OH^{\cdot} + NO_2^{\cdot}] \rightarrow {}^{\cdot}OH + {}^{\cdot}NO_2 \quad (15)$$

$$[{}^{\cdot}OH + {}^{\cdot}NO_2] \rightarrow NO_3^- + H^+ \quad (16)$$

$${}^{\cdot}OH + Tyr \rightarrow Tyr^{\cdot} + H_2O \quad (17)$$

$$Tyr^{\cdot} + {}^{\cdot}NO_2 \rightarrow TyrNO_2 \quad (18)$$

$$Tyr + {}^{\cdot}NO_2 \rightarrow Tyr^{\cdot} + NO_2^- + H^+ \quad (19)$$

$$Tyr^{\cdot} + Tyr^{\cdot} \rightarrow Tyr{-}Tyr \quad (20)$$

$${}^{\cdot}NO_2 + {}^{\cdot}NO_2 \rightarrow NO_2^- + NO_3^- \quad (21)$$

It can be seen that the yield of nitrotyrosine [reaction (18)] depends on pH [through reaction (15)] and the relative concentrations of peroxynitrite and tyrosine [through reactions (17) to (19)] and on the concentrations of radicals formed and its side reactions [reactions (20) and (21)] (Goldstein *et al.,* 2000; Pfeiffer *et al.,* 2000). The overall nitration mechanism can be summarized as a one-electron oxidation of the aromatic ring [reaction (17) and (19)] followed by a coupling of the resulting phenoxyl radical with nitrogen dioxide. The first reaction is H^+-catalyzed, as the yield of nitration decreases at alkaline pH.

Besides H^+, other Lewis acids (LA) including CO_2 and transition metal centers (Table II) catalyze peroxynitrite-mediated tyrosine nitration. In general the reaction can be represented as:

$$ONOO^- + LA \rightarrow LA{-}O^{\cdot-} + NO_2^{\cdot} \quad (22)$$

where $LA{-}O^{\cdot-}$ is the oxidizing intermediate (Table II).

Nitration yields as a function of initial peroxynitrite added vary widely depending on the Lewis acid (Table II).

It can be seen from Table II that the redox potential of the oxidizing intermediate is an important factor in determining the nitration yield in the presence of catalysts.

Direct Reactions versus the Hydroxyl Radical Pathway

When peroxynitrite reacts directly with a target molecule, the rate of the reaction will be proportional to the product of the rate constant times the concentration (Fig. 6). On the other hand, when the oxidant is the $^{\cdot}OH$, the apparent rate of this process is the proton-catalyzed decomposition of peroxynitrite: 0.9 s^{-1} at pH 7.4 and 37°C. In biological systems, it can be calculated that most peroxynitrite will be consumed by direct reactions. The contribution of the hydroxyl radical pathway to peroxynitrite reactivity in biology is a rather minor route (<5%): peroxynitrite will react before evolving to secondary reactive intermediates (Fig. 6).

Diffusion

Oxidizing intermediates tend to have limited diffusion in the biological milieu because of their high reactivity toward biomolecules, which determines that most of the oxidant will

Table II Nitration of Tyrosine Catalyzed by Lewis Acids

Lewis acid	Oxidizing intermediate	Redox potential V/NHE	Nitration yield (%)	References
H^+	$^{\cdot}O-H$	2.3	7–10	Beckman *et al.* (1992)
Cu^{2+}	$^{\cdot}O-Cu$	N.D.[a]	12–20	Beckman *et al.* (1992); Ferrer-Sueta *et al.* (1997)
CO_2	$^{\cdot}O-CO_2^-$	1.8	20–35	Denicola *et al.* (1996)
Fe^{III}EDTA	$O=Fe^{IV}$EDTA	1.1–1.6	34	Beckman *et al.* (1992)
Fe^{III}TMPyP	$O=Fe^{IV}$TMPyP	~1.1	N.D.[a]	
Mn^{III}TMPyP	$O=Mn^{IV}$TMPyP	~1.1	25–30	Ferrer-Sueta *et al.* (1999)
Mn^{III}TBAP	$O=Mn^{IV}$TBAP	> 0.9	50	Ferrer-Sueta *et al.* (1997)

[a] N.D. not determined.

be consumed within the compartment where it is formed. A striking example of this concept is the case of $^{\cdot}OH$ that reacts within three to four molecular diameters from its site of formation (Hutchinson, 1957).

Thus, to account for the biological actions of peroxynitrite, it becomes important to define how far it can diffuse and reach critical cellular targets. A related question is whether the cellular membrane is permeable to this oxidant.

Early evidence suggested that peroxynitrite was able to cross cell membranes, as intact cells exposed to peroxynitrite exhibited oxidation of intracellular proteins and inactivation of enzymes, indirectly indicating the ability of peroxynitrite to permeate membranes (Denicola *et al.,* 1993; Hu *et al.,* 1994; Radi *et al.,* 1994; Rubbo *et al.,* 1994a; Soszynski and Bartosz, 1996; Szabo *et al.,* 1996).

Mechanisms of Peroxynitrite Diffusion

Recent studies have directly proved the ability of peroxynitrite to diffuse across biological membranes (Denicola *et al.,* 1998; Marla *et al.,* 1997). Using model phospholipid vesicular systems, it has been demonstrated that peroxynitrite freely crosses phospholipid membranes (Marla *et al.,* 1997). Diffusion of peroxynitrite from extra- to intracellular compartments was also demonstrated using red blood cells as a model (Denicola *et al.,* 1998). Peroxynitrite can cross the erythrocyte membrane by a mechanism involving $ONOO^-$ transport through the HCO_3^-/Cl^- exchanger (band 3) and also by passive diffusion of ONOOH (Denicola *et al.,* 1998). At physiological pH, most peroxynitrite would be in the anionic form and therefore diffusion through the anion channel becomes relevant. Red blood cells are particularly abundant in this anion channel (band 3) but there are also proteins homologous to band 3 in nonerythroid cells that promote anion transport and that might serve to transport $ONOO^-$ as well (Jennings, 1989). Superoxide anion also diffuses across membranes through the erythrocyte anion channel (Lynch and Fridovich, 1978). However, a significant difference is that passive diffusion of its conjugated acid $HO_2^{\cdot}$ ($pK_a = 4.8$) is not feasible at physiological pH. In contrast, membranes scarce in anion channels and cellular compartments under low pH conditions will still be permeable to ONOOH.

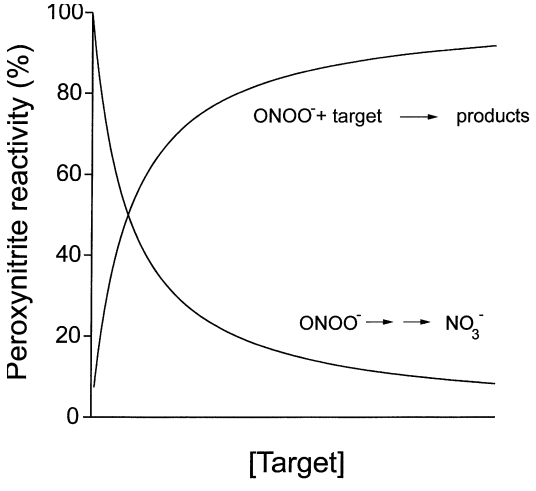


Figure 6 Direct reaction versus the hydroxyl radical pathway. As the concentration of target molecules that react directly with peroxynitrite increases, peroxynitrite will react preferentially with them instead of evolving to $^{\cdot}OH$ and $^{\cdot}NO_2$ and isomerizing to nitrate.

While peroxynitrite is diffusing through the membrane, it can also react with some of its components, resulting in protein and/or lipid oxidation. For instance, in red blood cells nitration of the cytoplasmic domain of band 3 was observed at high concentrations of the oxidant (Mallozzi *et al.,* 1997). Thus, appropriate membrane targets can interact with a fraction of the peroxynitrite travelling across cell compartments.

Peroxynitrite Diffusion in Biology

The reported studies of peroxynitrite diffusion across membranes (Denicola *et al.,* 1998; Marla *et al.,* 1997) have been performed under conditions in which the decay of peroxynitrite in the extracellular milieu was only due to the proton-catalyzed decomposition, a relatively slow process in the diffusional time scale. However, from a biological perspective, an important question is whether the presence of

extracellular targets that will accelerate the consumption of peroxynitrite would preclude its diffusion to intracellular compartments. For instance, the reaction between peroxynitrite and CO_2 represents a major route of peroxynitrite consumption in biological systems, particularly in extracellular compartments. However, the diffusion of peroxynitrite across cells can occur even in the presence of extracellular CO_2 (Romero *et al.,* 1999). The concept is that the apparent rate of peroxynitrite diffusion across membranes can be comparable to and even larger than the rate of peroxynitrite reaction with CO_2 and other bioreductants (Fig. 7). Whereas at low cell densities the diffusion time of peroxynitrite will be too long to compete with extracellular target molecule reactions, at high cell densities the diffusion distances will be shorter and so an important percentage of peroxynitrite can reach and cross the membranes. For example, peroxynitrite production by activated macrophages can reach a pathogen cell and cause cytotoxic effects.

Biological Targets

Peroxynitrite reacts with a limited number of targets *in vivo* (Radi, 1998). This is due in part to the large variation of rate constants in the reactions of peroxynitrite against biological targets (Table I) and target concentrations. In addition, the formation sites of peroxynitrite differ depending on cell types, tissues, and pathophysiological conditions and therefore compartmentalization and diffusion properties of peroxynitrite also play a central role in determining the biological fate and principal reactions of this reactive intermediate.

Even though we still have partial information regarding the biological targets of peroxynitrite, we will describe some well-established events, which show some of the central pathways of peroxynitrite reactivity *in vivo* which account for its biological actions.

Proteins

Proteins are key biological targets of peroxynitrite due to their ubiquity, their concentration and the reactivity of some of their constituent amino acids and prosthetic groups.

Some predictions can be made regarding the reactivity of peroxynitrite toward proteins and peptides:

1. Transition metal centers such as reduced heme and metal–sulfur clusters will determine the rate constants when present.
2. Cysteine, methionine, and tryptophan (in that order) will account for the vast majority of the reactivity of the polypeptide.
3. The protein environment can enhance the reactivity of cys, met, and trp in proteins [such as the essential thiol in GAPDH (Souza and Radi, 1998)].
4. Selenium-containing amino acids (selenocysteine and selenomethionine) have enhanced reactivities respective to their sulfur analogs (Briviba *et al.,* 1996; Padmaja *et al.,* 1996a, 1997; Sies *et al.,* 1998).

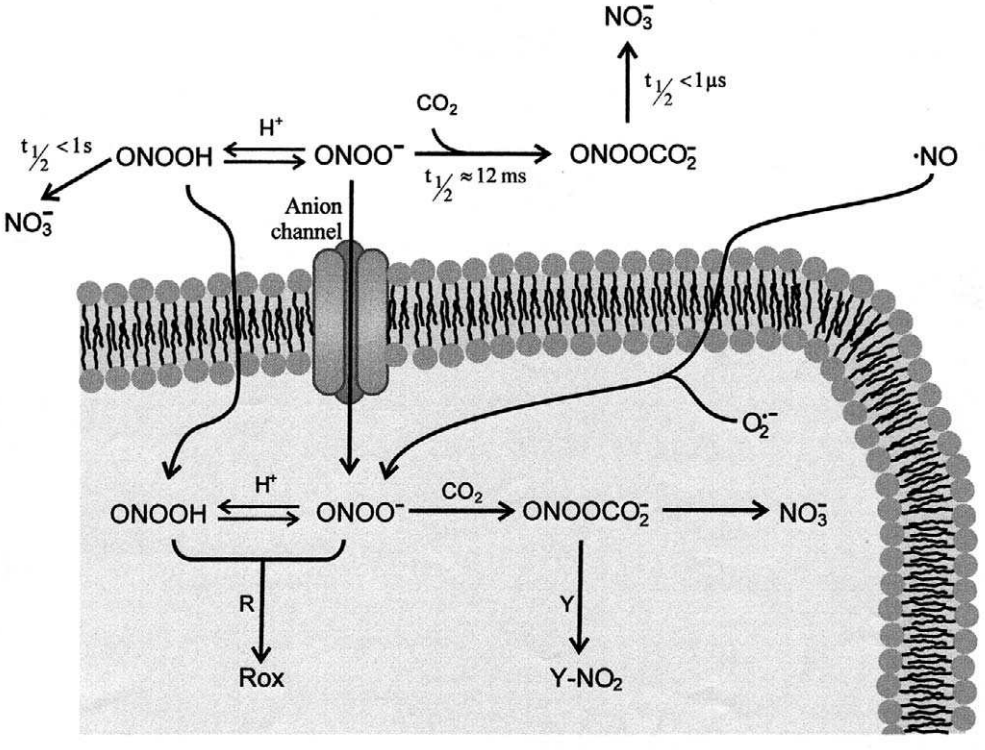


Figure 7 Scheme representing competition between peroxynitrite diffusion and reaction with extracellular CO_2.

5. Tyrosine nitration is a slow process and must be catalyzed by CO_2 or metals to occur *in vivo.*

ENZYME MODIFICATION

A number of enzymes are inactivated by reaction with peroxynitrite (Table III). The inactivations observed during exposure of the isolated enzyme have in many cases (Castro *et al.,* 1994, 1998), but not always (Cassina and Radi, 1996; Sharpe and Cooper, 1998b), been reproduced at the cellular or subcellular level where several other competing reactions exist.

Typically, peroxynitrite causes the irreversible oxidation or nitration of a critical residue in the protein. In contrast, in other cases the oxidation of the enzyme by peroxynitrite can be regarded as a step in the enzymatic cycle promoting the catalytic activity. Examples are prostaglandin endoperoxide synthase (Landino *et al.,* 1996) and several peroxidases (Floris *et al.,* 1993; Grace *et al.,* 1998; Sies *et al.,* 1997) that are oxidized by peroxynitrite and then reduced by endogenous substrates.

The only reported case of an enzyme activated by reaction with peroxynitrite is procollagenase (Okamoto *et al.,* 1997), in which peroxynitrite apparently oxidizes a cysteine residue in the autoinhibitory domain of the proenzyme, thus liberating a Zn coordination position in the active site and rendering the enzyme active.

It is important to remark that the ''activation'' of some enzymatic functions such as poly(ADP–ribose) synthetase (PARS) and caspase 3 during peroxynitrite exposure or formation *in vivo* (Cuzzocrea *et al.,* 1997; Endres *et al.,* 1998; Kennedy *et al.,* 1998; Lin *et al.,* 1998; Szabo *et al.,* 1996, 1997b) is not due to direct interactions of peroxynitrite with these enzymes, but rather to cellular responses associated with oxidative stress, and constitutes downstream events associated to peroxynitrite toxicity.

Enzyme Sensitivity and IC_{50} Enzyme reactions with peroxynitrite are usually determined through the effect of the oxidant on enzyme activity. Peroxynitrite concentrations inhibiting 50% of initial enzyme activity (IC_{50}) are used as a

Table III Enzymes Inactivated by Peroxynitrite

Enzyme	Modified residue	Reference
Aconitase[c]	[4Fe–4S]	Castro *et al.* (1994, 1998)
Ca^{2+}-ATPase[b]	Cys	Klebl *et al.* (1998), Viner *et al.* (1996)
Caspase 3[a]	Cys	Haendeler *et al.* (1997)
Creatine kinase[a]	Cys	Konorev *et al.* (1998), Stachowiak *et al.* (1998)
Cyclooxygenase	Tyr	Boulos *et al.,* 2000
Fumarate reductase[b]	Cys	Rubbo *et al.* (1994a)
Glutamine synthetase[a]	Tyr	Berlett *et al.* (1998)
Glutathione peroxidase[a]	Se-Cys	Asahi *et al.* (1997), Briviba *et al.* (1998a), Padmaja *et al.* (1998)
Glyceraldehyde-3-phosphate dehydrogenase[a]	Cys	Souza and Radi, (1998)
Inducible nitric oxide synthase[a]	Heme	Huhmer *et al.* (1997)
Iron–sulfur dehydratases[b]	[4Fe–4S]	Keyer and Imlay (1997)
Mn Superoxide dismutase[c]	Tyr	MacMillan-Crow *et al.* (1996), Yamakura *et al.* (1998)
Nicotinamide nucleotide transhydrogenase[a]	Tyr	Forsmark-Andree *et al.* (1996)
Prostacyclin synthase[a]	Tyr	Zou and Ullrich (1996)
Ribonucleotide reductase	Tyr	Guittet *et al.* (2000)
Succinate dehydrogenase[b]	Cys	Radi *et al.* (1994)
Tryptophase hydroxylase	Cys	Khun and Geddes (1999)
Tyrosine hydroxylase[b]	Tyr/Cys	Ara *et al.* (1998), Khun *et al.* (1999)
Tyrosine phosphatases	Cys	Takakura *et al.* (1999)
Xantine oxidase[a]	Mo center	Houston *et al.* (1998), Lee *et al.* (2000)
Yeast alcohol dehydrogenase[a]	Cys	Crow *et al.* (1995)
Zn^{2+}-glycerophosphocholine cholinephosphodiesterase[a]	Tyr	Sok (1998)

[a] Inactivation was observed with the isolated enzyme.
[b] Inactivation was observed with the enzyme present in a cellular or subcellular compartment.
[c] Inactivation was observed under both conditions.

measure of the enzyme sensitivity to peroxynitrite (Castro *et al.,* 1994). Nevertheless, IC_{50} values do not provide a true measure of sensitivity unless they are determined with extreme care. Many factors influence IC_{50}, including enzyme concentration, stoichiometry of the reaction with peroxynitrite, the rate constants for the reaction of $ONOO^-$ with critical and noncritical residues in the enzyme, and the amount of peroxynitrite that escapes (via isomerization) during the reaction.

The following equation has been proposed to evaluate the rate constant of peroxynitrite reacting with an enzyme (Padmaja *et al.,* 1998):

$$[ONOO^-]_i = z(A_i - A_f)_E - \left(\frac{k_1}{k_E} \ln \frac{A_f}{A_i}\right)_E \qquad (23)$$

where A_i and A_f are initial and final enzyme activities, z is the molar activity of the enzyme, k_1 is the rate constant of peroxynitrite isomerization to nitrate, and k_E is the rate of the bimolecular reaction between peroxynitrite and the critical residue of the enzyme. This constant must not be confused with the overall rate constant of peroxynitrite with the whole protein molecule that is obtained through direct kinetic methods.

Equation (23) can be rearranged to give the dependence of IC_{50} on initial enzyme concentration.

$$IC_{50} = \frac{[E]_i}{2} + \frac{k_1}{k_E} \ln 2 \qquad (24)$$

Thus, by determining IC_{50} values at different initial enzyme concentrations and plotting them as a function of $[E]_i$ the value of k_E can be obtained from the intercept.

The reaction stoichiometry and the protection of the enzyme by reaction of peroxynitrite with noncritical residues affect the slope of an IC_{50} versus $[E]_i$ plot; thus, a single determination of IC_{50} can lead to erroneous values of k_E.

A potential pitfall of this method is that it considers peroxynitrite as affecting only the turnover number of the enzyme. If the oxidation alters other enzymatic parameters, such as K_M and K_S (Forsmark-Andree *et al.,* 1996), the measurement of enzymatic activity might not yield reliable results if only one substrate concentration is used throughout the study. Another problem arises at low enzyme concentration because a significant fraction of peroxynitrite may evolve to $\cdot OH$ and $\cdot NO_2$, which may or may not affect enzyme activity.

In summary, k_E values are more reliable than IC_{50} for measuring the enzyme sensitivity to peroxynitrite, but they should be measured thoroughly over a range of high enzyme concentrations, and other enzymatic parameters should be considered.

PROTEIN NITRATION

Biological protein nitration is an event related to excess $^{\cdot}NO$ production. Initially thought to depend exclusively on peroxynitrite reactions (Beckman *et al.,* 1994), it has more recently been shown that other biologically relevant mechanisms of protein nitration may exist. Some of these mechanisms depend on the formation of nitrating species derived from interactions of hemeperoxidases with nitrite and peroxides (Eiserich *et al.,* 1998; van der Vliet *et al.,* 1997) and others on the oxidation of nitrosotyrosine with the intermediate formation of iminoxyl radicals (Gunther *et al.,* 1997).

Thus, since the formation of nitrotyrosine is not a totally specific ''footprint'' of peroxynitrite reactions, the accumulation of nitrated proteins can only be unambiguously attributed to a peroxynitrite-dependent mechanism with the aid of pharmacological tools.

Cellular Studies Indicating Peroxynitrite Formation and Protein Nitration For peroxynitrite to be formed, $^{\cdot}NO$ and $O_2^{\cdot-}$ should encounter each other in a given biological compartment. Since the biological half-lives and diffusion properties of $^{\cdot}NO$ and $O_2^{\cdot-}$ are rather different, with $^{\cdot}NO$ being significantly longer lived and more diffusible than $O_2^{\cdot-}$, peroxynitrite formation will occur closer to the $O_2^{\cdot-}$-producing sites. Once generated, peroxynitrite can nitrate proteins in the same or adjacent cell or tissue compartments, as peroxynitrite can diffuse some distance in a cellular length scale (Denicola *et al.,* 1998).

The first study demonstrating cellular peroxynitrite-dependent nitration (Ischiropoulos *et al.,* 1992b) was performed in alveolar rat macrophages, in which $^{\cdot}NO$ production by iNOS in concert with the formation of $O_2^{\cdot-}$ by activation of the membrane-bound NADPH oxidase resulted in nitration of *p*-hydroxyphenylacetate present in the extracellular milieu. The nitration process was inhibitable by *N*-monomethyl arginine (L-NMMA). Externally added Cu,Zn-SOD was unable to inhibit nitration, possibly due to the fact that peroxynitrite was being formed in regions not readily accesible to SOD. In more recent studies in $^{\cdot}NO$- and $O_2^{\cdot-}$-producing macrophages, protein nitration was also detected intracellularly (Shigenaga *et al.,* 1997).

Other cellular models in which $^{\cdot}NO$ and $O_2^{\cdot-}$ were cogenerated also resulted in protein nitration (Estevez *et al.,* 1998; Estevez *et al.,* 1999, 2000). Growth factor deprivation and the presence of Zn-deficient SOD to rat embryo motor neurons induced protein nitration. Motor neuron nitration was inhibitable by either the NOS inhibitor L-nitroarginine methyl ester (L-NAME) or the cell permeable SOD mimic manganese(III) tetrakis(4'-benzoic acid)porphyrin (Mn-TBAP). Growth factor deprivation promoted the induction of nNOS, and excess $O_2^{\cdot-}$ arose from a yet unidentified cellular source. The pharmacological inhibition of cellular nitration correlated with protection against cell death.

Normal human lymphocytes activated by antigen binding to the TCR/CD3 membrane complex and cultured in the presence of autologous monocytes presented protein tyrosine nitration (Brito *et al.,* 1999). Activated lymphocytes promoted the production of nitrating species by monocytes through soluble mediators such as cytokines or cell-to-cell interactions. The monocyte-derived nitrating species were able to nitrate their own cellular proteins and also cellular proteins of the neighboring lymphocytes. Nitration was almost completely inhibited by aminoguanidine, an inhibitor of the inducible isoform of nitric oxide synthase, or Mn-

TBAP. The combination of these two inhibitors completely abrogated nitrotyrosine detection in cellular proteins. These results indicate that peroxynitrite was the main nitrating agent and support the concept that peroxynitrite can diffuse from source to target cells.

Peroxynitrite-dependent intracellular nitration can be also seen during exposure of cells overproducing $O_2^{\cdot-}$ to NO donors (Castro *et al.,* 1998). Hamster fibroblasts exposed to a low ·NO flux had a small amount of intracellular protein nitration, which was attributable to the reactions of ·NO with the basal levels of endogenous $O_2^{\cdot-}$. When fibroblasts were poisoned to overproduce $O_2^{\cdot-}$ at the mitochondrial electron transport chain, cellular nitration significantly increased. Exogenous addition of peroxynitrite also resulted in protein nitration, in agreement with the capacity of peroxynitrite to permeate membranes and react in different cell compartments. However, the nitration pattern observed in the Western blot analysis differed from that seen during exposure to the ·NO donor, indicating that compartmentalization plays a role in targeting protein nitration. The critical role of glutathione in modulating nitration yields became evident in GSH-depleted fibroblasts. Indeed, cell depletion of GSH led to enhancement of protein nitration by peroxynitrite, indicating that GSH can trap, at least partially, nitrating intermediates arising from peroxynitrite, $ONOOCO_2^-$, and/or peroxynitrite–metal center adducts (Denicola *et al.,* 1996; Zhang *et al.,* 1997).

In conclusion, various cellular models responding to rather different stimuli which ultimately lead to ·NO, $O_2^{\cdot-}$, and then peroxynitrite formation, resulted in protein nitration. Nitration appears to be targeted to a restricted number of proteins and modulated by intracellular factors such as GSH. Identification of critical and preferentially nitrated proteins awaits further studies. Of note is the fact that the antioxidant enzyme Mn-superoxide dismutase that is located in the mitochondrial matrix is nitrated and inactivated *in vivo* during chronic rejection of human renal allografts (MacMillan-Crow *et al.,* 1996). This chronic inflammatory process progresses with an overproduction of both ·NO and $O_2^{\cdot-}$, and targets nitration in a specific cellular compartment, affecting a specific protein function.

Lipid Peroxidation

Unsaturated membrane lipids are critical targets of oxygen-derived reactive species. $O_2^{\cdot-}$ does not react at significant rates with polyunsaturated fatty acids (Bielski *et al.,* 1983), and $O_2^{\cdot-}$-mediated damage to membranes has been explained through the Haber–Weiss reaction [reactions (25) and (26)] which requires catalysis by transition metal ions (Girotti, 1985; Prutz *et al.,* 1985):

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (25)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad (26)$$

The mechanisms of lipid oxidation by reactive oxygen species involve initiation by ·OH and propagation reactions involve oxygen consumption and formation and reactions of peroxyl radicals (reactions *d* and *e*). End products of lipid peroxidation typically include lipid hydroperoxides, aldehydes, and other products derived from decomposition of unstable oxidized intermediates. Iron would further participate in lipid peroxidation processes by increasing the chain length of propagation by decomposing lipid hydroperoxides to either LO· and LOO· radicals in a chain-branching redox cycling mechanism:

$$ROOH + Fe^{3+} \rightarrow ROO^{\cdot} + H^+ + Fe^{2+} \quad (27)$$

$$ROOH + Fe^{2+} \rightarrow RO^{\cdot} + OH^- + Fe^{3+} \quad (28)$$

Peroxynitrite is able to initiate lipid peroxidation. Indeed, in model membrane systems such as phosphatidylcholine liposomes, lipid peroxidation was evidenced by formation of malondialdehyde, conjugated dienes, oxygen consumption, and liquid chromatography–mass spectrometry (LC–MS) analysis of lipid hydroperoxides formation (Radi *et al.,* 1991b; Rubbo *et al.,* 1994b). Peroxynitrite oxidation of linolenic acid yields significant amounts of hydroperoxo- and hydroxy-linolenate (Rubbo *et al.,* 1994b).

Peroxynitrite initiated lipid peroxidation most effectively at acidic pH, which is consistent with ONOOH rather than $ONOO^-$ as the oxidizing species (Radi *et al.,* 1991b; Shustov *et al.,* 2000). It is possible that lipid peroxidation also involves the participation of low concentrations of ·NO_2 or ·NO produced in these reactions:

$$ONOO^- + H^+ \rightleftharpoons ONOOH \rightarrow {}^{\cdot}OH + {}^{\cdot}NO_2 \quad (29)$$

$$ONOO^- + {}^{\cdot}OH \rightarrow ONOO^{\cdot} + OH^- \quad (30)$$

$$ONOO^- \rightarrow O_2 + {}^{\cdot}NO \quad (31)$$

Hydroxyl radical reacts with unsaturated fatty acids at near diffusion-controlled rates ($k \approx 10^9\ M^{-1}\ s^{-1}$) (Fig. 8, reaction *a*) and the ·NO_2 formed during ONOOH decomposition is a lipophilic radical, capable of initiating fatty acid oxidation as well ($k = 10^5\ M^{-1}\ s^{-1}$) by forming a carbon-centered alkyl radical (Fig. 8, reaction *b*). At low oxygen tensions, the carbon-centered alkyl radical combines with a second ·NO_2 to form allylic nitro (LNO_2) products (reaction *c*).

In addition, both ·NO_2 and ·NO can react with peroxyl radicals to form $LOONO_2$ and LOONO, respectively, giving rise to nonradical end products of lipid peroxidation (reactions *f* and *g*). Indeed, in addition to the predominant hydroperoxy and hydroxy derivatives formed after treatment of linolenic acid with peroxynitrite, the formation of nitrogen-containing lipid species has been also evidenced (Rubbo *et al.,* 1994b). These products have been tentatively identified from their molecular weight as nitritolinolenate, nitrosoperoxolinolenate, hydroxylnitrosoperoxolinolenate, and hydroperoxonitrosoperoxolinolenate. More recently, it was shown that the products of the reaction of peroxynitrite plus linoleic acid displayed mass/charge characteristics of nitrated lipid derivatives (O'Donnell, 1999). Some nitrogen-containing lipid intermediates appear to be highly unstable and may decompose to reinitiate radical processes. In particular, the product of the peroxyl radical/·NO condensation reaction (LOONO) may be cleaved by homolysis (reaction

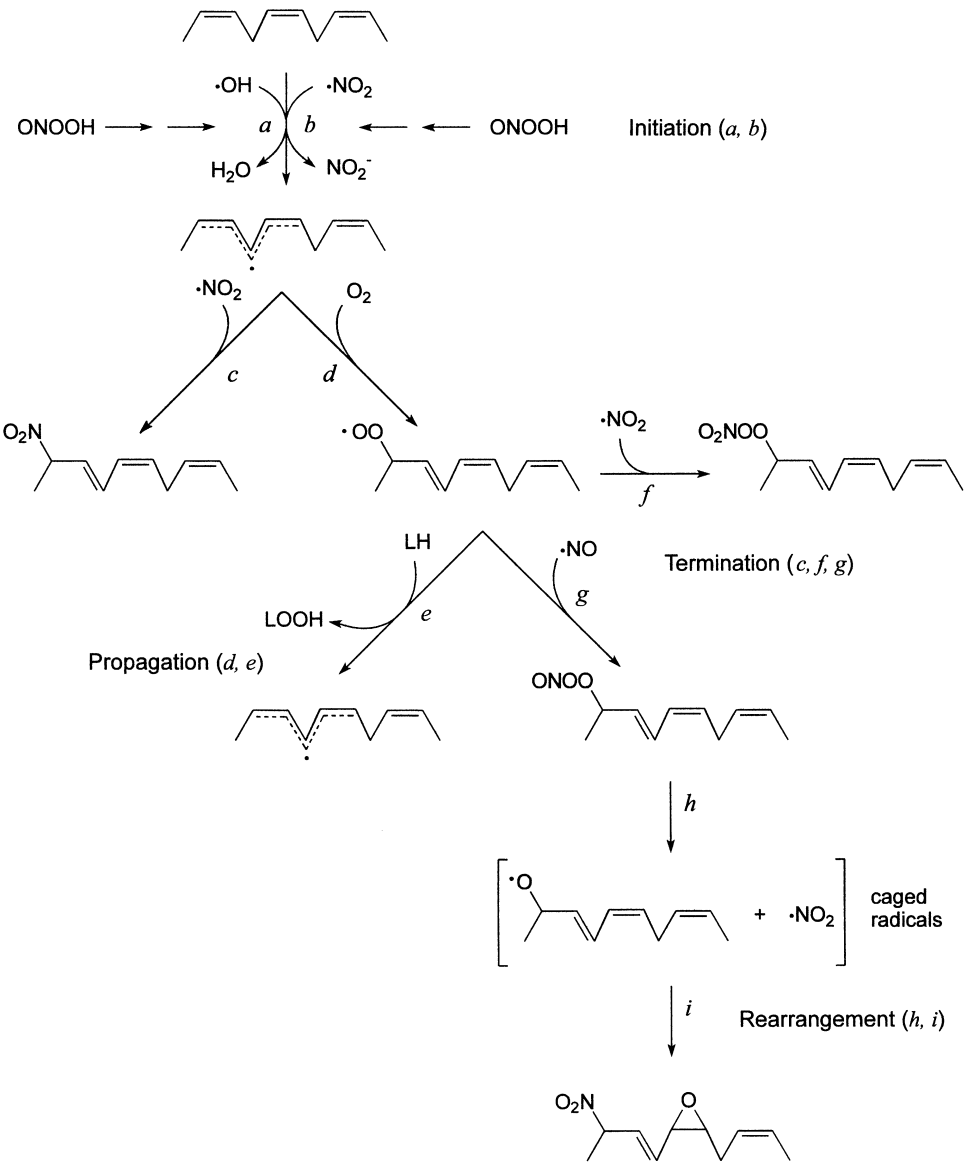


Figure 8 Scheme showing the major events occurring during peroxynitrite-dependent lipid oxidation.

h) to LO· and $^{\cdot}NO_2$, with rearrangement of LO· to an epoxyallylic acid radical L(O)· followed by recombination of L(O)· with $^{\cdot}NO_2$ (reaction i, [O'Donnell, 1999]).

Importantly, while iron is often required to initiate lipid peroxidation in many oxidizing systems, peroxynitrite induces lipid oxidation in model systems without the requirement of transition metals, since the metal chelator diethylenetriaminopentaacetic acid (DTPA) and iron depletion of reaction systems are only partially inhibitory (Radi *et al.,* 1991b).

Nucleic Acids

Peroxynitrite is able to cause significant DNA damage, including base modification, sugar oxidation, and strand breaks (Burney *et al.,* 1999).

On exposure of bases to peroxynitrite, several oxidative modifications were identified. The most reactive base seems to be guanine and the formation of 8-oxoguanine has been detected (Kennedy *et al.,* 1997a). 8-Oxoguanine is easily monitored for DNA damage and is known to cause GC–AT transitions. But further reaction of 8-oxoguanine with peroxynitrite may complicate its detection (Uppu *et al.,* 1996b). Remarkably, 8-nitroguanine has also been detected after peroxynitrite exposure (Yermilov *et al.,* 1995), which may be a useful marker of nucleic acid interactions with peroxynitrite. Most studies on peroxynitrite-dependent DNA damage have been conducted *in vitro* using nucleosides or isolated DNA. In addition, oxidative damage of bases has also been observed in the DNA of immunostimulated macrophages (deRojas-Walker *et al.,* 1995), as well as in spleen DNA of mice induced to produce high

levels of ·NO (Nair *et al.,* 1998). These processes were prevented by NOS inhibitors.

Peroxynitrite can cause DNA strand breaks (King *et al.,* 1993), which have been detected both in isolated DNA (Salgo *et al.,* 1995a) and in cells exposed to exogenous peroxynitrite (Salgo *et al.,* 1995b). Mechanistically, the strand breaks seem to arise both from sugar damage and from base modification (Burney *et al.,* 1999). The formation of strand breaks has been shown to activate poly(ADP-ribose) synthetase (PARS), leading to NAD^+ consumption followed by energy depletion (Szabo and Ohshima, 1997).

Carbon dioxide increases formation of nitroguanine but decreases DNA strand breaks (Yermilov *et al.,* 1996) and it should be an important modulator of nucleic acid modifications by peroxynitrite *in vivo.*

Peroxynitrite in Action

The outcome observed after peroxynitrite interactions with cellular structures is a consequence of its physicochemical, biochemical, and toxicological properties. We will outline actions promoted by peroxynitrite in two rather different and relevant biostructures (mammalian mitochondria and low density lipoprotein), with the aim of integrating various aspects of the biological chemistry of peroxynitrite.

PEROXYNITRITE INTERACTIONS WITH MITOCHONDRIA

Peroxynitrite interactions with mitochondrial components account for much of the mitochondrial toxicity previously ascribed to ·NO and it represents an important mechanism leading to mitochondrial dysfunction (Cassina and Radi, 1996; Lizasoain *et al.,* 1996; Radi *et al.,* 1994). Peroxynitrite present in mitochondria may arise from either direct diffusion from extramitochondrial compartments or from intramitochondrial formation. In turn, peroxynitrite inside the mitochondria is formed from mitochondrial $O_2^{\cdot-}$ reacting either with ·NO diffusing from the cytosol or with ·NO formed by NOS that may be present in mitochondria (Giulivi *et al.,* 1998; Radi *et al.,* 1994).

At the mitochondrial electron transport chain, peroxynitrite causes inactivation of complex II (succinate dehydrogenase) and to a lesser extent complex I (NADH dehydrogenase) (Radi *et al.,* 1994); in addition it inactivates complex V (ATP synthase). Thus, peroxynitrite leads to a profound inhibition of electron transport and ATP synthesis (Richter *et al.,* 1999). It is important to remark that peroxynitrite does not affect complex IV-dependent respiration (cytochrome *c* oxidase) in intact mitochondria, in line with its known resistance to inactivation by biological oxidants (Zhang *et al.,* 1990). Moreover, reduced cytochrome *c* oxidase map decompose peroxynitrite by a two-electron redox reaction (Pearce *et al.,* 1999). Cytochrome *c* oxidase is sensitive to peroxynitrite only when reactions are carried out with the isolated complex IV (Sharpe and Cooper, 1998b), underscoring the importance of preferential competing reactions *in vivo.* In contrast, ·NO readily interacts with the metal centers of cytochrome a_3, leading to reversible inhibition of mitochondrial electron transfer and oxygen consumption (Cassina and Radi, 1996; Poderoso *et al.,* 1996; Takehara *et al.,* 1996).

Other critical enzymes inactivated by peroxynitrite in mitochondria include aconitase (Castro *et al.,* 1994; Hausladen and Fridovich, 1994; Kennedy *et al.,* 1997b) and creatine kinase (Konorev *et al.,* 1998; Stachowiak *et al.,* 1998), both linked to energy metabolism and ATP homeostasis. In addition, peroxynitrite inactivates Mn-SOD, which may play a critical role in propagating peroxynitrite-mediated mitochondrial damage. Since peroxynitrite interactions with mitochondrial electron transport chain components lead to enhanced $O_2^{\cdot-}$ production (Radi *et al.,* 1994), the inactivation of Mn-SOD further contributes to raise intramitochondrial steady-state concentrations of $O_2^{\cdot-}$. Increased mitochondrial $O_2^{\cdot-}$ contributes to mitochondrial and cell oxidative injury via combined mechanisms including reactions of reactive oxygen species with mitochondrial components, diffusion of its dismutation product hydrogen peroxide to the cytosol, and enhanced peroxynitrite formation in mitochondria.

The reaction of peroxynitrite with carbon dioxide would be particularly relevant in the mitochondrial matrix where CO_2 levels are high, due to its continuous production by the Krebs cycle. Glutathione consumption by peroxynitrite will lead to a decreased activity of mitochondrial glutathione peroxidase and therefore to an enhancement of H_2O_2 levels.

Peroxynitrite also influences mitochondrial calcium homeostasis, favoring calcium efflux from mitochondria (Gadelha *et al.,* 1997; Packer and Murphy, 1994; Schweizer and Richter, 1996). The egress of calcium from mitochondria challenged by peroxynitrite is partly due to the inhibition of energy metabolism and also to the stimulation of the pyridine nucleotide-linked calcium release pathway. Finally, peroxynitrite increases mitochondrial membrane permeability by (a) opening of the permeability transition pore which depolarizes mitochondria and (b) a less specific mechanism involving membrane lipid and protein oxidation, leading to protein thiol cross-linking. Increased membrane permeability by peroxynitrite may lead to the release of components to the cytosol, including cytochrome *c,* that can participate in the mitochondrial signaling of apoptosis (Richter, 1998). Interestingly, excess ·NO formation may lead to peroxynitrite-dependent cytochrome *c* nitration (Cassina *et al.,* 2000; Hortelano *et al.,* 1999) but the correlation between cytochrome *c* nitration, release, and apoptosis remains to be established.

Due to the multiciplicity of mitochondrial targets and actions, the mitochondrial half-life of peroxynitrite is expected to be rather short (e.g., <5 ms) and therefore peroxynitrite is unable to diffuse significantly to extramitochondrial compartments.

LOW DENSITY LIPOPROTEIN OXIDATION

Peroxynitrite is a mediator of vascular injury in multiple forms of cardiovascular disease. First, peroxynitrite formation indirectly promotes atherogenesis by abrogating the physiological actions of ·NO, since peroxynitrite acts only as a comparatively weak stimulus for guanylate cyclase activity

in smooth muscle cells. Second, due to its potent oxidizing properties, peroxynitrite can participate in the oxidation of lipoproteins, whose modification is involved in the fatty streak production and subsequent plaque formation characteristic of the atherosclerotic lesion.

The low density lipoprotein (LDL) particle consists of an apolar core of cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipids, unesterified cholesterol, lipophilic antioxidants (α-tocopherol, ubiquinol 10, β-carotene), and one molecule of apolipoprotein B-100. Cholesteryl esters are the most abundant lipid class found in LDL (Schuster *et al.,* 1995). Thus, both the lipid and protein components of LDL could potentially be oxidized and nitrated by peroxynitrite. Peroxynitrite oxidizes LDL, causing a rapid depletion of endogenous antioxidants such as α-tocopherol (Hogg *et al.,* 1993a) and carotenoids (Panasenko *et al.,* 2000; Pannala *et al.,* 1998). The simultaneous production of $^{\cdot}NO$ and $O_2^{\cdot-}$ by 1,3-morpholino-sydnonimine hydrochloride (SIN-1) has been shown to oxidize LDL to a potentially atherogenic form via formation of $ONOO^-$ (Darley-Usmar *et al.,* 1992; Hogg *et al.,* 1993b; White *et al.,* 1994). Peroxynitrite converts the LDL to a form readily recognized by macrophage scavenger receptors (Graham *et al.,* 1993). More recently, it has been reported that exposure to peroxynitrite or SIN-1 resulted in time-dependent oxidation of both LDL lipids and protein (Thomas *et al.,* 1998).

Antibodies to nitrotyrosine show immunoreactivity in fatty streaks of coronary arteries of young autopsy subjects (Beckman *et al.,* 1994). In older patients, nitrotyrosine immunoreactivity is found in close association with foam cells and vascular endothelium, and in the neointima of advanced atherosclerotic lesions. In addition, measurements of 3-nitrotyrosine in LDL isolated from human atherosclerotic lesions show that there is a striking 90-fold increase compared with circulating LDL (Leeuwenburgh *et al.,* 1997).

Nitrotyrosine immunoreactivity may indicate peroxynitrite-dependent reactions during both early and chronic stages of atherosclerotic disease, which result in the formation of highly immunogenic and potentially proinflammatory protein oxidation products. However, it is important to note that myeloperoxidase may also play a role in nitrotyrosine formation during LDL modification because it can convert nitrite to a reactive intermediate that nitrates the aromatic ring of tyrosine (Eiserich *et al.,* 1996). Moreover, catalytically active myeloperoxidase is a component of human atherosclerotic tissue, where it colocalizes with foamy macrophages in the cellular-rich regions of lesions (Daugherty *et al.,* 1994). Oxidation products of the enzyme have been detected by immunohistochemistry in atherosclerotic vascular lesions, suggesting that myeloperoxidase promotes LDL oxidation *in vivo* (Hazell *et al.,* 1996; Podrez *et al.,* 1999). Thus, it is possible to envision a physiopathological scenario where endothelial and inflammatory cells contribute to LDL nitration via peroxynitrite-dependent and -independent mechanisms.

These observations raise the possibility that $^{\cdot}NO$, by the formation of secondary nitrogen oxides (i.e., $ONOO^-$, $^{\cdot}NO_2$), may promote atherogenesis, in contrast to its well-established direct antioxidant effects (Bolton *et al.,* 1994; Goss *et al.,* 1995; Hogg *et al.,* 1993c; Rubbo *et al.,* 1995; Rubbo and Radi, 2000).

Peroxynitrite Scavengers and Pharmacology

Pharmacological Approaches against Peroxynitrite-Mediated Toxicity

Peroxynitrite toxicity can be counteracted through several lines and direct scavenging is just one of them. In principle, any compound that inhibits $^{\cdot}NO$ production will yield an effective reduction of peroxynitrite formation, and the same principle is applicable to superoxide dismutation catalysis (Fig. 9). Therefore, NOS inhibitors and SOD mimics can be used to prevent peroxynitrite-mediated toxicity.

Once formed, peroxynitrite can be catalytically isomerized to nitrate or directly scavenged by sacrificial reductants; alternatively, the reactivity can be directed toward the oxidation of easily recoverable reductants such as glutathione, ascorbate, or uric acid.

Finally, if peroxynitrite damage is accomplished, some of its side effects may be reverted, such as reduction of radical intermediates and repair of oxidized moieties (Castro *et al.,* 1998) and potential reduction and elimination of nitrated groups (Kamisaki *et al.,* 1998; Grune *et al.,* 1998).

The design of peroxynitrite inactivators can involve more than one of the above-mentioned activities.

Peroxynitrite Scavengers

The scavenging of peroxynitrite in a biological milieu implies competition with other potential targets of oxidation.

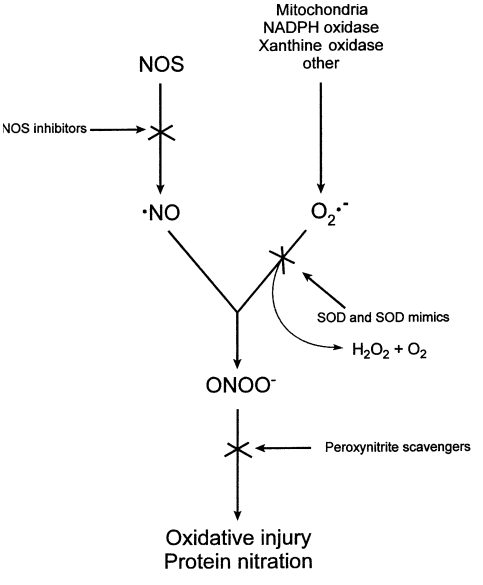


Figure 9 Pharmacological interventions to prevent peroxynitrite-mediated injury. Peroxynitrite formation can be inhibited by dismutation of superoxide or inhibition of NOS, or it can be scavenged directly.

Success in that competition will depend on the concentration of targets, their reactivity, and properties of the medium such as pH.

It is clear that in many cases the reaction with CO_2 dictates a significant part of the fate of peroxynitrite. It is also clear that under conditions of oxidative stress the natural antioxidant systems (such as GSH) cannot directly cope with $ONOOCO_2^-$ overproduction. Therefore, an efficient peroxynitrite scavenger would be able to divert $ONOO^-$ oxidative potential toward ''expendable'' reductants and could inhibit $ONOOCO_2^-$ formation, reactivity, or both.

Two different approaches have been devised to find a suitable decomposition catalyst: catalytic isomerization and catalytic reduction.

CATALYSIS OF ISOMERIZATION

Catalysis of peroxynitrite isomerization to nitrate represents an important strategy, given that relatively less toxic intermediates are formed during the catalytic process (Fig. 10, reaction *a*). Three iron porphyrins were reported to catalyze the isomerization to nitrate *in vitro,* with k_{cat} ranging from 10^5 to 10^6 M^{-1} s^{-1}, and showed promising results in cell culture and animal models of inflammation (Misko *et al.,* 1998; Salvemini *et al.,* 1998). It is important to note, however, that a reactive intermediate is generated during the catalytic cycle, implying that these iron porphyrins redirect, more than abolish, peroxynitrite reactivity.

CATALYSTS OF REDUCTION

The rationale behind these scavengers is that they can be oxidized by peroxynitrite very rapidly and then be reduced back to the original compound by endogenous reductants in another fast reaction (Fig. 10, *b*). The selenium compound ebselen was the first proposed cyclic scavenger (10^6 M^{-1} s^{-1}) (Masumoto and Sies, 1996b) using glutathione as reductant.

More recently, SOD mimic manganese and iron porphyrins were found to act as catalysts of peroxynitrite reduction using ascorbate, glutathione, Trolox, or urate as reducing substrates (Ferrer-Sueta *et al.,* 1999; Lee *et al.,* 1997, 1998) with rate constants ranging from 10^6 to 10^7 M^{-1} s^{-1}. However, the mechanism of this catalysis is not as simple as initially proposed; the metal (M) redox cycling between three 3+ and 4+ oxidation states should be expanded to include the 2+ state formed by reaction of the metal complexes with reductants. Studies are currently under way to understand the potential of these compounds as peroxynitrite scavengers.

OTHER SCAVENGERS

Hydroxamates Desferrioxamine has attracted attention as a potential scavenger although it does not react rapidly with peroxynitrite (Deliconstantinos and Villiotou, 1996; Denicola *et al.,* 1995; Lamarque and Whittle, 1995; Oury *et al.,* 1993; Radi *et al.,* 1991b). Desferrioxamine reaction with peroxynitrite leads to the formation of a desferrioxamine-derived nitroxide radical (Denicola *et al.,* 1995). This inhibition of peroxynitrite reactivity by desferrioxamine is independent of chelation of transition metals. Since there is no direct reaction between desferrioxamine and peroxynitrite, it is proposed to react with ·OH but the mechanism of inhibition remains to be established (Beckman *et al.,* 1990; Radi *et al.,* 1991b; Alvarez *et al.,* 1995; Denicola *et al.,* 1995).

Guanidine Derivatives Guanidine derivatives integrate two functions; they serve as inhibitors of iNOS, thus inhibiting ·NO overproduction under conditions of inflammation. Additionally, a reactive group, such as a thiol (e.g., mercaptoethyl guanidine) can be attached to the guanidine moiety to provide some direct scavenging of peroxynitrite

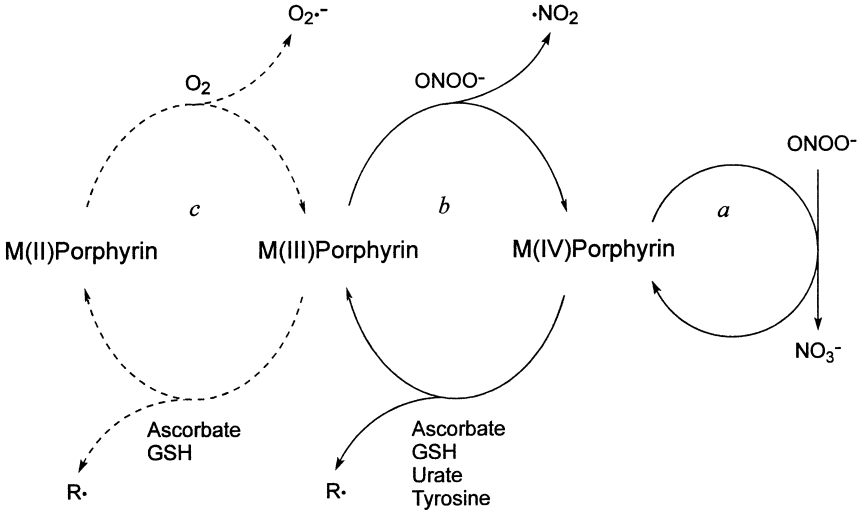


Figure 10 Catalysis of isomerization and reduction of peroxynitrite. (*a*) Fe^{IV} porphyrins can catalyze the isomerization of peroxynitrite. (*b*) The one-electron redox-cycling of Mn and Fe porphyrins has been shown to catalyze the reduction of peroxynitrite. (*c*) Metal porphyrins can also catalyze the autooxidation of glutathione and ascorbate.

(Brahn *et al.,* 1998; Cuzzocrea *et al.,* 1998; Lohinai *et al.,* 1998; Panas *et al.,* 1998; Szabo, 1998; Szabo *et al.,* 1997a; Zingarelli *et al.,* 1997, 1998) and proved to be efficient in different models of peroxynitrite-mediated injury.

Conclusions

Peroxynitrite, the reaction product of $^{\cdot}NO$ and $O_2^{\cdot-}$, is a strong oxidizing biomolecule. Both $ONOO^-$ and ONOOH participate in a variety of reactions including one- and two-electron oxidations, nitrations, and to a lesser extent nitrosation. Biologically, direct reactions of peroxynitrite with biomolecules predominate over homolysis of ONOOH. Preferential reactions *in vivo* include those with CO_2, metal centers, and thiols. Formation of $ONOOCO_2^-$ and peroxynitrite–transition metal adducts redirects peroxynitrite reactivity and promote nitrations (e.g., tyrosine nitration). Glutathione plays a central role in inhibiting oxidation processes mediated by peroxynitrite. The biological half-life of peroxynitrite is short, typically less than 20 ms, due to target molecule reactions. However, this time is long enough for peroxynitrite to travel one or two cell diameters and exert toxic effects in target cells or extracellular milieu. In addition to its direct cytotoxic effects by interactions with biomolecules (e.g., proteins, lipids, DNA) and subcellular structures (e.g., mitochondria), the role and mechanisms of peroxynitrite modulation of signal transduction pathways and signaling of cell death remain to be elucidated. Pharmacological strategies against peroxynitrite toxicity are being developed and will help both to unravel the contribution of peroxynitrite to oxidant-mediated pathologies and to neutralize its deleterious effects.

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References

Alayash, A. I., Ryan, B. A., and Cashon, R. E. (1998). Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins. *Arch. Biochem. Biophys.* **349,** 65–73.

Alvarez, B., Denicola, A., and Radi, R. (1995). Reaction between peroxynitrite and hydrogen peroxide: formation of oxygen and slowing of peroxynitrite decomposition. *Chem. Res. Toxicol.* **8,** 859–864.

Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B. A., and Radi, R. (1996). Peroxynitrite-dependent tryptophan nitration. *Chem. Res. Toxicol.* **9,** 390–396.

Alvarez, B., Ferrer-Sueta, G., and Radi, R. (1998). Slowing of peroxynitrite decomposition in the presence of mannitol and ethanol. *Free Radicals Biol. Med.* **24,** 1331–1337.

Alvarez, B., Ferrer-Sueta, G., Freeman, B. A., and Radi, R. (1999). Kinetics of peroxynitrite reaction with amino acids and human serum albumin. *J. Biol. Chem.* **274,** 842–848.

Anbar, M., and Taube, H. (1954). Interaction of nitrous acid with hydrogen peroxide and with water. *J. Am. Chem. Soc.* **76,** 6243–6247.

Ara, J., Przedborski, S., Naini, A. B., Jackson-Lewis, V., Trifiletti, R. R., Horwitz, J., and Ischiropoulos, H. (1998). Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7659–7663.

Asahi, M., Fujii, J., Takao, T., Kuzuya, T., Hori, M., Shimonishi, Y., and Taniguchi, N. (1997). The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor. *J. Biol. Chem.* **272,** 19152–19157.

Augusto, O., Gatti, R. M., and Radi, R. (1994). Spin-trapping studies of peroxynitrite decomposition and of 3-morpholinosydnonimine *N*-ethylcarbamide autooxidation: Direct evidence for metal-independent formation of free radical intermediates. *Arch. Biochem. Biophys.* **310,** 118–125.

Balavoine, G. G., Geletti, Y. V., and Bejan, D. (1997). Catalysis of peroxynitrite reactions by manganese and iron porphyrins. *Nitric Oxide* **1,** 507–521.

Balazy, M., Kaminski, P. M., Mao, K., Tan, J., and Wolin, M. S. (1998). *S*-Nitroglutathione, a product of the reaction between peroxynitrite and glutathione that generates nitric oxide. *J. Biol. Chem.* **273,** 32009–32015.

Bartlett, D., Church, D. F., Bounds, P. L., and Koppenol, W. H. (1995). The kinetics of the oxidation of L-ascorbic acid by peroxynitrite. *Free Radicals Biol. Med.* **18,** 85–92.

Beckman, J. S. (1990). Ischaemic injury mediator. *Nature* **345,** 27–28.

Beckman, J. S. (1996). ''The Physiological and Pathological Chemistry of Nitric Oxide.'' Academic Press, New York.

Beckman, J. S., Carson, M., Smith, C. D., and Koppenol, W. H. (1993). ALS, SOD and peroxynitrite. *Nature* **364,** 584.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992). Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch. Biochem. Biophys.* **298,** 438–445.

Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol. Chem. Hoppe-Seyler* **375,** 81–88.

Berlett, B. S., Levine, R. L., and Stadtman, E. R. (1998). Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase: Both modifications mimic effects of adenylylation. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2784–2789.

Bielski, B. H., Arudi, R. L., and Sutherland, M. W. (1983). A study of the reactivity of HO_2/O_2^- with unsaturated fatty acids. *J. Biol. Chem.* **258,** 4759–4761.

Bohle, S. D., and Hansert, B. (1997). O-atom scrambling in the aqueous isomerization of pernitrous acid. *Nitric Oxide: Biol. Chem.* **1,** 502–506.

Bohle, D. S., Hansert, B., Paulson, S. C., and Smith, B. D. (1994). Biomimetic synthesis of the putative cytotoxin peroxynitrite, $ONOO^-$, and its characterization as a tetramethylammonium salt. *J. Am. Chem. Soc.* **116,** 7423–7424.

Bohle, D. S., Glassbrenner, P. A., and Hansert, B. (1996). Syntheses of pure tetramethylammonium peroxynitrite. *Methods Enzymol.* **269,** 302–311.

Bolton, E. J., Jessup, W., Stanley, K. K., and Dean, R. T. (1994). Enhanced LDL oxidation by murine macrophage foam cells and their failure to secrete nitric oxide. *Atherosclerosis* **106,** 213–223.

Bonini, M. G., Radi, R., Ferrer-Sueta, G., Ferreira, A. M. D. C., and Augusto, O. (1999). Direct epr detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J. Biol. Chem.* in press.

Boulos, C., Jiang, H., and Balazy, M. (2000). Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues. *J. Pharmacol. Exp. Ther.* **293,** 229–229.

Brahn, E., Banquerigo, M. L., Firestein, G. S., Boyle, D. L., Salzman, A. L., and Szabo, C. (1998). Collagen induced arthritis: Reversal by mercaptoethylguanidine, a novel antiinflammatory agent with a combined mechanism of action. *J. Rheumatol.* **25,** 1785–1793.

Brito, C., Naviliat, M., Tiscornia, A. C., Vuillier, F., Gualco, G., Dighiero, G., Radi, R., and Cayota, A. M. (1999). Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. *J. Immunol.* **162,** 3356–3366.

Briviba, K., Roussyn, I., Sharov, V. S., and Sies, H. (1996). Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. *Biochem. J.* **319**(Pt. 1), 13–15.

Briviba, K., Kissner, R., Koppenol, W. H., and Sies, H. (1998a). Kinetic study of the reaction of glutathione peroxidase with peroxynitrite. *Chem. Res. Toxicol.* **11,** 1398–1401.

Briviba, K., Tamler, R., Klotz, L. O., Engman, L., Cotgreave, I. A., and Sies, H. (1998b). Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. *Biochem. Pharmacol.* **55,** 817–823.

Burney, S., Caulfield, J. L., Niles, J. C., Wishnok, J. S., and Tannenbaum, S. R. (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat. Res.* **424,** 37–49.

Cassina, A., and Radi, R. (1996). Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* **328,** 309–316.

Cassina, A., Hodara, R., Souza, J., Thomson, L., Castro, L., Ischiropoulos, H., Freeman, B. A., and Radi, R. (2000). Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.,* in press.

Castro, L., Rodriguez, M., and Radi, R. (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.* **369),** 29409–29415.

Castro, L. A., Robalinho, R. L., Cayota, A., Meneghini, R., and Radi, R. (1998). Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. *Arch. Biochem. Biophys.* **359,** 215–224.

Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59,** 527–605.

Chen, S. N., and Hoffman, M. Z. (1973). Rate constants for the reaction of carbonate radical with compounds of biochemical interest in neutral aqueous solution. *Radiat. Res.* **56,** 40–47.

Chen, S., and Hoffman, M. Z. (1974). Reactivity of the carbonate radical in aqueous solution. Trytophan and its derivatives. *J. Phys. Chem.* **78,** 2099–2101.

Cheng, B., Lee, J., and Lee, Y. (1991). Photolysis of nitric acid in solid argon: the infrared absorption of peroxynitrous acid (HOONO). *J. Phys. Chem.* **95,** 2814–2817.

Coddington, J. W., Hurst, J. K., and Lymar, S. V. (1999). Hydroxyl radical formation during peroxynitrous acid decomposition. *J. Am. Chem. Soc.* **121,** 2438–2443.

Crow, J. P., Beckman, J. S., and McCord, J. M. (1995). Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry* **34**(11), 3544–3552.

Cuzzocrea, S., Zingarelli, B., M., O. C., Salzman, A. L., Caputi, A. P., and Szabo, C. (1997). Role of peroxynitrite and activation of poly (ADP-ribose) synthase in the vascular failure induced by zymosan-activated plasma. *Br. J. Pharmacol.* **122,** 493–503.

Cuzzocrea, S., Zingarelli, B., Hake, P., Salzman, A. L., and Szabo, C. (1998). Antiinflammatory effects of mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, in carrageenan-induced models of inflammation. *Free Radical Biol. Med.* **24,** 450–459.

Darley-Usmar, V. M., Hogg, N., O Leary, V. J., Wilson, M. T., and Moncada, S. (1992). The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radicals Res. Commun.* **17,** 9–20.

Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994). Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J. Clin. Invest.* **94,** 437–444.

Deliconstantinos, G., and Villiotou, V. (1996). NO synthase and xanthine oxidase activities of rabbit brain synaptosomes: Peroxynitrite formation as a causative factor of neurotoxicity. *Neurochem. Res.* **21,** 51–56.

Denicola, A., Rubbo, H., Rodriguez, D., and Radi, R. (1993). Peroxynitrite-mediated cytotoxicity to *Trypanosoma cruzi. Arch. Biochem. Biophys.* **304,** 279–286.

Denicola, A., Souza, J. M., Gatti, R. M., Augusto, O., and Radi, R. (1995). Desferrioxamine inhibition of the hydroxyl radical-like reactivity of peroxynitrite: Role of the hydroxamic groups. *Free Radicals Biol. Med.* **19,** 11–19.

Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* **333,** 49–58.

Denicola, A., Souza, J. M., and Radi, R. (1998). Diffusion of peroxynitrite across erythrocyte membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 3566–3571.

deRojas-Walker, T., Tamir, S., Ji, H., Wishnok, J. S., and Tannenbaum, S. R. (1995). Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chem. Res. Toxicol.* **8,** 473–477.

Donald, C. E., Hughes, M. N., Thompson, J. M., and Bonner, F. T. (1986). Photolysis of th N=N bond in trioxodinitrato: Reactions between triplet NO^- and O_2 to form peroxynitrite. *Inorg. Chem.* **25,** 2676–2677.

Doyle, M. P., and Hoekstra, J. W. (1981). Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Biochem.* **14,** 351–358.

Eiserich, J. P., Butler, J., van der Vliet, A., Cross, C. E., and Halliwell, B. (1995). Nitric oxide rapidly scavenges tyrosine and tryptophan radicals. *Biochem. J.* **310,** 745–749.

Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and van der Vliet, A. (1996). Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. *J. Biol. Chem.* **271,** 19199–19208.

Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B., A., Halliwell, B., and van der Vliet, A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature,* **391,** 393–397.

Endres, M., Scott, G., Namura, S., Salzman, A. L., Huang, P. L., Moskowitz, M. A., and Szabo, C. (1998). Role of peroxynitrite and neuronal nitric oxide synthase in the activation of poly(ADP-ribose) synthetase in a murine model of cerebral ischemia-reperfusion. *Neurosci. Lett.* **248,** 41–44.

Estevez, A. G., Radi, R., Barbeito, L., Shin, J. T., Thompson, J. A., and Beckman, J. S. (1995). Peroxynitrite-induced cytotoxicity in PC12 cells: Evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. *J. Neurochem.* **65,** 1543–1550.

Estevez, A. G., Spear, N., Manuel, S. M., Radi, R., Henderson, C. E., Barbeito, L., and Beckman, J. S. (1998). Nitric oxide and superoxide contribute to motor neuron apoptosis induced by trophic factor deprivation. *J. Neurosci.* **18,** 923–931.

Estevez, A. G., Crow, J. P., Sampson, J. B., Reiter, C., Zhuang, Y., Richardson, G. J., Tarpey, M. M., Barbeito, L., and Beckman, J. S. (1999). Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase, *Science* **286,** 2498–2500.

Estevez, A. G., Sampson, J. B., Zhuang, Y., Spear, N., Richardson, G. J., Crow, J. P., Tarpey, M. M., Barbeito, L., and Beckman, J. S. (2000). Liposome-delivered superoxide dismutase prevents nitric oxide-dependent motor neuron death induced by trophic factor withdrawal. *Free Radic. Biol. Med.* **28,** 437–446.

Exner, M., and Herold, S. (2000). Kinetic and mechanistic studies of the peroxynitrite-mediated oxidation of oxymyoglobin and oxyhemoglobin. *Chem. Res. Toxicol.* **13,** 287–293.

Ferrer-Sueta, G., Ruiz-Ramirez, L., and Radi, R. (1997). Ternary copper complexes and manganese (III) tetrakis(4-benzoic acid) porphyrin

catalyze peroxynitrite-dependent nitration of aromatics. *Chem. Res. Toxicol.* **10,** 1338–1344.

Ferrer-Sueta, G., Batinic-Haberle, I., Spasojevic, I., Fridovich, I., and Radi, R. (1999). Catalytic scavenging of peroxynitrite by Isomeric Mn(III) *N*-methylpyridylporphyrins in the presence of reductants. *Chem. Res. Toxicol.,* 12, 442–449.

Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993). Interaction of myeloperoxidase with peroxynitrite. A comparison with lactoperoxidase, horseradish peroxidase and catalase. *Eur. J. Biochem.* **215,** 767–775.

Forsmark-Andree, P., Persson, B., Radi, R., Dallner, G., and Ernster, L. (1996). Oxidative modification of nicotinamide nucleotide transhydrogenase in submitochondrial particles: Effect of endogenous ubiquinol. *Arch. Biochem. Biophys.* **336,** 113–120.

Freeman, B. A., and Crapo, J. D. (1982). Biology of disease: Free radicals and tissue injury. *Lab. Invest.* **47,** 412–426.

Gadelha, F. R., Thomson, L., Fagian, M. M., Costa, A. D., Radi, R., and Vercesi, A. E. (1997). Ca^{2+}-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. *Arch. Biochem. Biophys.* **345,** 243–250.

Gatti, R. M., Radi, R., and Augusto, O. (1994). Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. *FEBS Lett.* **348,** 287–290.

Gatti, R. M., Alvarez, B., Vasquez-Vivar, J., Radi, R., and Augusto, O. (1998). Formation of spin trap adducts during the decomposition of peroxynitrite. *Arch. Biochem. Biophys.* **349,** 36–46.

Gerasimov, O. V., and Lymar, S. V. (1999). The yield of hydroxyl radical from the decomposition of perxynitrous acid. *Inorg. Chem.* **38,** 4317–4321.

Girotti, A. W. (1985). Mechanisms of lipid peroxidation. *J. Free Radicals Biol. Med.* **1,** 87–95.

Giulivi, C., Poderoso, J. J., and Boveris, A. (1998). Production of nitric oxide by mitochondria. *J. Biol. Chem.* **273,** 11038–11043.

Goldstein, S., and Czapski, G. (1995a). The reaction of NO· with $O_2^{\cdot-}$ and $HO_2^{\cdot}$: A pulse radiolysis study. *Free Radicals Biol. Med.* **19,** 505–510.

Goldstein, S., and Czapski, G. (1995b). Direct and indirect oxidations by peroxynitrite. *Inorg. Chem.* **34,** 4041–4048.

Goldstein, S., and Czapski, G. (1997). The effect of bicarbonate on oxidation by peroxynitrite: Implication for its biological activity. *Inorg. Chem.* **36,** 5113–5117.

Goldstein, S., Saha, A., Lymar, S. V., & Czapski, G. (1998). Oxidation of peroxynitrite by inorganic radicals: A pulse radiolysis study. *J. Am. Chem. Soc.* **120,** 5549–5554.

Goldstein, S., and Czapski, G. (1998). Formation of peroxynitrate from the reaction of peroxynitrite with CO2: Evidence for carbonate radical production. *J. Am. Chem. Soc.* **120,** 3458–3463.

Goldstein, S., Meyerstein, D., van Eldik, R., and Czapski, G. (1999). Peroxynitrous acid decomposes via homolysis: Evidence from high-pressure pulse radiolysis. *J. Phys. Chem. A* **103,** 6587–6590.

Goldstein, S., Czapski, G., Lind, J., and Merenyi, G. (2000). Tyrosine nitration by simultaneous generation of ·NO and O_2 under physiological conditions. How the radicals do the job. *J. Biol. Chem.* **275,** 3031–3036.

Goss, S. P., Hogg, N., and Kalyanaraman, B. (1995). The antioxidant effect of spermine NONOate in human low-density lipoprotein. *Chem. Res. Toxicol.* **8,** 800–806.

Gow, A., Duran, D., Thom, S. R., and Ischiropoulos, H. (1996). Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch. Biochem. Biophys.* **333,** 42–48.

Grace, S. C., Salgo, M. G., and Pryor, W. A. (1998). Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA. *FEBS Lett.* **426,** 24–28.

Graham, A., Hogg, N., Kalyanaraman, B., O Leary, V., Darley-Usmar, V., and Moncada, S. (1993). Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **330,** 181–185.

Grune, T., Blasig, I. E., Sitte, N., Roloff, B., Haseloff, R., and Davies, K. J. (1998). Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J. Biol. Chem.* **273,** 10857–10862.

Gryglewski, R. J., J., P. R. M., and S., M. (1986). Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. *Nature* **320,** 454–456.

Guittet, O., Decottignies, P., Serani, L., Henry, Y., Le Marechal, P., Laprevote, O., and Lepoivre, M. (2000). Peroxynitrite-mediated nitration of the stable free radical tyrosine residue of the ribonucleotide reductase small subunit. *Biochemistry* **39,** 4640–4648.

Gunther, M. R., Hsi, L. C., Curtis, J. F., Gierse, J. K., Marnett, L. J., Eling, T. E., and Mason, R. P. (1997). Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation. *J. Biol. Chem.* **272,** 17086–17090.

Haendeler, J., Weiland, U., Zeiher, A. M., and Dimmeler, S. (1997). Effects of redox-related congeners of NO on apoptosis and caspase-3 activity. *Nitric Oxide* **1,** 282–293.

Hausladen, A., and Fridovich, I. (1994). Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.* **269,** 29405–29408.

Hazell, L. J., Arnold, L., Flowers, D., Waeg, G., Malle, E., and Stocker, R. (1996). Presence of hypochlorite-modified proteins in human atherosclerotic lesions. *J. Clin. Invest.* **97,** 1535–1544.

Herold, S. (1999). Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin. *FEBS Lett.* **443,** 81–84.

Hodges, G. R., and Ingold, K. U. (1999). Cage-escape of geminate radical pairs can produce peroxynitrate from peroxynitrite under a wide variety of experimental conditions. *J. Am. Chem. Soc.* **121,** 10695–10701.

Hogg, N., Darley-Usmar, V. M., Wilson, M. T., and Moncada, S. (1993a). The oxidation of α-tocopherol in human low density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Lett.* **326,** 199–203.

Hogg, N., Darley-Usmar, V. M., Graham, A., and Moncada, S. (1993b). Peroxynitrite and atherosclerosis. *Biochem. Soc. Trans.* **21,** 358–362.

Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., and Parthasarathy, S. (1993c). Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett.* **334,** 170–174.

Hogg, N., Singh, R. J., and Kalyanaraman, B. (1996). The role of glutathione in the transport and catabolism of nitric oxide. *FEBS Lett.* **382,** 223–228.

Hortelano, S., Alvarez, A. M., and Bosca, L. (1999). Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages. *FASEB J.* **13,** 2311–2317.

Houk, K. N., Condroski, K. R., and Pryor, W. A. (1996). Radical and concerted mechanisms in oxidations of amines, sulfides, and alkenes by peroxynitrite, peroxynitrous acid, and the peroxynitrite-CO_2 adduct. *J. Am. Chem. Soc.* **118,** 13002–13006.

Houston, M., Chumley, P., Radi, R., Rubbo, H., and Freeman, B. A. (1998). Xanthine oxidase reaction with nitric oxide and peroxynitrite. *Arch. Biochem. Biophys.* **355,** 1–8.

Hu, P., Ischiropoulos, H., Beckman, J. S., and Matalon, S. (1994). Peroxynitrite inhibition of oxygen consumption and sodium transport in alveolar type II cells. *Am. J. Physiol.* **266**(Pt. 1), L628–L634.

Hughes, M. N., and Nicklin, H. G. (1968). The chemistry of pernitrites. Part I. Kinetics of decomposition of pernitrous acid. *J. Chem. Soc. (A),* 450–452.

Huhmer, A. F., Nishida, C. R., Ortiz de Montellano, P. R., and Schoneich, C. (1997). Inactivation of the inducible nitric oxide synthase by peroxynitrite. *Chem. Res. Toxicol.* **10,** 618–626.

Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radicals Res. Commun.* **18,** 195–199.

Hunt, J. A., Lee, J., and Groves, J. T. (1997). Amphiphilic peroxynitrite decomposition catalysts in liposomal assemblies. *Chem Biol.* **4,** 845–858.

Hutchinson, F. (1957). The distance that a radical formed by ionizing radiation can difuse in a yeast cell. *Radiat. Res.* **7,** 473–483.

Ignarro, L. J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* **30,** 535–560.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D., and Beckman, J. S. (1992a). Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298,** 431–437.

Ischiropoulos, H., Zhu, L., and Beckman, J. S. (1992b). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* **298,** 446–451.

Ischiropoulos, H., Nelson, J., Duran, D., and Al-Mehdi, A. (1996). Reactions of nitric oxide and peroxynitrite with organic molecules and ferrihorseradish peroxidase: Interference with the determination of hydrogen peroxide. *Free Radicals Biol. Med.* **20,** 373–381.

Jennings, M. L. (1989). Structure and function of the red blood cell anion transport protein. *Annu. Rev. Biophys. Biophys. Chem.* **18,** 397–430.

Jiang, H., Kruger, N., Rana, D., Wang, D., Vatele, J., and Balazy, M. (1999). Nitrogen dioxide induces cis-trans-isomerization of arachidonic acid within cellular phospholipids. *J. Biol. Chem.* **274,** 16235–16241.

Jursic, B. S., Klasinc, L., Pecur, S., and Pryor, W. A. (1997). On the mechanism of HOONO to $HONO_2$ conversion. *Nitric Oxide: Biol. Chem.* **1,** 494–501.

Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 11584–11589.

Keith, W. G., and Powell, R. E. (1969). Kinetics of decompositions of peroxynitrous acid. *J. Chem. Soc. A,* 90.

Kennedy, L. J., Moore, K., Jr., Caulfield, J. L., Tannenbaum, S. R., and Dedon, P. C. (1997a). Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents. *Chem. Res. Toxicol.* **10,** 386–392.

Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997b). An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. *J. Biol. Chem.* **272,** 20340–20347.

Kennedy, M., Denenberg, A. G., Szabo, C., and Salzman, A. L. (1998). Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBe cells. *Gastroenterology,* **114,** 510–518.

Keyer, K., and Imlay, J. A. (1997). Inactivation of dehydratase [4Fe–4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J. Biol. Chem.* **272,** 27652–27659.

Kuhn, D. M., Aretha, C. W., and Geddes, T. J. (1999). Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. *J. Neurosci.* **19,** 10289–10294.

Kuhn, D. M., and Geddes, T. J. (1999). Peroxynitrite inactivates tryptophan hydroxylase via sulfhydryl oxidation. Coincident nitration of enzyme tyrosyl residues has minimal impact on catalytic activity. *J. Biol. Chem.* **274,** 29726–29732.

King, P. A., Jamison, E., Strahs, D., Anderson, V. E., and Brenowitz, M. (1993). 'Footprinting' proteins on DNA with peroxonitrous acid. *Nucleic Acids Res.* **21,** 2473–2478.

Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem. Res. Toxicol.* **10,** 1285–1292.

Klebl, B. M., Ayoub, A. T., and Pette, D. (1998). Protein oxidation, tyrosine nitration, and inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase in low-frequency stimulated rabbit muscle. *FEBS Lett.* **422,** 381–384.

Konorev, E. A., Hogg, N., and Kalyanaraman, B. (1998). Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett.* **427,** 171–174.

Koppenol, W. H. (1989). Generation and thermodynamic properties of oxyradicals. *In* V. P. C (Ed.), ''Focus on Membrane Lipid Oxidation'' (V.P.S., ed.), Vol. 1, pp. 1–13. CRC Press, Boca Raton, Florida.

Koppenol, W. H. (1998). The chemistry of peroxynitrite and its relevance to biological systems. Interrelations between free radicals and metal ions in life processes. *In* ''Metal Ions in Biological Systems'' (A. Sigel & H. Sigel eds.), Vol. 36. Dekker, New York.

Koppenol, W. H., and Kissner, R. (1998). Can O=NOOH undergo homolysis? *Chem. Res. Toxicol.* **11,** 87–90.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5,** 834–842.

Lamarque, D., and Whittle, B. J. (1995). Role of oxygen-derived metabolites in the rat gastric mucosal injury induced by nitric oxide donors. *Eur. J. Pharmacol.* **277,** 187–194.

Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996). Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15069–15074.

Lee, J., Hunt, J., and Groves, J. T. (1997). Rapid decomposition of peroxynitrite by manganese porphyrin-antioxidant redox couples. *Bioorg. Med. Chem. Lett.* **7,** 2913–2918.

Lee, J., Hunt, J. A., and Groves, J. T. (1998). Manganese porphyrins as redox-coupled peroxynitrite reductases. *J. Am. Chem. Soc.* **120,** 6053–6061.

Lee, C., Liu, X., and Zweier, J. L. (2000). Regulation of xanthine oxidase by nitric oxide and peroxynitrite. *J. Biol. Chem.* **275,** 9369–9376.

Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P., and Heinecke, J. W. (1997). Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J. Biol. Chem.* **272,** 1433–1436.

Lemercier, J. N., Squadrito, G. L., and Pryor, W. A. (1995). Spin trap studies on the decomposition of peroxynitrite. *Arch. Biochem. Biophys.* **321,** 31–39.

Lemercier, J. N., Padmaja, S., Cueto, R., Squadrito, G. L., Uppu, R. M., and Pryor, W. A. (1997). Carbon dioxide modulation of hydroxylation and nitration of phenol by peroxynitrite. *Arch. Biochem. Biophys.* **345,** 160–170.

Lin, K. T., Xue, J. Y., Nomen, M., Spur, B., and Wong, P. Y. (1995). Peroxynitrite-induced apoptosis in HL-60 cells. *J. Biol. Chem.* **270,** 16487–16490.

Lin, K. T., Xue, J. Y., Lin, M. C., Spokas, E. G., Sun, F. F., and Wong, P. Y. (1998). Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. *Am. J. Physiol.* **274** (Pt. 1), C855–C860.

Lizasoain, I., Moro, M. A., Knowles, R. G., Darley-Usmar, V., and Moncada, S. (1996). Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *Biochem. J.* **314**(Pt. 3), 877–880.

Lo, W., Lee, Y., Tsai, J. M., and Beckman, J. S. (1995). Ultraviolet absorption spectra of cis and trans potassium peroxynitrite (KOONO) in solid argon. *Chem. Phys. Lett.* **242,** 147.

Lohinai, Z., Benedek, P., Feher, E., Gyorfi, A., Rosivall, L., Fazekas, A., Salzman, A. L., and Szabo, C. (1998). Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. *Br. J. Pharmacol.* **123,** 353–360.

Lymar, S. V., and Hurst, J. K. (1995). Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J. Am. Chem. Soc.* **117,** 8867–8868.

Lymar, S. V., and Hurst, J. K. (1996). Carbon dioxide: Physiological catalyst for peroxynitrite-mediated cellular damage or cellular protectant? *Chem. Res. Toxicol.* **9,** 845–850.

Lymar, S. V., and Hurst, J. K. (1998a). Radical nature of peroxynitrite reactivity. *Chem. Res. Toxicol.* **11,** 714–715.

Lymar, S. V., and Hurst, J. K. (1998b). CO_2-Catalyzed one-electron oxidations by peroxynitrite: Properties of the reactive intermediate. *Inorg. Chem.* **37,** 294–301.

Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996). Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry.* **35,** 7855–7861.

Lynch, R. E., and Fridovich, I. (1978). Permeation of the erythrocyte stroma by superoxide radical. *J. Biol. Chem.* **253,** 4697–4699.

MacMillan-Crow, L. A., Crow, J. P., Kerby, J. D., Beckman, J. S., and Thompson, J. A. (1996). Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11853–11858.

Mallozzi, C., Di Stasi, A. M., and Minetti, M. (1997). Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *FASEB J.* **11,** 1281–1290.

Manuszak, M., and Koppenol, W. H. (1996). The enthalpy of isomerization of peroxynitrite to nitrate. *Thermochim. Acta* **273,** 11–15.

Marla, S. S., Lee, J., and Groves, J. T. (1997). Peroxynitrite rapidly permeates phospholipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 14243–14248.

Masumoto, H., and Sies, H. (1996a). The reaction of 2-(methylseleno)benzanilide with peroxynitrite. *Chem. Res. Toxicol.* **9,** 1057–1062.

Masumoto, H., and Sies, H. (1996b). The reaction of ebselen with peroxynitrite. *Chem. Res. Toxicol.* **9,** 262–267.

Masumoto, H., Kissner, R., Koppenol, W. H., and Sies, H. (1996). Kinetic study of the reaction of ebselen with peroxynitrite. *FEBS Lett.* **398,** 179–182.

Merenyi, G., and Lind, J. (1997). Thermodynamics of peroxynitrite and its CO_2 adduct. *Chem. Res. Toxicol.* **10,** 1216–1220.

Merényi, G., Lind, J., Goldstein, S., and Czapski, G. (1999). Mechanism and thermochemistry of peroxynitrite decomposition in water. *J. Phys. Chem. A* **103,** 5685–5691.

Misko, T. P., Highkin, M. K., Veenhuizen, A. W., Manning, P. T., Stern, M. K., Currie, M. G., and Salvemini, D. (1998). Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. *J. Biol. Chem.* **273,** 15646–15653.

Moncada, S., Palmer, R. M. J., and Gryglewski, R. J. (1986). Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 9164–9168.

Moncada, S., Palmer, R. M., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Nair, J., Gal, A., Tamir, S., Tannenbaum, S. R., Wogan, G. N., and Bartsch, H. (1998). Etheno adducts in spleen DNA of SJL mice stimulated to overproduce nitric oxide. *Carcinogenesis* **19,** 2081–2084.

O'Donnell, V. B., Eiserich, J. P., Chumley, P., Jablonsky, M., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999). Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12,** 83–92.

Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M., Ichimori, K., and Maeda, H. (1997). Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: A novel mechanism for procollagenase activation involving nitric oxide. *Arch. Biochem. Biophys.* **342,** 261–274.

Oury, T. D., Piantadosi, C. A., and Crapo, J. D. (1993). Cold-induced brain edema in mice. Involvement of extracellular superoxide dismutase and nitric oxide. *J. Biol. Chem.* **268,** 15394–15398.

Packer, M. A., and Murphy, M. P. (1994). Peroxynitrite causes calcium efflux from mitochondria which is prevented by Cyclosporin A. *FEBS Lett.* **345,** 237–240.

Padmaja, S., and Huie, R. E. (1993). The reaction of nitric oxide with organic peroxyl radicals. *Biochem. Biophys. Res. Commun.* **195,** 539–544.

Padmaja, S., Squadrito, G. L., Lemercier, J. N., Cueto, R., and Pryor, W. A. (1996a). Rapid oxidation of DL-selenomethionine by peroxynitrite. *Free Radicals Biol. Med.* **21,** 317–322.

Padmaja, S., Ramezanian, M. S., Bounds, P. L., and Koppenol, W. H. (1996b). Reaction of peroxynitrite with L-tryptophan. *Redox Rep.* **2,** 173–177.

Padmaja, S., Squadrito, G. L., Lemercier, J. N., Cueto, R., and Pryor, W. A. (1997). Peroxynitrite-mediated oxidation of D,L-selenomethionine: Kinetics, mechanism and the role of carbon dioxide. *Free Radicals Biol. Med.* **23,** 917–926.

Padmaja, S., Squadrito, G. L., and Pryor, W. A. (1998). Inactivation of glutathione peroxidase by peroxynitrite. *Arch. Biochem. Biophys.* **349,** 1–6.

Palmer, R. M., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Panas, D., Khadour, F. H., Szabo, C., and Schulz, R. (1998). Proinflammatory cytokines depress cardiac efficiency by a nitric oxide-dependent mechanism. *Am. J. Physiol.* **275**(Pt. 2), H1016–H1023.

Panasenko, O. M., Sharov, V. S., Briviba, K., and Sies, H. (2000). Interaction of peroxynitrite with carotenoids in human low density lipoproteins. *Arch. Biochem. Biophys.* **373,** 302–305.

Pannala, A. S., Rice-Evans, C., Sampson, J., and Singh, S. (1998). Interaction of peroxynitrite with carotenoids and tocopherols within low density lipoprotein. *FEBS Lett.* **423,** 297–301.

Pearce, L. L., Pitt, B. R., and Peterson. J. (1999). The peroxynitrite reductase activity of cytochrome c oxidase involves a two-electron redox reaction at the heme a(3)-Cu(B) site. *J. Biol. Chem.* **274,** 35763–35767.

Pfeiffer, S., Gorren, A. C. F., Schmidt, K., Werner, E. R., Hansert, B., Bohle, D. S., and Mayer, B. (1997). Metabolic fate of peroxynitrite in aqueous solution. Reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2:1 stoichiometry. *J. Biol. Chem.* **272,** 3465–3470.

Pfeiffer, S., Schmidt, K., and Mayer, B. (2000). Dityrosine formation outcompetes tyrosine nitration at low steady-state concentrations of peroxynitrite. Implications for tyrosine modification by nitric oxide/superoxide *in vivo. J. Biol. Chem.* **275,** 6346–6352.

Pietraforte, D., and Minetti, M. (1997a). Direct ESR detection or peroxynitrite-induced tyrosine-centred protein radicals in human blood plasma. *Biochem. J.* **325**(Pt. 3), 675–684.

Pietraforte, D., and Minetti, M. (1997b). One-electron oxidation pathway of peroxynitrite decomposition in human blood plasma: Evidence for the formation of protein tryptophan-centred radicals. *Biochem. J.* **321**(Pt. 3), 743–750.

Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996). Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch. Biochem. Biophys.* **328,** 85–92.

Podrez, E. A., Schmitt, D., Hoff, H. F., and Hazen, S. L. (1999). Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J. Clin. Invest.* **103,** 1547–1560.

Pou, S., Nguyen, S. Y., Gladwell, T., and Rosen, G. M. (1995). Does peroxynitrite generate hydroxyl radical? *Biochim. Biophys. Acta* **1244,** 62–68.

Prutz, W. A., Monig, H., Butler, J., and Land, E. J. (1985). Reactions of nitrogen dioxide in aqueous model systems: Oxidation of tyrosine units in peptides and proteins. *Arch. Biochem. Biophys.* **243,** 125–134.

Pryor, W. A., and Squadrito, G. L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.* **268**(Pt. 1), L699–L722.

Pryor, W. A., Jin, X., and Squadrito, G. L. (1994). One- and two-electron oxidations of methionine by peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11173–11177.

Pryor, W. A., Jin, X., and Squadrito, G. L. (1996). Insensitivity of the rate of decomposition of peroxynitrite to changes in viscosity. *J. Am. Chem. Soc.* **118,** 3125–3128.

Pryor, W. A., Lemercier, J. N., Zhang, H., Uppu, R. M., and Squadrito, G. L. (1997). The catalytic role of carbon dioxide in the decomposition of peroxynitrite. *Free Radicals Biol. Med.* **23,** 331–338.

Quijano, C., Alvarez, B., Gatti, R. M., Augusto, O., and Radi, R. (1997). Pathways of peroxynitrite oxidation of thiol groups. *Biochem. J.* **322** (Pt. 1), 167–173.

Radi, R. (1998). Peroxynitrite reactions and diffusion in biology. *Chem. Res. Toxicol.* **11,** 720–721.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991a). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991b). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288,** 481–487.

Radi, R., Cosgrove, T. P., Beckman, J. S., and Freeman, B. A. (1993). Peroxynitrite-induced luminol chemiluminescence. *Biochem. J.* **290** (Pt. 1), 51–57.

Radi, R., Rodriguez, M., Castro, L., and Telleri, R. (1994). Inhibition of mitochondrial electron transport by peroxynitrite. *Arch. Biochem. Biophys.* **308,** 89–95.

Radi, R., Denicola, A., and Freeman, B. A. (1999). Peroxynitrite reactions with carbon dioxide–bicarbonate. *Methods Enzymol.* **301,** 353–367.

Ramezanian, M. S., Padmaja, S., and Koppenol, W. H. (1996). Nitration and hydroxylation of phenolic compounds by peroxynitrite. *Chem. Res. Toxicol.* **9,** 232–240.

Richeson, C. E., Mulder, P., Bowry, V. W., and Ingold, K. U. (1998). The complex chemistry of peroxynitrite decomposition: new insights. *J. Am. Chem. Soc.* **120,** 7211–7219.

Richter, C. (1998). Nitric oxide and its congeners in mitochondria: Implications for apoptosis. *Environ. Health Perspect.* **106** (Suppl. 5), 1125–1130.

Richter, C., Schweizer, M., and Ghafourifar, P. (1999). Mitochondria, nitric oxide, and peroxynitrite. *Methods Enzymol.* **301,** 381–393.

Roberts, E. S., Lin, H., Crowley, J. R., Vuletich, J. L., Osawa, Y., and Hollenberg, P. F. (1998). Peroxynitrite-mediated nitration of tyrosine and inactivation of the catalytic activity of cytochrome P450 2B1. *Chem. Res. Toxicol.* **11,** 1067–1074.

Romero, N., Denicola, A., Souza, J. M., and Radi, R. (1999). Diffusion of peroxynitrite in the presence of carbon dioxide. *Arch. Biochem. Biophys.* **368,** 23–30.

Rubanyi, G. M., and Vanhoutte, P. M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.* **250,** H822–H827.

Rubbo, H., Denicola, A., and Radi, R. (1994a). Peroxynitrite inactivates thiol-containing enzymes of *Trypanosoma cruzi* energetic metabolism and inhibits cell respiration. *Arch. Biochem. Biophys.* **308,** 96–102.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994b). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **269,** 26066–26075.

Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaraman, B., and Freeman, B. A. (1995). Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324,** 15–25.

Rubbo, H., Radi, R., Anselmi, D., Kirk, M., Barnes, S., Butler, J., Eiserich, J. P., and Freeman, B. A. (2000). Nitric oxide reaction with lipid peroxyl radicals spares α-tocopherol during lipid peroxidation. Greater oxidant protection from the pair nitric oxide/α-tocopherol than α-tocopherol/ascorbate. *J. Biol. Chem.* **275,** 10812–10818.

Salgo, M. G., Stone, K., Squadrito, G. L., Battista, J. R., and Pryor, W. A. (1995a). Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem. Biophys. Res. Commun.* **210,** 1025–1030.

Salgo, M. G., Bermudez, E., Squadrito, G. L., and Pryor, W. A. (1995b). Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes. *Arch. Biochem. Biophys.* **322,** 500–505.

Salvemini, D., Wang, Z. Q., Stern, M. K., Currie, M. G., and Misko, T. P. (1998). Peroxynitrite decomposition catalysts: Therapeutics for peroxynitrite-mediated pathology. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2659–2663.

Santos, C. X., Anjos, E. I., and Augusto, O. (1999). Uric acid oxidation by peroxynitrite: multiple reactions, free radical formation, and amplification of lipid oxidation. *Arch. Biochem. Biophys.* **372,** 285–294.

Schuster, B., Prassl, R., Nigon, F., Chapman, M., and Laggner, P. (1995). Core lipid structure is a major determinant of the oxidative resistance of low density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 2509–2513.

Schweizer, M., and Richter, C. (1996). Peroxynitrite stimulates the pyridine nucleotide-linked Ca^{2+} release from intact rat liver mitochondria. *Biochemistry* **35,** 4524–4528.

Sharpe, M. A., and Cooper, C. E. (1998a). Reactions of nitric oxide with mitochondrial cytochrome c: A novel mechanism for the formation of nitroxyl anion and peroxynitrite. *Biochem. J.* **332**(Pt. 1), 9–19.

Sharpe, M. A., and Cooper, C. E. (1998b). Interaction of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity. *J. Biol. Chem.* **273,** 30961–30972.

Shigenaga, M. K., Lee, H. H., Blount, B. C., Christen, S., Shigeno, E. T., Yip, H., and Ames, B. N. (1997). Inflammation and NO(X)-induced nitration: Assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3211–3216.

Shustov, G. V., Spinney, R., and Rauk, A. (2000). Mechanism of peroxynitrite oxidation of aliphatic CH bonds in saturated and unsaturated hydrocarbons. A theoretical model for the CH oxidation of lipids. *J. Am. Chem. Soc.* **122,** 1191–1199.

Sies, H., and Masumoto, H. (1997). Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. *Adv. Pharmacol.* **38,** 229–246.

Sies, H., Sharov, V. S., Klotz, L. O., and Briviba, K. (1997). Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *J. Biol. Chem.* **272,** 27812–27817.

Sies, H., Klotz, L. O., Sharov, V. S., Assmann, A., and Briviba, K. (1998). Protection against peroxynitrite by selenoproteins. *Z. Naturforsch. (C),* **53,** 228–232.

Smith, C. D., Carson, M., van der Woerd, M., Chen, J., Ischiropoulos, H., and Beckman, J. S. (1992). Crystal structure of peroxynitrite-modified bovine Cu,Zn superoxide dismutase. *Arch. Biochem. Biophys.* **299,** 350–355.

Sok, D. E. (1998). Active site of brain Zn^{2+}-glycerophosphocholine cholinephosphodiesterase and regulation of enzyme activity. *Neurochem. Res.* **23,** 1061–1067.

Soszynski, M., and Bartosz, G. (1996). Effect of peroxynitrite on erythrocytes. *Biochim. Biophys. Acta* **1291,** 107–114.

Souza, J. M., and Radi, R. (1998). Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. *Arch. Biochem. Biophys.* **360,** 187–194.

Stachowiak, O., Dolder, M., Wallimann, T., and Richter, C. (1998). Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J. Biol. Chem.* **273,** 16694–16699.

Stern, M. K., Jensen, M. J., and Kramer, K. (1996). Peroxynitrite decomposition catalysts. *J. Am. Chem. Soc.* **118,** 8735–8736.

Szabo, C. (1998). Potential role of the peroxynitrate-poly(ADP-ribose) synthetase pathway in a rat model of severe hemorrhagic shock. *Shock* **9,** 341–344.

Szabo, C., and Ohshima, H. (1997). DNA damage induced by peroxynitrite: Subsequent biological effects. *Nitric Oxide* **1,** 373–385.

Szabo, C., Ferrer-Sueta, G., Zingarelli, B., Southan, G. J., Salzman, A. L., and Radi, R. (1997a). Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. *J. Biol. Chem.* **272,** 9030–9036.

Szabo, C., Zingarelli, B., O'Connor, M., and Salzman, A. L. (1996). DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1753–1758.

Szabo, C., Saunders, C., O'Connor, M., and Salzman, A. L. (1997b). Peroxynitrite causes energy depletion and increases permeability via activation of poly (ADP-ribose) synthetase in pulmonary epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* **16,** 105–109.

Takakura, K., Beckman, J. S., MacMillan-Crow, L. A., and Crow, J. P. (1999). Rapid and irreversible inactivation of protein tyrosine phospha-

tases PTP1B, CD45, and LAR by peroxynitrite. *Arch. Biochem. Biophys.* **369,** 197–207.

Takehara, Y., Nakahara, H., Inai, Y., Yabuki, M., Hamazaki, K., Yoshioka, T., Inoue, M., Horton, A. A., and Utsumi, K. (1996). Oxygen-dependent reversible inhibition of mitochondrial respiration by nitric oxide. *Cell Struct. Funct.* **21,** 251–258.

Thomas, S. R., Davies, M. J., and Stocker, R. (1998). Oxidation and antioxidation of human low-density lipoprotein and plasma exposed to 3-morpholinosydnonimine and reagent peroxynitrite. *Chem. Res. Toxicol.* **11,** 484–494.

Thomson, L., Trujillo, M., Telleri, R., and Radi, R. (1995). Kinetics of cytochrome c^{2+} oxidation by peroxynitrite: Implications for superoxide measurements in nitric oxide-producing biological systems. *Arch. Biochem. Biophys.* **319,** 491–497.

Tsai, H. H., Hamilton, T. P., Tsai, J. M., van der Woerd, M., Harrison, J. G., Jablonsky, M. J., Beckman, J. S., and Koppenol, W. H. (1996). *Ab initio* and NMR study of peroxynitrite and peroxynitrous acid: Important biological oxidant. *J. Phys. Chem.* **100,** 15087–15095.

Tsai, J. M., Harrison, J. H., Martin, J. C., Hamilton, T., van der Woerd, M., Jablonski, M. J., and Beckman, J. S. (1994). Role of conformation of peroxynitrite anion ($ONOO^-$) in its stability and toxicity. *J. Am. Chem. Soc.* **116,** 4115–4116.

Turrens, J. F., and Boveris, A. (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem. J.* **191,** 421–427.

Uppu, R. M., Squadrito, G. L., and Pryor, W. A. (1996a). Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327,** 335–343.

Uppu, R. M., Cueto, R., Squadrito, G. L., Salgo, M. G., and Pryor, W. A. (1996b). Competitive reactions of peroxynitrite with 2'-deoxyguanosine and 7,8-dihydro-8-oxo-2'-deoxyguanosine (8–oxodG): Relevance to the formation of 8-oxodG in DNA exposed to peroxynitrite. *Free Radicals Biol. Med.* **21,** 407–411.

Uppu, R. M., Winston, G. W., and Pryor, W. A. (1997). Reactions of peroxynitrite with aldehydes as probes for the reactive intermediates responsible for biological nitration. *Chem. Res. Toxicol.* **10),** 1331–1337.

van der Vliet, A., O'Neill, C. A., Halliwell, B., Cross, C. E., and Kaur, H. (1994). Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett.* **339,** 89–92.

van der Vliet, A., Eiserich, J. P., O'Neill, C. A., Halliwell, B., and Cross, C. E. (1995). Tyrosine modification by reactive nitrogen species: A closer look. *Arch. Biochem. Biophys.* **319,** 341–349.

van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997). Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272,** 7617–7625.

van der Vliet, A., t Hoen, P. A. C., Wong, P. S. Y., Bast, A., and Cross, C. E. (1998). Formation of S-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J. Biol. Chem.* **273,** 30255–30262.

Vasquez-Vivar, J., Denicola, A., Radi, R., and Augusto, O. (1997). Peroxynitrite-mediated decarboxylation of pyruvate to both carbon dioxide and carbon dioxide radical anion. *Chem. Res. Toxicol.* **10,** 786–794.

Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998). Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9220–9225.

Viner, R. I., Huhmer, A. F., Bigelow, D. J., and Schoneich, C. (1996). The oxidative inactivation of sarcoplasmic reticulum $Ca^{(2+)}$-ATPase by peroxynitrite. *Free Radicals Res.* **24,** 243–259.

Wade, R. S., and Castro, C. E. (1996). Reactions of oxymyoglobin with NO, NO_2, and NO_2^- under argon and in air. *Chem. Res. Toxicol.* **9,** 1382–1390.

White, C. R., Brock, T. A., Chang, L. Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A., and Tarpey, M. M. (1994). Superoxide and peroxynitrite in atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1044–1048.

Wink, D. A., Feelisch, M., Fukuto, J., Chistodoulou, D., Jourd'heuil, D., Grisham, M. B., Vodovotz, Y., Cook, J. A., Krishna, M., DeGraff, W. G., Kim, S., Gamson, J., and Mitchell, J. B. (1998). The cytotoxicity of nitroxyl: Possible implications for the pathophysiological role of NO. *Arch. Biochem. Biophys.* **351,** 66–74.

Winterbourn, C. C. (1987). The ability of scavengers to distinguish OH. production in the iron-catalyzed Haber–Weiss reaction: Comparison of four assays for OH. *Free Radicals Biol. Med.* **3,** 33–39.

Wong, P. S., Hyun, J., Fukuto, J. M., Shirota, F. N., DeMaster, E. G., Shoeman, D. W., and Nagasawa, H. T. (1998). Reaction between *S*-nitrosothiols and thiols: Generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry,* **37,** 5362–5371.

Worle, M., Latal, P., Kissner, R., Nesper, R., and Koppenol, W. H. (1999). Conformation of peroxynitrite: determination by crystallographic analysis. *Chem. Res. Toxicol.* **12,** 305–307.

Xia, Y., and Zweier, J. L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 6954–6958.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6770–6774.

Xia, Y., Roman, L. J., Masters, B. S., and Zweier, J. L. (1998a). Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J. Biol. Chem.* **273,** 22635–22639.

Xia, Y., Tsai, A. L., Berka, V., and Zweier, J. L. (1998b). Superoxide generation from endothelial nitric-oxide synthase. A Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J. Biol. Chem.* **273,** 25804–25808.

Yamakura, F., Taka, H., Fujimura, T., and Murayama, K. (1998). Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **273,** 14085–14089.

Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995). Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis,* **16,** 2045–2050.

Yermilov, V., Yoshie, Y., Rubio, J., and Ohshima, H. (1996). Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. *FEBS Lett.* **399,** 67–70.

Zhang, H., Squadrito, G. L., Uppu, R. M., Lemercier, J. N., Cueto, R., and Pryor, W. A. (1997). Inhibition of peroxynitrite-mediated oxidation of glutathione by carbon dioxide. *Arch. Biochem. Biophys.* **339,** 183–189.

Zhang, Y., Marcillat, O., Giulivi, C., Ernster, L., and Davies, K. J. (1990). The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *J. Biol. Chem.* **265,** 16330–16336.

Zhu, L., Gunn, C., and Beckman, J. S. (1992). Bactericidal activity of peroxynitrite. *Arch. Biochem. Biophys.* **298,** 452–457.

Zingarelli, B., Ischiropoulos, H., Salzman, A. L., and Szabo, C. (1997). Amelioration by mercaptoethylguanidine of the vascular and energetic failure in haemorrhagic shock in the anesthetised rat. *Eur. J. Pharmacol.* **338,** 55–65.

Zingarelli, B., Cuzzocrea, S., Szabo, C., and Salzman, A. L. (1998). Mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, reduces trinitrobenzene sulfonic acid- induced colonic damage in rats. *J. Pharmacol. Exp. Ther.* **287,** 1048–1055.

Zou, M. H., and Ullrich, V. (1996). Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett.* **382,** 101–104.

CHAPTER 5

A Comparison of the Biological Reactivity of Nitric Oxide and Peroxynitrite

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THE MAIN GOAL OF THIS CHAPTER IS TO COMPARE AND CONTRAST THE BIOLOGICAL REACTIVITY OF NITRIC OXIDE AND PEROXYNITRITE WITH PROTEINS. NITRIC OXIDE AND PEROXYNITRITE REACT SELECTIVELY WITH DIFFERENT PROTEINS, CAUSING LOCAL OR EVEN LONG-RANGE STRUCTURAL ALTERATIONS THAT MODIFY PROTEIN FUNCTION. THIS CHAPTER REVIEWS FACTORS THAT DETERMINE THE GENERAL BIOLOGICAL REACTIVITY OF NITRIC OXIDE AND PEROXYNITRITE WITH BIOMOLECULES AND THE FACTORS THAT DETERMINE THE REACTIVITY WITH PROTEINS.

Introduction

The proteins that are selectively modified by nitric oxide fall into four major categories: (1) heme and non-heme iron-containing proteins (Murad, 1996; Moore and Gibson, 1986; Craven and DeRubertis, 1978; Lancaster *et al.,* 1992; Ribeiro *et al.,* 1993; Gardner *et al.,* 1998; Hausladen *et al.,* 1998; Tsubaki *et al.,* 1987; Khatsenko *et al.,* 1993), (2) proteins with iron–sulfur clusters (Kennedy *et al.,* 1997; Cassina and Radi, 1996; Clementi *et al.,* 1998; Bouton *et al.,* 1986; Pantopoulos *et al.,* 1994), (3) proteins with liable cysteine residues (Simon *et al.,* Molina y Vedis *et al.,* 1992; Lander *et al.,* 1996; Xu *et al.,* 1998; Lipton *et al.,* 1993; Gow and Stamler, 1998; Kim *et al.,* 1997), and (4) proteins containing tyrosine residues that undergo one-electron oxidation to form tyrosyl radical (Gunthers *et al.,* 1997; Goodwin *et al.,* 1998; Guittet *et al.,* 1998). Examples for the first category include guanylate cyclase, hemoglobin, cytochrome P-450, insect salivary heme protein, and bacterial flavohemoglobin (Murad, 1996; Moore and Gibson, 1976; Craven and DeRubertis, 1978; Lancaster *et al.,* 1992; Ribeiro *et al.,* 1993; Gardner *et al.,* 1998; Hausladen *et al.,* 1998; Tsubaki *et al.,* 1987; Khatsenko *et al.,* 1993). Among proteins in the second group are aconitase, mitochondrial electron transport chain complexes, and the iron regulatory element, all of which represent iron–sulfur targets for nitric oxide (Kennedy *et al.,* 1997; Cassina and Radi, 1996; Clementi *et al.,* 1998; Bouton *et al.,* 1996; Pantopoulos *et al.,* 1994). In the third group, glyceraldehyde-3-phospate dehydrogenase (GAPDH), ryodine receptor, p21ras hemoglobin, and caspase 3 are all modified by S-nitrosylation of cysteine residues (Table 1). Prostagladin H synthase-2, prostaglandin endoperoxidase synthase, and ribonucleotide reductase are examples of proteins in which nitric oxide has been shown to react with the tyrosyl radical (Gunthers *et al.,* 1997; Goodwin *et al.,* 1998; Guittet *et al.,* 1998).

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Table 1 Proteins Modified by S-Nitrosylation

Protein	Function	Model	S-nitrosylated function	Site of S-nitrosylation
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	Rat liver of *C. parvum* model of chronic hepatic inflammation (Molina y Vedia *et al.,* 1992)	Inhibits enzymatic activity and increases endogenous ADP-ribosylation	Possibly C_{149} in the active site
p21ras	Signal transduction	Cells exposed to nitric oxide donors (Lander *et al.,* 1996)	Activation of MAP kinase, signal transduction	C_{118}
Ryanodine receptor	Cardiac calcium regulation	Canine hearts (Xu *et al.,* 1998)	Reversible channel activation	Multiple residues
NMDA receptor	Synaptic transmission, Ca^{2+} homeostasis	Neuronal cell models exposed to nitric oxide donors (Lipton *et al.,* 1993)	Increases Ca^{2+} efflux and neurotoxicity	nd[a]
Hemoglobin	Oxygen transport	Rat blood (Gow and Stamler, 1998)	Control of blood pressure	C_{93}
Caspase-3	Cysteine protease, execution of apoptosis	Cells exposed to nitric oxide donors (Kim *et al.,* 1997)	Inhibits activity	nd

[a] nd, not determined.

Two amino acids have been shown to be the major targets for peroxynitrite in proteins, tyrosine and cysteine (Ischiropoulos, 1998; Radi *et al.,* 1991). The reaction with tyrosine results in the formation of 3-nitrotyrosine and the reaction with cysteine generates oxidized forms of cysteine, and to a lesser extent, *S*-nitrosocysteine (Ischiropoulos, 1998; Radi *et al.,* 1991; Vliet *et al.,* 1998; Balazy *et al.,* 1998). Peroxynitrite also selectively reacts with zinc–sulfur and iron–sulfur centers of proteins (Crow *et al.,* 1995; Keyer and Imlay, 1997). *In vivo,* a number of proteins have been found to be modified by nitration: Mn superoxide dismutase, prostacyclin synthase, Ca^{2+}-ATPase, tyrosine hydroxylase, and the plasma proteins, ceruloplasmin, transferrin, a1 antichymotrypsin, a1-protease inhibitor, and fibrinogen (Table II, MacMillan-Crow *et al.,* 1996; Yamakura *et al.,* 1998; Zhou *et al.,* 1998; Viner *et al.,* 1996; Klebl *et al.,* 1998; Ara *et al.,* 1998). Peroxynitrite has been implicated as the proximal nitrating species for the *in vivo* nitration of prostacyclin synthase and tyrosine hydroxylase (Zhou *et al.,* 1998; Ara *et al.,* 1998).

Currently, there are no examples of specific proteins modified by S-nitrosylation of cysteine(s) via the reaction with peroxynitrite. Although both nitric oxide and peroxynitrite have been also shown to react with and modify tryptophan residues, no specific proteins and targets have been identified *in vivo* (Alvarez *et al.,* 1996; Zhang *et al.,* 1996).

A number of specific proteins are modified by either nitric oxide or peroxynitrite, and the factors which determine this selectivity are currently being investigated. Based on this limited new information, as well as on the known biochem-

Table II Nitrated Proteins in Human Disorders and Animal or Cellular Models of Disease[a]

Protein	Function	Human pathology/model	Effect of nitration	Site of nitration
Mn-Superoxide dismutase	Decrease superoxide steady-state levels	Rejected human renal allografts (MacMillan-Crow *et al.,* 1996)	Loss of function	Residue Y34 (Yamakura *et al.,* 1998)
Prostacyclin synthase	Prostacyclin synthesis	IL-1β-treated mesanglial cells (Zhou *et al.,* 1998)	Loss of function	nd[b]
Sarcoplasmic reticulum Ca^{2+}-ATPase	Calcium and energy homeostasis	Aged rat skeletal muscle (Viner *et al.,* 1996), low-frequency stimulation of muscle (Klebl *et al.,* 1998)	Loss of function	nd
Tyrosine hydroxylase	Rate limiting step in catecholamine synthesis	MPTP mouse model of Parkinson's disease (Ara *et al.,* 1998)	Loss of function	One residue per molecule

[a] Evidence that the proximal species for nitration is peroxynitrite or $ONO(O)CO_2^-$ is provided for prostacyclin synthase and tyrosine hydroxylase. Prostacyclin synthase was nitrated only upon exposure to nitric oxide and superoxide donors but not to nitric oxide donors without a source of superoxide (Zhou *et al.,* 1998). IL-1β was also shown to nitrate the cytoskeleton of smooth muscle cells but no specific proteins have been determined (Boota *et al.,* 1996). Tyrosine hydroxylase was not nitrated in mice overexpressing Cu,Zn-superoxide dismutase in their brains (Ara *et al.,* 1998). These mice as well as NOS1 knock-out mice are resistant to MPTP neurotoxicity (Przedborski *et al.,* 1992, 1996).

[b] nd, not determined.

ical properties of nitric oxide and peroxynitrite, we will outline and discuss factors that may play a critical role in the selective biological reactivity of nitric oxide and peroxynitrite with proteins. These factors include the chemical nature of the reactants, the rate constant and concentration of the reactants, compartmentalization, specific pathways of removal or scavenging, and biophysical properties of the target protein.

Chemical Nature of the Reactant

Both nitric oxide and peroxynitrite are simple molecules. However, despite their simplicity in structure, the diversity of the biological reactivity of these two molecules is daunting—covering the range from physical chemistry to human physiology and pathology in nearly all major organs. In part, their biological reactivity is determined by their chemical and physical properties, nitric oxide being a free radical and peroxynitrite an oxidant. The free radical nature of nitric oxide is important for its interaction with heme iron-containing proteins, iron–sulfur clusters, and with other radical species. In general, nitric oxide is a mild oxidant and reductant, therefore the oxidoreductive chemistry of nitric oxide may be limited to specific environments and with specific biological targets. The biophysical properties of nitric oxide are similar to oxygen (Meyer *et al.,* 1990). Both are hydrophobic, gaseous molecules with similar solubility in biological fluids and ability to establish diffusion gradients. Both oxygen and nitric oxide are capable of undergoing reactions that will cause stress and in the extreme, a pathogenic outcome. Oxygen toxicity has been studied extensively over the past 40 years. The partial reduction of oxygen by one electron to form superoxide and/or by two electrons to form hydrogen peroxide is the biochemical reason for the toxicity of oxygen (Fridovich, 1997; Freeman and Crapo, 1982). Despite the presence of specific enzymatic antioxidant defenses, superoxide and hydrogen peroxide are capable of causing tissue pathology. Similarly, nitric oxide can undergo reactions with oxygen to produce nitrogen species with higher oxidation potential, or with superoxide to generate peroxynitrite. The reactive nitrogen species and peroxynitrite are capable of reacting with proteins, lipids, and DNA to produce a pathogenic outcome (Beckman and Koppenol, 1996).

In contrast to nitric oxide, peroxynitrite is not a free radical but an oxidant and nitrating agent. Peroxynitrite has been studied in the context of a mineral salt found on the planet Mars, as well as in the pathophysiology of most neurological, pulmonary, and circulatory disorders. The physical properties of peroxynitrite (Beckman and Koppenol, 1996; Squadrito and Pryor, 1998) allow it to transverse biological membranes and to be surprisingly selective in reacting with biological targets. Contrary to previous assumptions that biological membranes may act as a formidable barrier to limit the reactivity of peroxynitrite, two recent studies clearly establish that peroxynitrite is capable of diffusing through cell membranes (Marla *et al.,* 1997; Denicola *et al.,* 1998). Possibly the most important reaction of peroxynitrite in biology is the reaction with carbon dioxide (Lymar *et al.,* 1996; Uppu *et al.,* 1996; Denicola *et al.,* 1996; Gow *et al.,* 1996a). The reaction of peroxynitrite with CO_2 leads to the formation of a nitrocarbonate ($ONO(O)CO_2^-$) intermediate. This intermediate is a less potent one- or two-electron oxidant compared to peroxynitrite (Denicola *et al.,* 1996). However, the $ONO(O)CO_2^-$ adduct is an efficient and potent nitrating agent (Denicola *et al.,* 1996; Gow *et al.,* 1996a). Recent data indicate that the $ONO(O)O_2^-$ adduct is also capable of nitrosylating biomolecules such as thiols and uric acid (possible biological targets modified by nitration are shown in Fig. 1) (Skinner *et al.,* 1998; White *et al.,* 1999; Koppenol, 1998; Uppu *et al.,* 1998). Nitrosated adducts of reduced thiols and uric acid as well as mononitrated or dinitrated adducts of glycerol are biologically active nitric oxide donors (Vliet *et al.,* 1998; Balazy *et al.,* 1998; Skinner *et al.,* 1998; White *et al.,* 1999). These adducts provide a mechanism for the formation of endogenous vasodilators and may play a role in the cellular adaptation to stress. The reaction of peroxynitrite with CO_2 is an emerging area of research that has important ramifications in determining the outcome from the biological reactivity of peroxynitrite.

Aspects of the chemical reactivity of peroxynitrite and of $ONO(O)CO_2^-$, such as the formation of radical species ($\cdot OH$ and $\cdot NO_2$) and the formation of oxidized and nitrosated phenolics, remain controversial (Koppenol, 1998; Uppu *et al.,* 1998). In reviewing the literature, however, it appears that much of this controversy results from a remarkably simple variable: the method used for the synthesis of peroxynitrite. Peroxynitrite can be synthesized by a variety of methods, each producing solutions with different contaminants and possibly different isomers of peroxynitrite with different reactivity. Moreover, methodologies used for *in situ* synthesis are also plagued by problems that will be discussed below.

Rate of Reaction with Biological Targets

The second factor that may determine the reactivity of nitric oxide and peroxynitrite is the rate of reaction with the biological targets. The rate is a function of the rate constant and the concentration of the reactants. Kinetically favored and thermodynamically feasible reactions need to be consid-

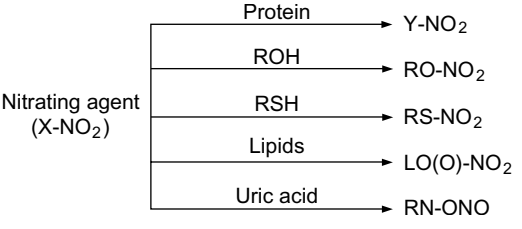


Figure 1 Possible biological targets modified by nitration.

ered first. The reactions of nitric oxide with heme iron, nonheme iron, iron–sulfur clusters, and tyrosyl radical are kinetically and thermodynamically favored (Koppenol, 1998). In contrast, the reaction of nitric oxide with cysteine is relatively slow and is not thermodynamically favored in the absence of an electron acceptor (Gow *et al.,* 1997). However, since the cysteine concentration in proteins and glutathione is higher than the concentration of all other putative targets, the overall rate of this reaction may be high enough to compete with the other pathways.

There is clear evidence for the formation of *S*-nitrosothiols *in vivo;* however, the nature of the nitrosating agent(s) remains unclear (Simon *et al.,* 1996; Molina y Vedra *et al.,* 1992; Lander *et al., 1996; Xu et al.,* 1998; Lipton *et al.,* 1993; Gow and Stamler, 1998; Kim *et al.,* 1997). Formation of *S*-nitrosothiols may proceed via a number of reactive pathways, catalyzed by metal, by the oxidation of thiol to thiyl radical followed by coupling of the radical with nitric oxide, or via the formation of a radical intermediate which donates the free electron to an electron acceptor (Gow *et al.,* 1997; Boese *et al.,* 1995). Another alternative is the reaction of nitric oxide with peroxynitrite to form dinitrogen trioxide (Wink *et al.,* 1997; Mayer *et al.,* 1998). However, the reaction between nitric oxide and peroxynitrite is not kinetically favored since the concentration of both reactants is low. The formation of higher nitrogen oxides by the trimolecular reaction of nitric oxide with oxygen generates nitrosating species. However, the reaction of nitric oxide with oxygen is relatively slow and hence it is unlikely to compete successfully with any of the other nitric oxide targets except in special environments. The reaction of nitric oxide with oxygen may take place in hydrophobic environments such as cell membranes where the concentration of both reactants is higher than in the cytosol or in the extracellular environment (Liu *et al.,* 1998). A six- to 10-fold increase in the concentration of nitric oxide and oxygen in the phospholipid bilayer may promote the formation of nitrogen oxides. Moreover, in the membranes, alternative targets are either absent or present in concentrations lower than that of O_2, favoring the reaction between nitric oxide and oxygen (Liu *et al.,* 1998). The nitrogen oxides are reactive and hydrate easily to give nitrite and nitrate, limiting their biological function to the membrane bilayer where they may be important for the formation of nitrosated and nitrated lipid adducts (O'Donnell *et al.,* 1998).

The predominant protein modification by peroxynitrite, even in the presence of alternative pathways such as reduce thiols, is with tyrosine residues to form 3-nitrotyrosine. This is a paradox since the apparent second-order rate constant of the reaction of peroxynitrite with the free amino acid is zero (Alvarez *et al.,* 1998). The same study determined the second-order rate constants of peroxynitrite reacting with each free amino acid. Cysteine had the highest second-order rate constant, suggesting that it should be the primary target reacting with peroxynitrite. Indeed, rendering the single cysteine residue of BSA inactive by derivatization prior to the reaction with peroxynitrite increased the yield of tyrosine nitration (Alvarez *et al.,* 1998). That nitration of protein tyrosine residues takes place, even in the presence of reduce thiols and other peroxynitrite scavengers (Gow *et al.,* 1996; Alvarez *et al.,* 1998), indicates that simple rate constants are not the only factors determining the biological reactivity of nitric oxide and peroxynitrite. Their modest rate of reaction with different targets extends their biological half-life. The consequence of extending the biological half-life of a reactive species is to increase its diffusion distance, which then amplifies the sphere of biological influence and permits reactions with selective targets. For nitric oxide, diffusion is not a problem, and it may establish large diffusion gradients as long as the various isoforms of nitric oxide synthases are continuously generating nitric oxide. Under this situation the diffusion distance is then limited by reactivity with targets, specific removal, and scavenging. The selectivity of reactivity determines which targets will be modified before the diffusion gradient is decimated.

A final point regarding studies of reactivity of nitric oxide and peroxynitrite pertains to using *in vitro* generating systems of nitric oxide and superoxide. These *in vitro* models have provided important information on the oxidizing, nitrosating and nitrative reactivity of nitric oxide and peroxynitrite (Wink *et al.,* 1997; Mayer *et al.,* 1998; Pfeiffer and Mayer, 1998). They were also useful in providing evidence for secondary reactions between nitric oxide and peroxynitrite (Wink *et al.,* 1997; Mayer *et al.,* 1998). However, much of the *in vitro* reactivity does not simulate *in vivo* conditions. For example, these systems typically use a single target to determine outcome, whereas in biology many targets will be present. Some models have been performed in the absence of CO_2; these play a major role in the reactivity of peroxynitrite (Wink *et al.,* 1997). Exposing targets, cells, or tissues to exogenous nitric oxide and/or peroxynitrite may not always produce the same outcome as with the endogenous generation of these species. A good example is our own experience with the detection of nitrated proteins in plasma after exposure to nitric oxide and superoxide as compared with the endogenous generation of nitrating agents in acute respiratory distress syndrome (ARDS). The major protein that is nitrated upon exposure of human plasma to nitrating agent is albumin. However, albumin was not one of the five plasma proteins that have been identified as nitrated in ARDS patients. At first glance it is surprising that a protein that comprises over 50% of the total protein in plasma and contains 18 tyrosine residues is not nitrated *in vivo.* Another surprising finding is that the yield and efficiency of nitration of the free amino acid tyrosine are significantly lower (no apparent second-order rate constant) than the yield of nitration of selective tyrosine residues in proteins (MacMillan *et al.,* 1996; Yamakura *et al.,* 1998; Zhou *et al.,* 1998; Viner *et al.,* 1996; Klebl *et al.,* 1998; Ara *et al.,* 1998). The local environment of tyrosine residues in proteins and the protein structure appear to facilitate nitration of specific tyrosine residues in proteins (discussed below). This is consistent with observations that stimulated neutrophils supplemented with nitrite failed to nitrate phagocytized small-aromatic sub-

strates (Jiang and Hurst, 1997) but effectively nitrated proteins in phagocytized bacteria (Evans *et al.,* 1996). Although test-tube experiments may be fundamental in understanding basic aspects of the chemical reactivity of nitric oxide and peroxynitrite, we should also consider the findings in biological models and human disease before drawing conclusions regarding the biological reactivity of nitric oxide and peroxynitrite.

Cellular Compartments and Biological Reactivity

The importance of magnitude of the sphere of biological influence is diminished when one considers issues of microenvironments and compartmentalization. Cellular microenvironments such as the membrane, mitochondria, and sites of superoxide generation allow for the formation of higher nitrogen oxides and peroxynitrite. We have reasoned that the biological reactivity of peroxynitrite is closely associated with the sites of superoxide formation. The superoxide dismutases maintain a low steady-state level of superoxide and thus prevent reactions between superoxide and iron–sulfur proteins or nitric oxide (Fridovich, 1997). However, in local environments where the superoxide dismutase is not efficient in removing superoxide, and under conditions where higher levels of superoxide are generated, peroxynitrite will be formed. This may be the case with the selective destruction of dopaminergic neurons in the MPTP model of Parkinson's disease (Ara *et al.,* 1998; Przedborski *et al.,* 1992, 1996). These neurons produce higher than normal superoxide during the metabolism of MPTP and thus are susceptible to peroxynitrite. Dopaminergic neurons in mice overexpressing Cu, Zn superoxide dismutase, or in mice without neuronal NOS1 (NOS1 knock-out), are protected from MPTP neurotoxicity (Przedborski *et al.,* 1992, 1996). The MPTP-induced nitration of a specific protein, tyrosine hydroxylase, is prevented in the mice overexpressing Cu,Zn-superoxide, providing evidence for the role of superoxide in nitration of proteins *in vivo* (Ara *et al.,* 1998).

Specific Pathways of Removal and Scavenging Pathways

The first line of cellular defense from reactive species is avoiding their formation. Maintaining coupling of electron transfer in the mitochondrial respiratory chain, within cytosolic proteins that catalyze different oxidations such as the cytochrome P-450 and monoxygenases, as well as nitric oxide synthases, prevents electron transfer to oxygen. The second line of defense is the presence of enzymatic defenses against the reactive species. Superoxide dismutases, glutathione peroxidase, and catalase remove superoxide and hydrogen peroxide. A mammalian equivalent to the recently described bacterial NO reductases has not been described. This flavohemoprotein is capable of specifically removing nitric oxide in bacteria (Gardner *et al.,* 1998; Hausladen *et al.,* 1998). The selenium-dependent glutathione peroxidase and possibly selenoprotein P may serve as peroxynitrite reductases in mammalian cells (Arteel *et al.,* 1999; Burk and Hill, 1999). The third line of defense is the repair mechanisms. Oxidized protein, and, to a certain extent, nitrated proteins, are proteolytically degraded and removed (Davies, 1987; Gow *et al.,* 1996b). Recent evidence also indicates that a nonproteolytic pathway can repair nitrated proteins (Gow *et al.,* 1996b; Kamisaki *et al.,* 1998). This activity is present in most rodent tissues and is induced by endotoxin or by nitrative stress (Kamisaki *et al.,* 1998). A similar activity may exist in human tissues. A decline in the lung biopsy levels of 3-nitrotyrosine has been reported in an infant who survived septic lung injury (Haddad *et al.,* 1994). The overall magnitude of plasma protein 3-nitrotyrosine declined in infants with improved oxygenation and clinical outcome after treatment to alleviate symptoms of bronchopulmonary dysplasia (Banks *et al.,* 1999).

Selection of Preferential Target

The following question remains: how do free radicals, oxidants, and nitrating agents select their biological targets? A number of the factors listed previously may determine the selective modification of biological targets. To answer this question we must also consider the factors that predispose a target to modification. The information appears to be embedded in the structure and local environment afforded by specific amino acids in the protein. For example, soluble guanylate cyclase does not bind oxygen, but it will bind nitric oxide and, to a lesser extent, carbon monoxide (Stone and Marletta, 1994). The coordination of the ligand nitric oxide in the heme iron active site is essential for the functional activation of the protein. Another example is the S-nitrosylation of cysteine 93 in hemoglobin. During the transition of hemoglobin from T to R state, the cysteine residue is exposed to solvent and is S-nitrosylated (Gow and Stamler, 1998). Nitration of tyrosine residues in a protein is also a selective process. Experimental evidence established that only a few proteins are nitrated *in vivo* (Ara *et al.,* 1998). Recent work from our laboratory determined the sites of tyrosine nitration on different proteins. Several factors appear to be critical for determining the selectivity of nitration: (1) location of the tyrosine on a loop but not in α-helical or β-sheet structure (usually near turn-inducing residues such as glycine and proline), (2) absence of nearby cysteine or disulfide bridge, (3) paucity of positive charges, (4) exposure to the surface, and (5) most importantly the presence of a nearby negative charges.

The selective modification of cysteine and tyrosine residues may have a profound effect in the biological function (Tables I and II). S-nitrosylation of cysteine residues is a reversible process and thus it does not have a long-lasting effect on biological function. Similarly, the repair of tyrosine nitration and proteolytic degradation may prevent the long-term effects on protein function induced by nitration. The

failure to repair modified proteins is likely to result in accelerated protein turnover and other disturbances in cell function that will lead to the development of a pathogenic phenotype.

Summary

Despite differences in structure and chemical reactivity, nitric oxide and peroxynitrite may share similar biological reactivity. Both species modify critical residues in proteins, and nitric oxide induces S-nitrosylation of cysteine residues and peroxynitrite, most likely via its reaction with CO_2, tyrosine nitration. In most cases, modification of a single residue per protein molecule is sufficient to induce a loss or gain of function (Tables I and II). To maintain normal physiological function, the protein modification is reversible or the modified protein is repaired. Failure to repair the modified proteins may result in a pathological outcome.

References

Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B. A., and Radi, R. (1996). Peroxynitrite-dependent tryptophan nitration. *Chem., Res. Toxicol.* **9,** 390–396.

Alvarez, B., Ferrer-Sueta, G., Freeman, B. A., and Radi, R. (1998). Kinetics of peroxynitrite reaction with amino acids and human serum albumin. *J. Biol. Chem.* **274,** 842–848.

Ara, J., Przedborski, S., Naini, A. B., Jackson-Lewis, V., Trifiletti, R. R., Horwitz, J., and Ischiropoulos, H. (1998). Inactivation of tyrosine hydroxylase following exposure to peroxynitrite and MPTP. *Proc. Natl. Acad. Sci. U.S.A.*. **95,** 7659–7663.

Arteel, G. E., Briviba, K., and Sies, H. (1999). Protection against peroxynitrite. *FEBS Lett.* **445,** 226–230.

Balazy, M., Kaminski, P. M., Mao, K., Tan, J., and Wolin, M. S. (1998). *S*-nitroglutathione, a product of the reaction between peroxynitrite and flutathione that generates nitric oxide. *J. Biol. Chem.* **273,** 32009–32015.

Banks, B. A., Seri, I., Ischiropoulos, H., Merrill J., Rychik J., and Ballard R. A. (1999). Improved oxygenation with inhaled nitric oxide in severe bronchopulmonary dysplasia. *Pediatrics* **103,** 610–618.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide and peroxynitrite: The good the bad and the ugly. *Am. J. Physiol.* **217,** C1424–1437.

Boese M., Mordvintcev, P. I., Vanin, A. F., Busse, R., and Mulsch, (1995). S-Nitrosation of serum albumin by dinitrosyl–iron complex. *J. Biol. Chem.* **270,** 29244–29249.

Boota, A., Zar, H., Kim, Y.-M., Johnson, B., Pitt, B., and Davies, P. (1996). IL-1β stimulates superoxide and delayed peroxynitrite production by pulmonary vascular smooth muscle cells. *Am. J. Physiol.* **271,** L932–L938.

Bouton, C., Raveau, M., and Drapier, J. C. (1996). Modulation of iron regulatory protein functions. *J. Biol. Chem.* **271,** 2300–2306.

Burk, R. F., and Hill, K. E. (1999). Orphan selenoproteins. *BioEssays* **21,** 1–8.

Cassina, A., and Radi, R. (1996). Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* **328,** 309–316.

Clementi, E., Brown, G. C., Feelisch, M., and Moncada, S. (1998). Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and Protective action of glutathione. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7631–7636.

Craven, P. A., and DeRubertis, F. R. (1978). Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemeproteins. *J Biol. Chem.* **253,** 8433–8443.

Crow, J. P., Beckman, J. S., and McCord J. M. (1995). Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypoclorite and peroxynitrite. *Biochemistry* **34,** 3544–3522.

Davies, K. J. A. (1987). Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **262,** 9895–9901.

Denicola, A., Trujillo, M., Freeman, B. A., and Radi, R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidation reactions. *Arch. Biochem. Biophys.* **333,** 49–58.

Denicola, A., Souza, J. M., and Radi, R. (1998). Diffusion of peroxynitrite across erythrocyte membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 3566–3571.

Evans, T. J., Buttery, L. D. K., Carpenter, A., Springall, D. R., Polak, J. M., and Cohen, J. (1996). Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 9553–9558.

Freeman, B. A., and Crapo, J. D. (1982). Biology of the disease: Free radicals and tissue injury. *Lab. Invest.* **47,** 412–426.

Fridovich, I. (1997). Superoxide anion radical, superoxide dismutases, and related matters. *J. Biol. Chem.* **272,** 18515–81517.

Gardner, P. R., Gardner, A. M., Martin, L. A., and Salzman, A. L. (1998). Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10378–10383.

Goodwin, D. C., Gunthers, M. R., Hsi, L. C., Crews, B. C., Eling, T. E., Mason, R. P., and Marnett, L. J. (1998). Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. *J. Biol. Chem.* **273,** 8903–8909.

Gow, A., and Stamler, J. (1998). Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* **391,** 169–173.

Gow, A. D., Duran, Thom, S. R., and Ischiropoulos, H. (1996a). Carbon dioxide catalyzed protein tyrosine nitration by peroxynitrite. *Arch. Biochem. Biophys.* **333,** 42–48.

Gow, A., Duran, D., Malcolm, S., and H. Ischiropoulos. (1996b). Effects of peroxynitrite induced modifications to signal transduction and protein degradation. *FEBS Lett.* **385,** 63–66.

Gow, A., Buerk, D. G., and Ischiropoulos, H. (1997). A novel reaction mechanism for the formation of *S*-nitrosothiols *in vivo*. *J. Biol. Chem.* **272,** 2841–2845.

Guittet, O., Ducastel, B., Salem, J. S., Henry, Y., Rubin, H., Lemaire, G., and Lepoivre, M. (1998). Differential sensitivity of the tyrosyl radical of mouse ribonucleotide reductase to nitric oxide and peroxynitrite. *J. Biol. Chem.* **273,** 22136–22144.

Gunthers, M. R., Hsi, L. C., Curtis, J. F., Giorse, J. K., Marnett, L. J., Eling, T. E., and Mason, R. P. (1997). Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation. *J. Biol. Chem.* **272,** 17086–17090.

Haddad, I. Y., Pataki, G., Hu, P., Galliani, C., Beckman, J. S., and Matalon S. (1994). Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J. Clin. Invest.* **94,** 2407–2413.

Hausladen, A., Gow, A. J., and Stamler, J. S. (1998). Nitrosative stress: Metabolic pathway involving the flavohemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14100–14105.

Ischiropoulos, H. (1998). Biological tyrosine nitration-A pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356,** 1–11.

Jiang, Q., and Hurst, J. K. (1997). Relative chlorinating, nitrating, and oxidizing capabilities of neutrophils determined with phagocytosable probes. *J. Biol. Chem.* **272,** 32767–32772.

Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y.-C., and Murad, F. (1998). An activity in rat tissues that modifies nitrotyrosine containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 11584–11589.

Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997). An EPR inves-

tigation of the products of the reaction of cytosolic and mitochondrial aconitase with nitric oxide. *J. Biol. Chem.* **272,** 20340–20347.

Keyer, K., and Imlay, J. A. (1997). Inactivation of deyhydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J. Biol. Chem.* **272,** 27652–27659.

Khatsenko, O. G., Gross, S. S., Pirkin, A. B., and Vane, J. R. (1993). Nitric oxide is a mediator of the decrease cytochrome P450-dependent metabolism caused by immunostimulants. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11147–11151.

Kim Y. M., Talanian R. V., and Billiar T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Klebl, B. M., Ayoub, A. T., and Pette, D. (1998). Protein oxidation, tyrosine nitration, and inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase in low-frequency stimulated rabbit muscle. *FEBS Lett.* **422,** 381–384.

Koppenol, W. H. (1998). The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radals. Biol. Med.* **25,** 385–391.

Lancaster Jr., J. R., Langrehr, J. M., Bergonia, H. A., Murase, N., Simmons, R. L., and Hoffman, R. A. (1992). EPR detection of heme and nonheme iron-containing protein nitrosylation by nitric oxide during rejection of rat heart allograft. *J. Biol. Chem.* **267,** 10994–10998.

Lander, H. M., Milbank, A. J., Tauras, J. M., Hajjar, D. P., Hempstead, B. L., Schwartz, G. D., Kraemer, R. T., Mirza, U. A., Chalt, B. T., Burk, S. C., and Quilliam, L. A. (1996). Redox regulation of cell signalling. *Nature* **381,** 380–381.

Lipton, S. A., Choi, Y.-B., Pan, Z.-H., Lei, S. Z., Chen, H.-S. V., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Liu, X., Miller, M. J., Johshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998). Accelerated reaction of nitric oxide with oxygen within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2175–2179.

Lymar, S., Jiang, Q., and Hurst, J. K. (1996). Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **35,** 7855–7861.

MacMillan-Crow, L. A., Crow, J. P., Kerby, J. D., Beckman, J. S., and Thompson, J. A. (1996). Nitration and inactivation of Mn superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. U.S.A.*. **93,** 11853–11858.

Marla, S. S., Lee, J., and Groves, J. T. (1997). Peroxynitrite rapidly permeates phospholipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 14243–14248.

Mayer, B., Pfeiffer, S., Schrammel, A., Koesling, D. Schmidt, K., and Brunner, F. (1998). A new pathway of nitric oxide/cyclic GMP signalling involving *S*-nitrosoglutathione. *J. Biol. Chem.* **273,** 3264–3270.

Meyer, M., Schuster, K.-D., Schulz, H., Mohr, M., and Piiper, J. (1990). Pulmonary diffusing capacities for nitric oxide and carbon monoxide determined by rebreathing in dogs. *J. Appl. Physiol.* **68,** 2344–2357.

Molina y Vedia, L., McDonald, B., Reep, B., Brune, B., Di Silvio, M., Billiar, T. R., and Lapetina, E. G. (1992). Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J Biol. Chem.* **267,** 24929–24932.

Moore, E. G., and Gibson, Q. H. (1976). Cooperativity in the dissociation of nitric oxide from hemoglobin. *J Biol. Chem.* **251,** 2788–2794.

Murad, F. (1996). Signal transduction using nitric oxide and cyclic guanosine monophosphate. *JAMA* **276,** 1189–1192.

O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1998). Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitric acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12,** 83–92.

Pantopoulos, K., Weiss, G., and Hentze, M. W. (1994). Nitric oxide and the post-transcriptional control of cellular iron traffic. *Trends Cell Biol.* **4,** 82–86.

Pfeiffer, S., and Mayer, B. (1998). Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J. Biol. Chem.* **273,** 27280–27285.

Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A. B., Simonetti, S., Fahn, S., Epstein, C. J., and Cadet, J. L., (1992). Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J. Neurosci.* **12,** 1658–1667.

Przedorski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T., Dawson, V. L., and Dawson, T. M. (1996). Role of neuronal nitric oxide in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced dopaminergic neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4565–4571.

Radi R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Sulfhydryl oxidation by peroxynitrite: The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Ribeiro, J. M. C., Hazzard, J. M. H., Nussenzveig, R. H., Champagne, D. E., and Walker, F. A. (1993). Reversible binding of nitric oxide by a salivary heme protein from a bloodsucking insect. *Science* **260,** 539–541.

Simon, D. I., Mullins, M. E., Jia, L., Gaston, B., Singel, D. J., and Stamler, J. S. (1996). Polynitrosylated proteins: Characterization, bioactivity, and functional consequences. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4736–4741.

Skinner, K. A., White, C. R., Patel, R., Tan, S., Barnes, S., Kirk, M., Darley-Usmar, V., and Parks, D. A. (1998). Nitrosation of uric acid by peroxynitrite. *J. Biol. Chem.* **273,** 24491–24497.

Squadrito, G. L., and Pryor, W. A. (1998). Oxidative chemistry of nitric oxide: The roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radicals. Biol. Med.* **25,** 392–403.

Stone, J. R., and Marletta, M. A. (1994). Soluble guanylate cyclase from bovine lung: Activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33,** 5636–5640.

Tsubaki, M., Hiwatashi, A., Ichikawa, Y., and Hori, H. (1987). EPR study of ferrous cytocrome P450scc-nitric oxide complexes: Effects of cholesterol and its anologues. *Biochemistry* **26,** 4527–4534.

Uppu, R. M., Squadrito, G. L., and Pryor, W. A. (1996). Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327,** 335–343.

Uppu, R. M., Lemercier, J. N., Squadrito, G. L., Zhang, H., Bolzan, R. M., and Pryor, W. A. (1998). Nitrosation by peroxynitrite: Use of phenol as a probe. *Arch. Biochem. Biophys.* **358,** 1–16.

Viner R. I., Ferrington, D. A., Huhmer, A. F. R,, Bigelow, D. J., and Schoneich, C. (1996). Accumulation of nitrotyrosine on the SERCA2a isoform of SR Ca-ATPase of rat skeletal muscle during aging: A peroxynitrite-mediated process? *FEBS Lett.* **379,** 286–290.

Vliet, A. van der, Chr.'t Hoen, P. A., Wong. P. S. Y., Bast, A., and Cross, C. E. (1998). Formation of S-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J. Biol. Chem.* **273,** 30255–30262.

White, C. R., Patel, R. P., and Darley-Usmar, V. (1999). Nitric oxide donor generation from reactions of peroxynitrite. *Methods Enzymol.* **301,** 288–298.

Wink, D. A., Cook, J. A., Kim, S. Y., Vodovotz, Y., Pacelli, R., Krishna, M. C., Russo, A., Mitchell, J. B., Jourd'heuil, D., Miles, A. M., and Grisham, M. B. (1997). Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide-derived reactive intermediates. *J. Biol. Chem.* **272,** 11147–11151.

Xu, L., Eu, J. P., Msissner, G., and Stamler, J. S. (1998). Activation of the cardiac calcium release channel (ryanodine receptor). by poly-S-nitrosylation. *Science* **279,** 234–237.

Yamakura, F., Taka, H., Fujimura, T., and Murayama, K. (1998). Inactivation of human Mn superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **273,** 14085–14089.

Zhang, Y. Y., Xu, A. M., Nomen, M., Walsh, M., Keaney, Jr., J. F., and Loscalzo, J. (1996). Nitrosation of tryptophan residue(s). in serum albumin and model dipeptides. *J. Biol. Chem.* **271,** 14271–14279.

Zhou, M.-H., Klein, T., Pasquet, J.-P., and Ullrich, V. (1998). Interleukin-1β decreases prostacyclin synthase activity in rat mesanglial cells via endogenous peroxynitrite formation. *Biochem. J.* **336,** 507–512.

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CHAPTER 6

Structural Variations to Accommodate Functional Themes of the Isoforms of NO Synthases

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NITRIC OXIDE SYNTHASES ARE RESPONSIBLE FOR THE ENZYMATIC FORMATION OF NITRIC OXIDE (NO·), THE DIATOMIC GAS RESPONSIBLE FOR A VARIETY OF CELLULAR SIGNALING EVENTS IN NEURONAL, SKELETAL MUSCLE, AND ENDOTHELIAL CELLS AND FOR THE PRODUCTION OF REACTIVE OXYGEN SPECIES RESPONSIBLE FOR DESTRUCTION OF MICROORGANISMS AND TUMOR CELLS BY PHAGOCYTIC CELLS. THE FOLLOWING DESCRIBES THE PRESENT STATE OF KNOWLEDGE OF THREE GENETICALLY DETERMINED ISOFORMS OF NITRIC OXIDE SYNTHASE. THESE ENZYMES SHARE A STRONG SIMILARITY WITH RESPECT TO PROSTHETIC GROUP COMPOSITION AND BASIC CHEMICAL MECHANISMS, BUT THEY DIFFER MARKEDLY IN AMINO ACID SEQUENCE, MOLECULAR MASS, AND REGULATORY CONTROL. ALTHOUGH NO REVIEW CAN BE TOTALLY COMPREHENSIVE, IT WILL BE THE GOAL OF THIS CHAPTER TO DESCRIBE THE UNIQUE FUNCTIONS OF THE NITRIC OXIDE SYNTHASES AS DETERMINED BY THEIR STRUCTURAL PROPERTIES WITH REFERENCE TO THEIR LOCATIONS WITHIN SPECIFIC CELLS. WHILE THERE IS MUCH TO BE LEARNED OF THE COMPLETE STRUCTURES OF THESE ISOFORMS, IT IS POSSIBLE TO REVIEW THE PERTINENT LITERATURE FOR INFORMATION LEADING TO THE UNDERSTANDING OF THE REGULATION OF FORMATION OF NO· IN VARIOUS ORGANS AND CELL TYPES. IT IS HOPED THAT BY DETERMINING THREE-DIMENSIONAL STRUCTURES, INVESTIGATORS WILL GAIN INFORMATION REGARDING POSSIBLE MECHANISMS FOR DIFFERENTIAL REGULATION OF THE NITRIC OXIDE SYNTHASES WITHIN THE CELLULAR MILIEU AND INSIGHTS INTO THE DESIGN OF POSSIBLE THERAPEUTIC AGENTS, WHICH CAN SPECIFICALLY INHIBIT ONE ISOFORM AS OPPOSED TO THE OTHER.

Nitric Oxide Synthases: Historical Introduction and Functional Aspects

Nitric oxide (NO·)-producing enzymes have been logically named nitric oxide synthases (NOS), but the derivation of the nitrogen of NO· from the guanidino nitrogen of L-arginine, one of the amino acid building blocks of cellular proteins, was unexpected. Before any of the enzymes were isolated and purified, first from rat brain cerebella (Bredt and Snyder, 1990), the activities were often referred to as guanylate cyclase-activating enzymes. In fact, the initial observations by Murad and colleagues (Arnold *et al.,* 1977) led to the proposal that NO·, generated by nitroglycerin and other nitric oxide-producing compounds, acted on guanylate

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cyclase to cause increases in the tissue levels of cGMP. This finding laid the chemical foundation for the possible function of NO· as a mediator in biological processes.

Early studies by Furchgott and Zawadski (1980), showing that endothelial cells serve an obligatory role in acetylcholine-mediated relaxation of arterial smooth muscle, were seminal in further examination of the factors involved in signaling vasorelaxation. When Furchgott (1988), Ignarro *et al.* (1987), and Palmer *et al.* (1987) subsequently independently reported that endothelium-derived relaxing factor (EDRF) was NO·, a new field exploded onto the scientific scene. These exciting discoveries formed the basis for the awarding of the Nobel Prize in Medicine or Physiology to Robert A. Furchgott, Louis J. Ignarro, and Ferid Murad in 1998. The studies of Furchgott (1988) and Moncada's group (Palmer *et al.,* 1987) produced strong evidence of a role for NO· in blood flow regulation, and experiments from Tannenbaum's laboratory in rodents and humans (Green *et al.,* 1981a,b; Leaf *et al.,* 1990) had shown that L-arginine was the precursor of nitrate (NO_3^- and nitrite (NO_2^-). The stage was now set to search for the enzymes that produce NO· in various tissues.

After Garthwaite *et al.* (1988) showed that NO· could act as an intracellular messenger in the brain, Bredt and Snyder reported (1990) the isolation and purification of neuronal nitric oxide synthase (nNOS). In these studies they showed the requirement for Ca^{2+}/calmodulin (Ca^{2+}/CaM) for the elicitation of L-arginine to L-citrulline conversion. Immediately thereafter, Snyder and colleagues (Bredt *et al.,* 1991) and Mayer *et al.* (1991) determined that nNOS contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in 1:1 stoichiometry, and Bredt *et al.* (1991) reported that the amino acid sequence of its 641-residue C terminus was highly homologous to NADPH–cytochrome P-450 reductase. These findings were to be portentous of mechanistic aspects of the NOS isoforms being similar to the interaction of NADPH–cytochrome P-450 reductase with a large number of heme-containing cytochromes P-450 in the endoplasmic reticulum of various mammalian organs. The laboratories of Marletta (Hevel *et al.,* 1991) and Nathan and colleagues (Stuehr *et al.,* 1991) also reported the flavoprotein nature of inducible NOS (iNOS), having already demonstrated that iNOS from mouse macrophages absolutely requires tetrahydrobiopterin [(1'*R*,2'*S*,6*R*)-5,6,7,8-tetrahydrobiopterin; H_4B] for activity (Tayeh and Marletta, 1989; Kwon *et al.,* 1989); a similar requirement for H_4B in nNOS was demonstrated by Mayer *et al.* (1990) and Murad and co-workers (Schmidt *et al.,* 1992). These discoveries led to some complacency in the field that all prosthetic groups required for enzymatic activity had been identified. This was not to be the case as, in 1992, the simultaneous discovery of a single iron protoporphyrin IX (heme) per monomer was made by four independent laboratories (McMillan *et al.,* 1992; White and Marletta, 1992; Stuehr and Ikeda-Saito, 1992; Klatt *et al.,* 1992), when spectral data showed a reduced, CO difference spectrum at approximately 450 nm, suggesting a cysteine thiolate-liganded heme.

Until this time, all experiments on purified nitric oxide synthases depended on purification of the enzymes from natural sources, that is, cerebellum for nNOS (Mayer *et al.,* 1991; White and Marletta, 1992) or murine macrophages for iNOS (White and Marletta, 1992; Stuehr and Ikeda-Saito, 1992), or an alternative source being the human kidney 293 cells into which the cDNA for rat neuronal NOS had been transfected (Bredt *et al.,* 1991; McMillan *et al.,* 1992; Stuehr and Ikeda-Saito, 1992). Later, Richards and Marletta (1994), using a baculovirus expression system, expressed the nNOS holoenzyme, and the Masters' laboratory (McMillan and Masters, 1995), using an *Escherichia coli* expression system to express the heme domain of nNOS, employed site-directed mutagenesis to identify the proximal heme ligand as cysteine-415, as predicted by McMillan *et al.* (1992) from sequence alignments of the isoforms. The introduction, soon thereafter, of heterologous expression systems by Roman *et al.* (1995) and Gerber and Ortiz de Montellano (1995) for the expression of nNOS holoenzyme in *E. coli* revolutionized the ability to obtain large-scale amounts of material with a high percentage of heme incorporation required for biophysical studies, such as electron paramagnetic resonance spectroscopy and crystallography.

In light of the sequence homology of the C-terminal half of nNOS to NADPH–cytochrome P-450 reductase (Bredt *et al.,* 1991), the discovery of the cysteine thiolate-liganded heme prosthetic group induced much speculation regarding the mechanism of monooxygenation catalyzed by the NOS isoforms (Marletta, 1993; Klatt *et al.,* 1993). Heme iron has been shown to be the site of oxygen binding in the cytochrome P-450-mediated systems, but it had been assumed that in the NOS isoforms, on the identification of the requirement for H_4B for catalysis (Tayeh and Marletta, 1989; Kwon *et al.,* 1989; Mayer *et al.,* 1990, 1991; Schmidt *et al.,* 1992), pterin would be the oxygen-activating prosthetic group. In addition, the enigma of the role of H_4B involvement in NOS function continues, having been confounded as a result of the finding of heme as a prosthetic group in the NOS isoforms. Possible roles for H_4B in NOS catalysis will be discussed in detail later in this review. As if the aforementioned prosthetic groups were not enough, during the X-ray crystallographic studies reported by Raman *et al.* (1998) on the bovine eNOS heme domain dimer, a metal tetrathiolate center containing zinc (ZnS_4) was found. Crystal structures of both human eNOS and human iNOS by Fischmann *et al.* (1999) and of human iNOS by Li *et al.* (1999) in Poulos' laboratory have also been shown to contain ZnS_4 centers. More recently, the Tainer, Getzoff, and Stuehr laboratories (Crane *et al.,* 1999) have confirmed the presence of zinc in crystal structures of murine iNOS but also reported the solution of a Zn-free structure. Subsequently, all three NOS isoforms have been found to contain zinc, based on biochemical studies of nNOS and eNOS by Miller *et al.* (1999) in Masters' laboratory and by the laboratories of Ortiz de Montellano (Rodríguez-Crespo *et al.,* 1999) and Mayer (Leber *et al.,* 1999) in eNOS. The possible significance of zinc as a metal center will be discussed from the aspects of struc-

ture and conformation, and various hypotheses regarding the role of zinc in NOS function will be presented later.

A summary of the properties of the three isoforms of nitric oxide synthase is found in Table I. A more comprehensive discussion of these structural and enzymatic parameters, not discussed in the limited space of this review chapter, can be found in the review by Raman *et al.* (2000).

Prosthetic Groups and Cofactors of Nitric Oxide Synthases

Flavin Adenine Dinucleotide and Flavin Mononucleotide

STOICHIOMETRY

The discovery of flavins in the partially purified preparations of nNOS, either from porcine cerebellum (Mayer *et al.,* 1991) or from stably transfected HK293 cells (Bredt *et al.,* 1991), or in preparations of iNOS from murine macrophages (Hevel *et al.,* 1991; Stuehr *et al.,* 1991) was the first indication that the NOS enzymes might resemble the cytochrome P-450 reductase-mediated systems. Hevel *et al.* (1991) and Stuehr *et al.* (1991) reported a stoichiometry of ~1 mol per mol each of FAD and FMN per 130-kDa subunit for iNOS, and Mayer *et al.* (1991) found between 0.6 and 0.9 mol per mol each of FAD and FMN per 160-kDa subunit of porcine nNOS. Bredt *et al.* (1991) did not present data supporting their contention that FAD and FMN are present in stoichiometric amounts in cloned nNOS expressed in HK293 cells, but they were the first to show the sequence homology between the C-terminal 641 residues of nNOS and NADPH–cytochrome P-450 reductase. These investigators also recognized that this 58% homology (36% identity) identified nNOS as a member of a class of flavoproteins, including sulfite reductase (Siegel and Kamin, 1968) and NADPH–cytochrome P-450 reductase, that contain binding sites for NADPH, FAD, and FMN within the same polypeptide (Porter and Kasper, 1986).

OXIDATION–REDUCTION AND MECHANISTIC ASPECTS

The mechanisms of action of NADPH–cytochrome P-450 reductase and sulfite reductase require a shuttling of electrons between the FAD and FMN prosthetic groups, and a similar mode of transfer was suggested for NOS in the Bredt *et al.* (1991) paper. At the same time, the authors stated that the N-terminal half of nNOS showed no resemblance to any protein known at that time. Perhaps this was a message to investigators that even more interesting findings were to come. It could have been surmised from the striking sequence homology between the C-terminal half of nNOS and NADPH–cytochrome P-450 reductase that the binding site for tetrahydrobiopterin, the only other cofactor known to be required for NOS activity in 1991, was more likely to be in the N terminus (see later).

Marletta's laboratory suggested a role for the flavin semiquinone in catalyzing the one-electron chemistry that must accommodate the formation of NO·, a five-electron oxidation process (Hevel *et al.,* 1991). In the first studies of an FAD- and FMN-containing enzyme, a mechanism for NADPH–cytochrome P-450 reductase catalysis was proposed (Masters *et al.,* 1965a,b) in which the semiquinone redox state was the most oxidized partner of the catalytic cycle and electrons were shuttled between one- and three- or

Table I Properties of Mammalian NOS Isoforms

Structural and enzymatic parameters	Active homodimers		
	nNOS (NOS I)	eNOS (NOS III)	iNOS (NOS II)
Subunit molecular mass	160 kDa	135 kDa	125–130 kDa
Inducibility	Constitutive	Constitutive	Inducible
Calmodulin binding	$\sim 30 \times 10^{-9}\,M$	$\sim 30 \times 10^{-9}\,M$	Subunit-like ($\gg 30 \times 10^{-9}\,M$)
Cofactors	H_4B, FAD, FMN, heme, Zn	H_4B, FAD, FMN, heme, Zn	H_4B, FAD, FMN, heme, Zn
Substrates	NADPH, L-arginine, oxygen	NADPH, L-arginine, oxygen	NADPH, L-arginine, oxygen
Protein variants	μ, α, β, γ tissue-specific isoforms	—	—
Posttranslational modifications	Specific phosphorylation sites present	Myristoylation, palmitoylation, phosphorylation sites present	Specific phosphorylation site present
Sources of superoxide formation	Heme domain, reductase domain	Mainly heme domain	Mainly reductase domain
Protein–protein interactions	PSD-95, caveolin3, phosphofructokinase M	Caveolin1, HSP 90, CAT-2, bradykinin receptor	—
Major physiological function	Neurotransmission	Vasodilatation	Cytotoxicity
Role in disease	Stroke, muscular dystrophy, ischemia–reperfusion injury	Endothelial dysfunction, hypercholesterolemia, hypertension	Toxic shock, inflammation, autoimmune disease

between two- and four-electron-reduced states. The possibility that such a mechanism might apply to the NOS isoforms was brought to light by the report of Stuehr and Ikeda-Saito (1992) in which they provided evidence for a $g = 2.0$ electron paramagnetic resonance (EPR) signal that disappeared on reoxidation of the sample by ferricyanide. This led this author (Masters, 1994) to propose an oxygenation cycle for NOS that involves redox states of the heme and flavins by analogy with the known interactions of NADPH–cytochrome P-450 reductase with cytochromes P-450 or artificial electron acceptors. In this scheme, the various redox states for the flavoprotein domain are proposed to interact with the appropriate substrate-bound form of NOS during the oxygenation process. Although this scheme did not include a redox role for H_4B, the cation radical form of $H_3B^{\cdot}$ could be invoked as an intermediate in the odd-electron chemistry of the overall NOS reaction mechanism (see later).

In a study of all three isoforms, Martásek *et al.* (1999) showed that the flavoprotein-catalyzed reduction of cytochrome *c* by all three NOS isoforms can be compared to that of NADPH–cytochrome P-450 reductase under similar conditions. The turnover numbers for nNOS and eNOS in the reduction of artificial electron acceptors, such as cytochrome *c,* are minimal in the absence of Ca^{2+}/CaM. The addition of Ca^{2+}/CaM to nNOS results in a 10- to 15-fold increase in cytochrome *c* reduction ($\sim$5000 min^{-1}), which is $\sim$1.6 times the rate of NADPH–cytochrome P-450 reductase-catalyzed cytochrome *c* reduction (Martásek *et al.,* 1999). On the other hand, although eNOS cytochrome *c* reductase activity is increased >2-fold in the presence of Ca^{2+}/CaM, its activity is only $\sim$16% that of the reductase (Martásek *et al.,* 1999, Fig. 1). Inducible NOS, in the absence of added Ca^{2+}/CaM, catalyzes cytochrome *c* reduction at the same rate as Ca^{2+}/CaM-activated nNOS. The rates of cytochrome *c* reduction can be ranked accordingly: iNOS = nNOS $\gg$ e NOS. In other experiments in which electron transfer to the heme domain is measured by reduced CO difference spectroscopy (binding of CO is possible only with reduced heme), it is apparent that eNOS is the most ''tightly coupled'' of the NOS isoforms: NADPH reduces the heme to the same extent as dithionite, whereas in the case of nNOS, approximately 70% of the heme is reduced by NADPH, and in iNOS, approximately 30% reduction occurs. These results have interesting implications with respect to the structure–function relationships among the various NOS isoforms due to the marked differences in sequence in specific regions of the flavoprotein domains of the three NOS isoforms.

Advantage was taken of sequence alignments by Salerno *et al.* (1997) to show additional sequences in the flavoprotein domains of the constitutive NOS isoforms that were not present in inducible NOS, flavodoxins, or NADPH–cytochrome P-450 reductase (Salerno *et al.,* 1997). Homology-based molecular modeling showed the superimposition of the backbone structures of iNOS and NADPH–cytochrome P-450 reductase on the backbone of *Desulfovibrio vulgaris* flavodoxin, the structural homolog of the FMN binding mod-

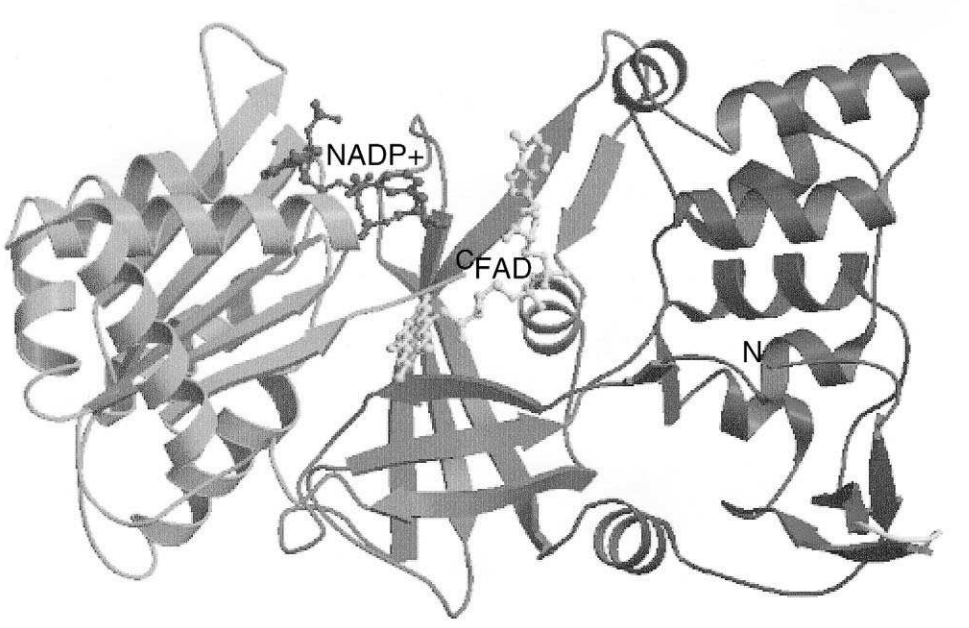


Figure 1 A ribbon diagram of the overall structure of the nNOS FAD-binding domain. The structure comprises three functional domains: the NADP(H)-binding domain is shown in olive green, the FAD-binding domain in green, and the connecting domain in red. The N and C termini are indicated, and the cofactors are shown in ball-and-stick form. See color insert.

ule of NOS that it most resembles. Most of the modeled eNOS and nNOS backbone structures can also be superimposed, except for the region representing an ~45-amino acid insertion in the constitutive isoforms, eNOS and nNOS. These comparisons suggested the possibility that a loop, consisting of a preponderance of positive charges and located within the FMN binding site only in eNOS and nNOS, could be involved in the binding interactions required for calmodulin activation of the constitutive NOS isoforms. The hypothesis was put forth that this insertion could represent an ''autoinhibitory loop'' that sterically hinders the binding of calmodulin through binding domain overlap or through allosteric effects.

To test this hypothesis, Salerno *et al.* (1997) used peptides, the sequences of which were derived from the 45-amino acid insert in eNOS, to inhibit the formation of NO· or the binding of ^{125}I-calmodulin or [^{3}H]N^{G}-nitro-L-arginine. The most potent inhibitory sequences were those containing the RRKRK motif. In a control experiment, limited trypsinolysis was performed, and it was determined that cleavage of exposed lysine residues (Lys-856 in nNOS and Lys-545 in eNOS) occurred in the presence of Ca^{2+}/calmodulin binding but not in its absence. Since these residues exist within the putative ''autoinhibitory loop,'' the latter experiment suggests that conformational changes involving this loop occur during calmodulin activation of constitutive NOS isoforms. This activation is precluded in the indicuble isoform due to the absence of this loop sequence. Additional support for such conformational changes has come from the reports of Sheta *et al.* (1994), Gachhui *et al.* (1996), Narayanasami *et al.* (1997), and Schmidt *et al.* (1992) in which Ca^{2+}/calmodulin-induced fluorescence changes were measured.

Subsequent to this report, several additional studies have appeared that support the role of such a modulatory sequence. In a study from Stuehr's laboratory (Adak *et al.,* 1999) in which the aim was to study the role of the nNOS reductase domain cluster of acidic residues, two conserved acidic residues (Asp-918 and Glu-919) and one conserved aromatic residue (Phe-892) were mutated. The authors examined flavin binding, conformational changes, electron transfer reactions, calmodulin regulation, and catalytic activities to determine the effects of these mutations. Although FMN binding was destabilized, the binding of substrate, cofactor, and calmodulin were unaffected when the nNOS was reconstituted with FMN, indicating that the defect was isolated to the FMN-binding module. Coincidentally, these studies established the flow of electrons from NADPH to FAD to FMN to heme. FMN binding seemed to suppress NADPH binding, but its absence did not affect electron transfer to 2,6-dichlorophenolindophenol (DCIP) or potassium ferricyanide. FMN removal increased the rate of ferricyanide reduction, as did the binding of Ca^{2+}/calmodulin, leading the authors to conclude that Ca^{2+}/calmodulin binding is equivalent to FMN removal and is consistent with the increase in flavin fluorescence being restricted to the FMN module of nNOS. These authors offer the conclusion that the FMN module is the key response element in nNOS responsible for regulating electron transfer to the various acceptors, including the NOS-bound heme. These authors also offer the possibility that their mechanism could involve the insert in nNOS proposed by Salerno *et al.* (1997) to regulate Ca^{2+}/calmodulin binding affinity.

Simultaneously, Daff *et al.* (1999) showed that deletion mutants of nNOS, in which 40 and 42 amino acids were removed from the putative autoinhibitory loop (Salerno *et al.,* 1997), retained maximal NO· synthesis activity at lower concentrations of Ca^{2+} than the wild-type nNOS. Also, remarkably, the mutants retained 30% of their activities in the absence of Ca^{2+}/calmodulin, suggesting that this insert is involved in inhibiting electron transfer from FMN to heme in the absence of Ca^{2+}/calmodulin and in destabilizing Ca^{2+}/calmodulin binding when Ca^{2+} levels are low in nNOS, supporting its role as an autoinhibitory loop.

The generation of chimeric constructs, utilizing sequences of the constitutive NOS isoforms and inducible NOS, by the laboratories of Nathan (Ruan *et al.,* 1996), Ortiz de Montellano (Nishida and Ortiz de Montellano, 1998), and Stull (Lee and Stull, 1998) also presented evidence that the Ca^{2+}/calmodulin binding domain as well as elements in both the reductase and oxygenase domains are responsible for the Ca^{2+} independence of iNOS. None of these studies is inconsistent with the existence of an autoinhibitory binding loop in the constitutive NOS isoforms.

The author's laboratory, in collaboration with Dr. J.-J. Kim, has been involved in crystallographic studies of flavin-containing constructs of the NOS isoforms to follow their reports of the crystallization (Djordjevic *et al.,* 1995) and structural determination (Wang *et al.,* 1997) of NADPH–cytochrome P-450 reductase. More recently, it has been possible to crystallize and solve the structure of the FAD binding domain containing nNOS residues 963–1412 obtained by trypsinolysis of the holoenzyme shown in Fig. 1. In support of the conclusions of Adak *et al.* (1999), the FAD binding domain is not unique structurally. In fact, when the structures of the nNOS–FAD binding domain and NADPH–cytochrome P-450 reductase are overlaid, the FAD binding domains are virtually superimposable. This means that this domain, which exhibits considerable sequence similarity with two NADPH-dependent flavoproteins, ferredoxin–$NADP^{+}$ reductase and NADH–cytochrome b_5 reductase, contains binding sites for both NADPH and FAD and binds FAD in an extended conformation. As in NADPH–cytochrome P-450 reductase, the isoalloxazine ring is between the FAD and NADP(H) binding subdomains, and the remainder of the FAD molecule lies between the FAD binding and connecting domains. There are aromatic stacking residues on both sides of the nNOS FAD isoalloxazine ring and on one side of the adenine ring.

Of course, the absence of the FMN binding domain in this crystal structure leaves many of the questions raised in the previous discussion unanswered. By default, the structural homology of the FAD binding domain with NADPH–

cytochrome P-450 reductase leaves the FMN binding domain highly suspect as the seat of the modulation of Ca^{2+}/calmodulin regulation. This suggestion is bolstered by the fact that the crystal structures of the heme domain dimers of the NOS isoforms have offered little insight into mechanistic differences.

Iron Protoporphyrin IX

Stoichiometry

When the simultaneous discovery by four separate laboratories of the presence in NOS of iron protoporphyrin IX (heme) was made (McMillan *et al.,* 1992; White and Marletta, 1992; Stuehr and Ikeda-Saito, 1992; Klatt *et al.,* 1992), the question of the site of substrate oxygenation was solved, but the role of tetrahydrobiopterin became even more mystifying. Since the stoichiometry of flavin binding was already known (see earlier), it was a simple matter to establish the relative stoichiometry of heme binding to NOS. In all studies reported in 1992, the binding of one heme group per monomer was determined, regardless of the NOS isoform. Of interest from a mechanistic viewpoint was the finding that the difference absorption spectrum, determined in the presence of reducing agent (dithionite) and CO and compared to the oxidized form, revealed an absorption maximum at ~445 nm. As is the case with all cytochromes P-450 known, this absorption maximum indicates the presence of a cysteine thiolate ligand to the heme iron.

Oxidation–Reduction, Mechanistic Aspects, and Structural Properties

Because the cytochromes P-450 are involved in the oxygenation of a variety of endogenous and exogenous substrates, the analogies with the nitric oxide synthesis reaction began to unfold, since it was already known that the C terminus of nNOS was highly homologous to NADPH–cytochrome P-450 reductase. Almost immediately, these analogies led to speculation regarding the oxidation–reduction mechanisms of NOS catalysis by a number of workers, including Marletta (1993; Hurshman *et al.,* 1999), Griffith and Stuehr (1995), Masters (1994), and Korth *et al.* (1994), among others. Evidence was presented by McMillan and Masters (1993) and Pufahl and Marletta (1993), using L-arginine or N^G-hydroxy-L-arginine perturbation of the heme spectrum of NOS (optical absorption difference spectroscopy), that the spectral signature of a high spin heme was obtained on the addition of L-arginine to NOS. These results, which were reminiscent of those obtained with cytochromes P-450 and various substrates and heme ligands (Lewis, 1996; Schenkman *et al.,* 1981), indicated that these compounds were perturbing the structures of NOS in the environment of the heme. The development of a high-spin (''type I'') spectrum precluded direct ligation to the heme iron. Other nitrogenous ligands, such as imidazole (McMillan and Masters, 1993), on the other hand, produced low-spin shifts in the binding spectra, indicating direct ligation to the heme iron. Given these interactions, it was concluded that the interaction of substrate with heme indicated its involvement in the oxygenation process, thus diminishing the role of tetrahydrobiopterin in this respect.

As mentioned in the review by Raman *et al.* (2000), there were actually four important observations that supported the notion that NOS isoforms might bear some similarity to the cytochrome P-450 family of enzymes: (1) the similarity of the domain structure to the cytochrome P-450 from *Bacillus megaterium,* a self-sufficient fatty acid-metabolizing monooxygenase consisting of a heme domain and flavin domain (FAD- and FMN-containing) in a single polypeptide (Narhi and Fulco, 1987); (2) the spectroscopic properties, similar to members of the cytochrome P-450 family, alluded to above; (3) sequence similarity between the NOS and cytochrome P-450 family members within a nine-residue sequence believed to be involved in heme ligation and containing the cysteine thiolate (McMillan *et al.,* 1992); and (4) the capability of the cytochromes P-4503A to catalyze NO· formation from N^G-hydroxy-L-arginine, the obligatory intermediate in NOS catalysis. It is well to bear in mind one major difference, however, in the binding sites for NOS enzymes as compared to those for cytochromes P-450. Catalysis by NOS of L-arginine monooxygenation already defines the environment of the substrate as being highly polar, due to the guanidinium function of L-arginine, whereas cytochromes P-450 catalyze the hydroxylation of hydrophobic substrates such as steroids, fatty acids, prostaglandins, and a myriad of drugs and environmental toxicants, all of which are hydrophobic compounds.

On the revelation of the structure of iNOS by Crane *et al.* (1998) and that of eNOS by Raman *et al.* (1998), it was apparent that a unique fold with no similarities to previous P-450 structures (all of bacterial origin) exists in iNOS and eNOS. The cysteine thiolate ligand resides on the proximal side of the heme in both cytochromes P-450 and NOS, but in cytochromes P-450, a helix containing residues that participate in catalysis occurs in proximity to the heme iron. NOS, in contrast, contains a β strand on the distal side of the iron protoporphyrin ring. As in many other enzymes that contain metal-bound thiolates, cytochromes P-450 (Poulos *et al.,* 1985) and NOS isoforms (Crane *et al.,* 1998; Raman *et al.,* 1998; Fischmann *et al.,* 1999; Li *et al.,* 1999) can accept hydrogen bonds from neighboring backbone NH groups due to the formal negative charge of these metal–thiolate complexes.

In examining the distal heme environment and the planar structure of the bound heme, it can be seen that the heme ring is distorted into a concave configuration with the bowl facing the distal heme pocket. The heme is buried within this pocket with little solvent accessibility except for one of the heme propionates, but a large substrate/cofactor access channel allows solvent access to both the active site and heme, as well as the tetrahydrobiopterin binding site. Tetrahydrobiopterin binding, however, has no effect on the nonplanarity of the heme, since H_4B-free eNOS (Raman *et al.,* 1998) reveals

the same ring distortion. Possible consequences of this heme ring distortion are discussed in detail by Raman *et al.* (2000). In addition to the obvious nonplanar configuration, NOS heme exhibits a different diastereomeric orientation relative to the methyl and vinyl side chain substituents of the porphyrin ring. The iNOS crystal structure shows that the heme plane facing the thiolate ligand (proximal) is flipped 180° compared with cytochromes P-450 (Crane *et al.,* 1997). In addition, the eNOS structure (Raman *et al.,* 1998) indicates that the heme orientation is similar to that seen in peroxidases, although these enzymes utilize a histidine residue as the proximal ligand. Despite these facts, chloroperoxidase and eNOS show similarities in EPR signatures for ligand binding, indicating polar distal heme environments in both types of enzymes. Comparison of the Soret circular dichroism structures showed that the spectra were inverted with respect to one another, suggesting a different heme orientation that was later confirmed in the structures (C. S. Raman, P. Martásek, and B. S. S. Masters, unpublished observations, 1995; Raman *et al.,* 2000; Andersson and Peterson, 1995).

The recognition of the substrate, L-arginine, is a far more important consideration in the NOS isoforms, because of the high degree of specificity of the active site of NOS compared to cytochromes P-450, which generally accommodate a large number of hydrophobic substrates of differing structures (with the exception of the steroid hydroxylases that exhibit stereo- and regioselectivity). L-Arginine is bulky and contains a large and extended surface (length 6.3 Å, volume 195 $Å^3$), and, of course, it is charged owing to its guanidinium side chain. Substrate recognition by NOS isoforms must accommodate the strong polarity of L-arginine, which is the most hydrophilic of all amino acids found in proteins.

With the exception of one residue, Val-338 in eNOS, which makes a van der Waals contact with the aliphatic portion of L-arginine, there are no other hydrophobic groups in spatial contiguity with the substrate. In eNOS, the guanidinium side chain of L-arginine is hydrogen bonded to the active site Glu-363, which is a typical side-chain interaction in many proteins; this result is predicted by the site-directed mutagenesis studies of Chen *et al.* (1997; in eNOS) and Gachhui *et al.* (1997; in iNOS). The binding of L-arginine is nearly identical in both iNOS and eNOS, as shown in the crystal structures of the substrate-bound heme domain dimers (Crane *et al.,* 1998; Raman *et al.,* 1998; Fischmann *et al.,* 1999; Li *et al.,* 1999), and the residues participating in hydrogen bonding are highly conserved. The Raman *et al.* (2000) review shows hydrogen bonding interactions of eNOS with L-arginine, seven of which are with protein residues, one with water, and one with the propionic side chain of heme. In addition, a number of van der Waals contacts contribute to substrate binding (Raman *et al.,* 2000). In experiments performed in collaboration with Hoffman's laboratory, the Masters' laboratory was able to determine the distance between the guanidinium nitrogen of both L-arginine (~4 Å) and N^G-hydroxy-L-arginine (3.8 Å) to the heme iron in nNOS (Tierney *et al.,* 1998, 1999) using Q-band pulsed ^{15}N electron nuclear double resonance (ENDOR) spectroscopy and employing ^{15}N-labeled substrates. The data for L-arginine were actually published before the crystal structures were revealed, exhibiting the power of this technique for obtaining structural information.

In addition to the binding of L-arginine, the heme domain also contains the binding site for H_4B. This site also appears to be identical among the various isoforms, based on the available crystal structure data.

Tetrahydrobiopterin

STOICHIOMETRY

The requirement for tetrahydrobiopterin (H_4B) for catalysis of NO· formation was established first for the inducible NOS from macrophages by Tayeh and Marletta (1989) and Kwon *et al.* (1989) and subsequently in neuronal NOS by Mayer *et al.* (1990) and Schmidt *et al.* (1992). Although the content of H_4B in tissue-purified NOS preparations varies greatly, it is now clear that the stoichiometry for binding is one H_4B per monomer, based on X-ray crystallographic studies to be discussed later. On the successful high-level expression of the NOS isoforms in *Escherichia coli* (McMillan and Masters, 1995; Roman *et al.,* 1995; Martásek *et al.,* 1996), in which the absence of the GTP cyclohydrolase precludes the biosynthesis of H_4B, it has been possible to examine H_4B-free preparations of the NOS isoforms. It is now well established from many studies that these preparations are inactive without the addition of exogenous H_4B (Roman *et al.,* 1995; Vásquez-Vivar *et al.,* 1998, 1999; Hurshman *et al.,* 1999). Many studies have attempted to delineate the role of H_4B in the mechanism of NOS, and certain aspects of the binding and action of this pterin cofactor have been compared to the aromatic amino acid hydroxylases and other pterin-utilizing enzymes. Raman *et al.* (1998) have discussed these comparisons in detail, and similarities have been drawn between H_4B binding by NOS and those enzymes that utilize pterin as a substrate (dihydroneopterin aldolase, dihydropteroate synthase, thymidylate synthase, and molybdopterin oxidoreductase) and not as a cofactor (aromatic amino acid hydroxylases). Also, in light of the fact that the cytochrome P-450 systems, with which the NOS isoforms share much similarity in mechanism, do not require H_4B, it is important to determine its role in these complex enzymes.

OXIDATION–REDUCTION AND/OR FUNCTIONAL ROLE

The actual functional significance of H_4B in NOS catalysis remains unsolved. However, studies utilizing different approaches to probe the structure–function aspects of NOS isoforms are invoking an oxidation–reduction (redox) role for H_4B, while not involving this cofactor in the activation of oxygen for the oxygenation of either L-arginine or *N*-hydroxy-L-arginine. It has been known for some time that the reduced form of pterin is the only redox state that supports NO· formation catalyzed by any of the NOS isoforms. Klatt *et al.* (1994) examined the binding site of tetrahy-

drobiopterin, using ^{3}H-labeled (6*R*)-5,6,7,8-tetrahydro-L-biopterin as a radioligand and a variety of pteridine derivatives as antagonists. These investigators also found that the affinity of porcine brain nNOS for H_4B was increased sixfold in the presence of L-arginine (K_D = 37 n*M*), a phenomenon that was not to be thoroughly understood until the structures of the heme domains of NOS were solved (see later).

On examination of the structures of the heme domains of inducible NOS or endothelial NOS (Crane *et al.,* 1998; Raman *et al.,* 1998; Fischmann *et al.,* 1999; Li *et al.,* 1999), the nature of the binding of H_4B to NOS may be better understood. In particular, the report of Raman *et al.* (1998) focused on the complex hydrogen-bonding system involving H_4B, including interactions with the heme prosthetic group and the various amino acid residues of NOS within hydrogen-bonding distance of H_4B, and the impact of these interactions with the substrate, L-arginine. In addition, Raman *et al.* (1998) noted that in one of their solved crystal structures, obtained with the inhibitor *S*-ethylisothiourea (SEITU) bound in the substrate binding site, L-arginine was unexpectedly found in the H_4B binding site (Fig. 2) (Raman *et al.,* 1998, 2000). This led the authors to suggest that this site accommodated a structure similar to a cation radical, mimicked by the planar guanidino group of L-arginine.

The prior report of Bec *et al.* (1998) had already suggested an enzymatic basis for implicating the $H_3B\cdot$ in electron transfer by showing differences in the decay of the $Fe^{II}O_2$ complex of nNOS in single turnover studies in the presence and absence of H_4B. The studies by Bec *et al.* (1998) were performed at lower temperature (−30°C) to stabilize the oxy complex of nNOS, and spectral intermediates were directly compared to cytochrome P-450 2B4 under the same experimental conditions. Spectral comparisons led to the conclusion that the decay of the oxyferrous complex of NOS produces a discernible proportion of high-spin ferric heme, not seen in the concurrent P-450 spectra obtained in the Bec *et al.* (1998) experiments but resembling those seen with other ferrous complexes of cytochromes P-450 (Dawson *et al.,* 1983; Dawson and Sono, 1987) for ''hyperporphyrin'' species. Bec *et al.* (1998) determined that H_4B-free and H_4B-bound NOS produced similar spectral changes on the binding of O_2 at −30°C, and the autooxidation of these species resulted in low-spin ferric heme. In contrast, although L-arginine alone did not produce the blue-shifted oxygen complex observed in H_4B-bound nNOS, and the spectra resembled those obtained with the H_4B-free nNOS in the absence of L-arginine, the presence of both substrate and cofactor resulted in the formation of high-spin ferric heme. Thus, the decay of the oxygenated, L-arginine-bound intermediate in the presence of added H_4B, but not in its absence, led to the conclusion that $H_3B\cdot$ could be formed as a result of a one-electron donation from the reduced pterin to the oxygenated species. This observation could also be explained by the unique interactions between H_4B, the heme prosthetic group, and the substrate, L-arginine, revealed in the crystal structures or, alternatively, by interaction with the flavins of NOS.

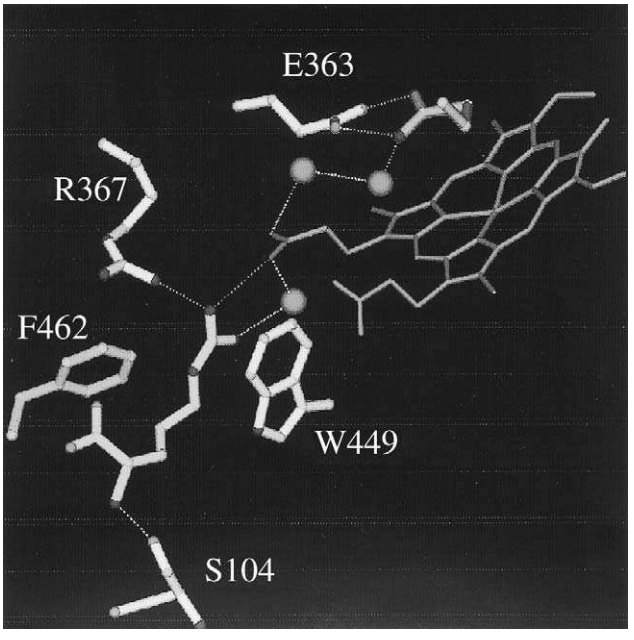


Figure 2 L-Arg is a structural mimic of H_4B at the pterin-binding site when SEITU is bound at the active site (−H_4B, +SEITU data). L-Arg binds to the pterin site and exquisitely mimics the H_4B interaction with eNOS (Fig. 3). The specific interaction of the potent inhibitor, SEITU, at the active site is mediated by a pair of bifurcated hydrogen bonds to Glu-363. Two water molecules bridge the inhibitor and heme propionate. The ethyl group of the inhibitor forms nonbonded contacts with Val-338 and Phe-355. The ureido sulfur is positioned 3.5 and 4.0 Å above the heme pyrrole B-ring nitrogen and the heme iron, respectively. See color insert.

Mayer and colleagues (Reithmuller *et al.,* 1999) have also reported that the 5-methyl analog of H_4B is functionally active in NOS but cannot react with molecular oxygen or stimulate phenylalanine hydroxylase activity. Although previous experiments from Kaufman and colleagues (Giovanelli *et al.,* 1991) had shown that H_4B in catalytic amounts could support NOS activity, these investigators also showed that no recycling of exogenous H_4B occurred during NOS turnover. Furthermore, these investigators later stated that their previous results did not preclude the possibility that tightly bound H_4B could participate in recycling (Campos *et al.,* 1992). Using limiting concentrations of NADPH and a regenerating system for NADPH, Kaufman's laboratory (Witteveen *et al.,* 1996) was able to determine H_4B levels formed from qH_2B during turnover of recombinant nNOS, by coupling its formation to the phenylalanine hydroxylase reaction and detecting the amount of H_4B-dependent conversion of [^{3}H]phenylalanine to [^{3}H]tyrosine. The reduction of qH_2B was inhibitable by diphenyleneiodonium, a potent, nonspecific flavoprotein inhibitor, but was slightly stimulated by inhibitors of the oxygenase (heme) domain, such as 7-nitroindazole or *N*-nitro-L-arginine, indicating that it was catalyzed by the flavoprotein domain of nNOS.

Witteveen *et al.* (1999), using the same method of measuring the formation of H_4B, purported to show that single-turnover oxidation of H_4B is stimulated by L-arginine in the presence of Ca^{2+}/CaM, and that reduction of the oxidation

product of H_4B by the flavoprotein activity is consistent with a role for pterin as an electron donor in product formation. Witteveen *et al.* (1999) were unable to define this role, however. The nNOS preparation used in these experiments (Witteveen *et al.,* 1996, 1999) was expressed in and purified from human kidney 293 cells, in which H_4B can be biosynthesized. Therefore, the preparations contain varying amounts of the pterin cofactor, and, in the later publication (Witteveen *et al.,* 1999), these authors state that the H_4B content is ''typically 0.5 mol/mol subunit'' from high-pressure liquid chromatography analysis of oxidized samples. These authors quoted the report of Bec *et al.* (1998), who suggested that H_4B is involved in supplying the single electron required for reduction of ferric heme iron superoxide to the ferric heme iron peroxo species during L-arginine conversion to *N*-hydroxy-L-arginine from their single-turnover experiments. On the basis of this requirement for only one electron, Bec *et al.* (1998) formally represented the intermediate pterin species as $H_3B\cdot$, the trihydrobiopterin radical.

Also in 1999, Hurshman *et al.* from Marletta's laboratory performed rapid freeze–quench electron paramagnetic resonance spectroscopy to monitor the reaction of reduced iNOS heme with O_2 in the presence and absence of substrate. These experiments revealed the formation of a transient species, while heme was oxidized at a rate of $\sim 15\ s^{-1}$, with a $g = 2.0$ EPR signal. The anisotropic properties of this signal are typical of an organic radical with $S = \frac{1}{2}$. When the iNOS heme construct is reconstituted in the presence of N-5 ^{14}N- and ^{15}N-substituted H_4B, the spectra reveal a hyperfine interaction of the unpaired electron with the pterin N-5 nitrogen, identifying the radical as the one-electron oxidized form of the bound H_4B (the trihydrobiopterin radical, $H_3B\cdot$). In the presence of L-arginine, the radical accumulates to ~80% of the total iNOS heme concentration, but *N*-hydroxy-L-arginine prevents its formation, limiting it to ~3%. The rate of formation of the radical is 15–20 s^{-1}, independent of substrate, and its subsequent decay rate is 0.12–0.7 s^{-1}, depending on the substrate. Furthermore, the $g = 2.0$ signal is not observed in the absence of pterin. The authors argue that all of their data implicate the $g = 2.0$ signal as a NOS-bound pterin radical. Pterin radicals have been produced chemically under acidic conditions by reacting oxidants with reduced pterins or by comproportionation of tetrahydropterin and dihydropterin. The chemical and electronic structure of the pterin organic radical formed in the studies of Hurshman *et al.* (1999), however, cannot be determined from the X-band EPR experiments, and the representation of the radical form as $H_3B\cdot$ does not designate whether it is cationic or neutral. In addition, three-syringe double mixing experiments by Hurshman *et al.* (1999) showed that the radical does not react with either L-arginine or *N*-hydroxy-L-arginine (NHA), thus negating the possibility that a fast reaction of the latter intermediate with the pterin radical occurs. On the other hand, the presence of NHA could prevent the one-electron oxidation of H_4B by reducing the ferrous–dioxygen complex ($Fe^{+3}O_2\cdot^-$) by single electron transfer, but there is no evidence for NHA$\cdot$ formation in these experiments. Finally, it should be cautioned, as the authors (Hurshman *et al.,* 1999) have correctly done, that these results, obtained with the iNOS heme domain dimer, may not be extrapolatable to the holoenzyme. The presence of reduced flavin cofactors in the holoenzyme could complicate the formation and/or decay rates of the putative $H_3B\cdot$ radical. Nevertheless, it is tempting to entertain the possibility, suggested by the turnover experiments of Bec *et al.* (1998) and the crystallographic results of Raman *et al.* (1998), that the $H_3B\cdot$ radical is involved in electron transfer in NOS to the $Fe^{2+}O_2$ complex. The answers await development of the appropriate technology to examine this mechanistic possibility more directly and to identify the nature of the radical unequivocally.

Zinc Tetrathiolate Metal Center

STOICHIOMETRY

The determination of the 1.9-Å-resolution X-ray crystal structure of the heme domain dimer of endothelial NOS by Raman *et al.* (1998), in a collaborative study between the laboratories of Poulos and Masters, resulted in the discovery of zinc as the metal constituent of a tetrathiolate center (Fig. 3; see earlier). The beautiful symmetry of the zinc-bound form of eNOS, that is, the equal distance (21.6 Å) of the ZnS_4 center to each of the hemes, which are 34 Å apart in the dimer, and the equal distance (12 Å) from each of the

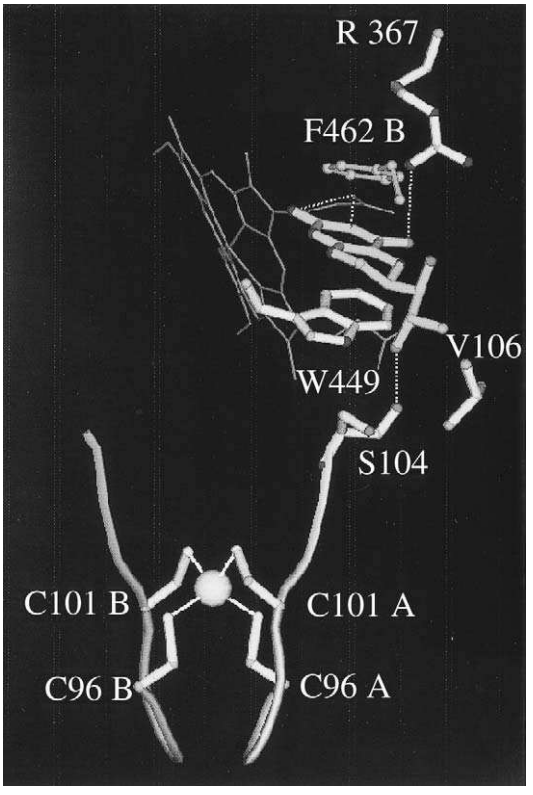


Figure 3 The ZnS_4 metal center and its relationship to H_4B. Ser-104 is part of the loop containing the cysteine ligands and hydrogen bonds to the C-6 side chain of pterin. The stereospecific recognition of H_4B by NOS is dictated by the substitution at the C-6 position. See color insert.

pterin cofactors, which form an equilateral triangle with zinc, argues for biological significance and chemical stability. On further examination, Raman *et al.* (1998) discovered that all NOS sequences presently known, from mosquito to human, contain the consensus sequence CX_4C, which constitutes an identical pair of cysteine residues in each of the monomers of NOS involved in the ligation of zinc. Other interesting aspects of this ZnS_4 center are its sequence proximity to the Ser-104 residue that hydrogen bonds to the dihydroxypropyl side chain of tetrahydrobiopterin, which probably ensures the stereospecific recognition of H_4B (see earlier in this review, and Raman *et al.,* 1998).

The report of Miller *et al.* (1999) verified the stoichiometry of one Zn atom per two hemes in holoenzyme preparations of both eNOS and nNOS, utilizing electrothermal vaporization–inductively coupled plasma–mass spectrometry (ETV-ICP-MS) and a spectrophotometric chelation assay with 4-(2-pyridylazo)resorcinol (Crow *et al.,* 1997). Rodríguez-Crespo *et al.* (1999) determined the zinc content of all three isoforms, and although the zinc content was low (~25%) in nNOS, both eNOS and iNOS contained ~70% zinc as measured by inductively coupled plasma atomic emission spectroscopy. Interestingly, these authors showed that treatment of iNOS with 50 m*M* EDTA during affinity column purification lowered the zinc content to below 15%. In addition, contrary to the results of Perry and Marletta (1998), who reported that reconstitution of iNOS and nNOS with iron increases NO-synthesizing activity, Rodríguez-Crespo *et al.* (1999) showed no effect of Fe^{2+} on eNOS, slightly enhanced activity of nNOS, and a lowering of iNOS activity to ~70%. Perry and Marletta (1998) also reported that both purified iNOS and nNOS contain approximately equal amounts of bound zinc and copper, respectively, and both were reconstituted, after desalting, with $FeCl_2$, yielding one nonheme iron per monomer. This result led to their proposal that a metal binding site in the vicinity of the H_4B binding site, involving His-652 and Asp-650 residues of nNOS, exists in the NOS isoforms similar to that seen, for example, in lysyl hydroxylase (Pirskanen *et al.,* 1996). This is now known not to be the case from the solved structures of eNOS and iNOS heme domain dimers (Crane *et al.,* 1998; Raman *et al.,* 1998; Fischmann *et al.,* 1999; Li *et al.,* 1999). Furthermore, the mutation of all five conserved histidine residues in endothelial NOS, undertaken by Rodríguez-Crespo *et al.* (1999) to determine if any of these histidines form a nonheme iron-binding site, did not affect the metal content, nor did it influence the ability of exogenous Fe^{2+} to modify the catalytic activity of endothelian NOS.

With the publication of Raman *et al.* (1998), the report of Chen *et al.* (1995) showing that a C99A mutation of human eNOS markedly affected the binding of H_4B peaked the interest of the Masters laboratory and prompted the generation of a comparable mutant, C331A, of rat nNOS (Martásek *et al.,* 1998). The homologous residue in each isoform fortuitously constituted one of the cysteine residues in each monomer involved in Zn binding. Miller *et al.* (1999) also reported the Zn content of the C331A nNOS mutant to be 5 or 19%, based on the stoichiometry of 1:2 zinc:heme in the H_4B-minus and H_4B-plus preparations, respectively, of this mutant. This result confirmed that the C331A mutant was defective in its zinc binding affinity owing to the loss of one-half of the residues involved in Zn binding.

In addition, following the Raman *et al.* (1998) study, a second crystallographic report appeared (Fischmann *et al.,* 1999) in which the crystal structures of human iNOS and eNOS heme domain dimers were determined and a zinc tetrathiolate was found to be a constituent of both isoforms. In their experiments, Fischmann *et al.* (1999) showed the near identity of the oxygenase (''catalytic'') domain structures of the human iNOS and eNOS isoforms. In addition, a second publication from Poulos' laboratory (Li *et al.,* 1999), showing Zn-free and Zn-replete human iNOS structures (Fig. 4), confirmed a disulfide bond between the symmetry-related Cys-115 residues in the Zn-free preparation. On Zn repletion, the preparation revealed the ZnS_4 tetrathiolate center reported by Raman *et al.* (1998). In their experiments, Li *et al.* (1999) reported that the addition of zinc, in the presence of a strong reducing agent, ''strengthened the crystal lattice as indicated by a decrease in cell dimensions and better diffraction'' (Li *et al.,* 1999). On the other hand, the original reports of Crane *et al.* (1997, 1998) did not reveal the presence of zinc in their crystallized preparations, perhaps due to the construct used for crystal growth, conditions for growth in the *E. coli* expression system, other factors during purification, and/or the presence of chelators in the buffers used (see Raman *et al.,* 2000). More recently, however, this group has produced a Zn-containing structure of murine iNOS and compared it to that of a Zn-free preparation (Crane *et al.,* 1999). The authors show ''swapped,'' disulfide-linked (Cys-109A–Cys-109B) intersubunit interactions across the dimer interface in the Zn-free structure and Zn-containing intrasubunit interactions involving N-terminal hairpin hooks in the ''unswapped'' structure. It is apparent that the Zn-free, swapped structure of Crane *et al.* (1999) has ordered conformation in the region of the Zn binding loop. The authors attribute these differences between their Zn-free structure and that of the disordered structure of Li *et al.* (1999) to the presence of ascorbate in the crystallization and synchrotron data collection buffer. The implications of these findings with respect to the functional role of iNOS, according to Crane *et al.* (1999) and Ghosh *et al.* (1999), will be discussed later. It should be pointed out, however, that in neither of these two publications is there an analysis of zinc content in the preparations utilized for crystallography, leaving the comparison of these structures with respect to zinc content somewhat conjectural.

FUNCTIONAL ROLE

Although there is considerable controversy regarding the function of zinc in the various NOS isoforms, it is generally agreed that profound structural changes occur on zinc binding (Li *et al.,* 1999; Crane *et al.,* 1999), at least in iNOS. Raman *et al.* (1998, 2000) have provided evidence for a structural role for zinc in the NOS isoforms. Their arguments

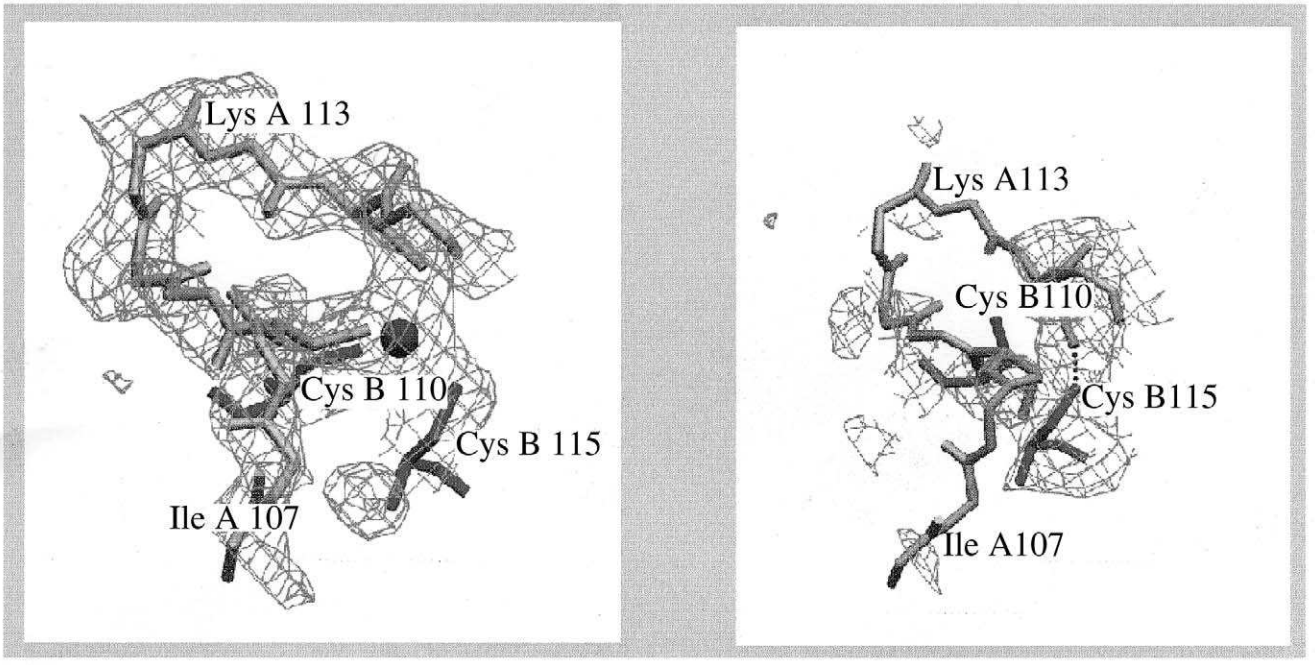


Figure 4 Stereo view of the $2F_0$-F_0 omit electron density map of zinc-free (right) and zinc-bound (left) structures contoured at 1σ. Maps were calculated by a simulated annealing protocol with the residues shown omitted. A disulfide bond is formed between two symmetry-related Cys^{-115} residues when zinc is lost. Residues 107–144 are disordered in the zinc-free structure, which is obviously indicated by the poor, broken density. For clarity, only side chains of Cys ligands are shown. See color insert.

are based on the location of the ZnS_4 center at the dimer interface and the roles of similar centers in at least six other enzymes in mediating protein–protein interactions or stabilizing intersubunit interactions or oligomerization, for example, aspartate transcarbamylase (Honzatko *et al.,* 1982), $p56^{lck}$–CD4 interaction (Huse *et al.,* 1998; Lin *et al.,* 1998), and Mss4 (Ostermeier and Brunger, 1999).

The Cys-331A mutant of nNOS, generated by Martásek *et al.* (1998), resulted in the replacement of one of the Zn-binding residues. This mutant was totally inactive until reactivated by overnight incubation with high concentrations of L-arginine, yielding activities comparable to that of wild-type nNOS. These results support the structural role of the intricate network connecting the zinc-binding cysteine residues with H_4B, heme, and L-arginine (Fig. 3). Not shown in this figure for the sake of clarity is the binding of L-arginine to the same propionic acid side chain of the heme that interacts with N-3 and the amine at C-2 of H_4B (Raman *et al.,* 1998).

Raman *et al.* (2000) have also presented a strong case for the unlikelihood that under normal cellular conditions disulfides would substitute for a metal center, including the fact that the cytosol exhibits a highly reducing environment, a situation that does not favor disulfide formation. In fact, except for a few instances in which sulfhydryl–disulfide interchange occurs in redox-active flavoproteins, such as glutathione reductase (Schulz *et al.,* 1978), most disulfide-containing proteins are extracellular. Raman *et al.* (2000) also point out in their review that, although there is no evidence at the present time that the ZnS_4-encompassing surfaces in NOS isoforms are involved in docking with other proteins, as has been shown for Mss4–rab interactions (Yu and Schreiber, 1995; Ostermeier and Brunger, 1999), the participation of zinc in the formation of a scaffold involved in molecular recognition is highly probable. In addition, the dissociation constants of three proteins binding zinc and one binding iron through $(Cys)_4$ ligands were shown to range from 10^{-9} to 10^{-15}, suggesting the very tight binding of zinc in these varied proteins that exhibit tetrahedral binding geometry.

It is important to keep these comparisons in mind in considering the reported swapped versus unswapped structures reported by Crane *et al.* (1999) and the accompanying biochemical and mutational studies of Ghosh *et al.* (1999). The tantalizing suggestion is made that ''correlation of metal ion release with swapping and the ability of Cys109 [in murine iNOS] to switch between zinc coordination and intermolecular disulfide formation may have implications for the regulation of NOS via alteration of dimeric stability and molecular surface properties.'' These authors support their suggestion further, stating, ''Reactive oxygen species and NO· produced during the oxidative burst of activated macrophages alter the cellular redox environment and react specifically with thiol-ligated metal centers (Demple, 1996; Lipinski and Drapier, 1997; Piedrafita and Liew, 1998; Wink and Mitchell, 1998). It will be interesting to determine if there are physiological or pathophysiological circumstances in which this type of switching could occur, in light

of the findings of Li *et al.* (1999) in human iNOS in which powerful reducing conditions, namely, tris(2-carboxyethyl) phosphine (TCEP) in lieu of reduced glutathione, were required to insert zinc into the disordered zinc-free construct prepared by trypsinolysis of the human iNOS heme domain (residues 74–703). In addition, Miller *et al.* (1999) were able to eject zinc from the eNOS or nNOS preparations only in the presence of guanidinium chloride and/or potent reduced oxygen species, such as hydrogen peroxide (H_2O_2). This, of course, does not preclude an isoform-specific response, but these circumstances are subject to confirmation by studies of more physiological situations and are difficult to substantiate.

References

Adak, S., Ghosh, S., Abu-Soud, H. M., and Stuehr, D. J. (1999). Role of reductase domain cluster 1 acidic residues in neuronal nitric-oxide synthase. Characterization of the FMN-free enzyme. *J. Biol. Chem.* **274,** 22313–22320.

Andersson, L. A., and Peterson, J. A. (1995). Active-site analysis of ferric P450 enzymes: Hydrogen-bonding effects on the circular dichroism spectra. *Biochem. Biophys. Res. Commun.* **211,** 389–395.

Arnold, W. P., Mittal, C. K., Katsuki, S., and Murad, F. (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3',5'-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3203–3207.

Bec, N., Gorren, A. C., Voelker, C., Mayer, B., and Lange, R. (1998). Reaction of neuronal nitric-oxide synthase with oxygen at low temperature. Evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin. *J. Biol. Chem.* **273,** 13502–13508.

Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 682–685.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P450 reductase. *Nature* **351,** 714–718.

Campos, K. L., Giovanelli, J., and Kaufman, S. (1992). Studies on the role of tetrahydrobiopterin in rat cerebellar nitric oxide synthase. *In* ''The Biology of Nitric Oxide'' (S. Moncada, M. A. Marletta, J. B. Hibbs, Jr., and E. A. Higgs, eds.), Vol. 2, pp. 47–50. Portland Press, London.

Chen, P.-F., Tsai, A.-L., and Wu, K. K. (1995). Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *Biochem. Biophys. Res. Commun.* **215,** 1119–1129.

Chen, P.-F., Tsai, A.-L., Berka, V., and Wu, K. K. (1997). Mutation of Glu-361 in human endothelial nitric-oxide synthase selectively abolishes L-arginine binding without perturbing the behavior of heme and other redox centers. *J. Biol. Chem.* **272,** 6114–6118.

Crane, B. R., Arvai, A. S., Gachhui, G., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1997). The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **278,** 425–431.

Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* **279,** 2121–2126.

Crane, B. R., Rosenfeld, R. J., Arvai, A. S., Ghosh, D. K., Ghosh, S., Tainer, J. A., Stuehr, D. J., and Getzoff, E. D. (1999). N-Terminal domain swapping and metal ion binding in nitric oxide synthase dimerization. *EMBO J.* **18,** 6271–6281.

Crow, J. P., Sampson, J. B., Zhuang, Y., Thompson, J. A., and Beckman, J. S. (1997). Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. *J. Neurochem.* **69,** 1936–1944.

Daff, S., Sagami, I., and Shimizu, T. (1999). The 42-amino acid insert in the FMN domain of neuronal nitric-oxide synthase exerts control over Ca^{2+}/calmodulin-dependent electron transfer. *J. Biol. Chem.* **274,** 30589–30595.

Dawson, J. H., and Sono, M. (1987). Cytochrome P450 and chloroperoxidase: Thiolate-ligated heme enzymes. Spectroscopic determination of their active site structures and mechanistic implications of thiolate ligation. *Chem. Rev.* **87,** 1255–1276.

Dawson, J. H., Andersson, L. A., and Sono, M. (1983). The diverse spectroscopic properties of ferrous cytochrome P450–CAM ligand complexes. *J. Biol. Chem.* **258,** 13637–13645.

Demple, B. (1996). Redox signaling and gene control in the *Escherichia coli* soxRS oxidative stress regulon—a review. *Gene* **179,** 53–57.

Djordjevic, S., Roberts, D. L., Wang, M., Shea, T., Camitta, M. G. W., Masters, B. S. S., and Kim, J. J. P. (1995). Crystallization and preliminary x-ray studies of NADPH-cytochrome P450 reductase. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 3214–3218.

Fischmann, T. O., Hruza, A., Niu, X.-D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999). Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat. Struct. Biol.* **6,** 233–242.

Furchgott, R. F. (1988). Studies on relaxation of rabbit aorta by sodium nitrite: The basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. *In* ''Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium'' (P. M. Vanhoutte, ed.), pp. 401–414. Raven, New York.

Furchgott, R. F., and Zawadski, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K., and Stuehr, D. J. (1996). Characterization of the reductase domain of rat neuronal nitric oxide synthase generated in the methylotrophic yeast *Pichia pastoris. J. Biol. Chem.* **271,** 20594–20602.

Gachhui, R., Ghosh, D. K., Wu, C.-Q., Parkinson, J., Crane, B. R., and Stuehr, D. J. (1997). Mutagenesis of acidic residues in the oxygenase domain of inducible nitric-oxide synthase identifies a glutamate involved in arginine binding. *Biochemistry* **36,** 5097–5103.

Garthwaite, J., Charles, S. J., and Chess-Williams, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336,** 385–388.

Gerber, N. C., and Ortiz de Montellano, P. R. (1995). Neuronal nitric oxide synthase. Expression in *Escherichia coli,* irreversible inhibition by phenyldiazene, and active site topology. *J. Biol. Chem.* **270,** 17791–17796.

Ghosh, S., Wolan, D., Adak, S., Crane, B. R., Kwon, N. S., Tainer, J. A., Getzoff, E. D., and Stuehr, D. J. (1999). Mutational analysis of the tetrahydrobiopterin-binding site in inducible nitric-oxide synthase. *J. Biol. Chem.* **274,** 24100–24112.

Giovanelli, J., Campos, K. L., and Kaufman, S. (1991). Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7091–7095.

Green, L. C., De Luzuriaga, K. R., Wagner, D. A., Rand, W., Istfan, N., *et al.* (1981a). Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7764–7768.

Green, L. C., Tannenbaum, S. R., and Goldman, P. (1981b). Nitrate synthesis and reduction in the germ-free and conventional rat. *Science* **212,** 56–58.

Griffith, O. W., and Stuehr, D. J. (1995). Nitric oxide synthases: Properties and catalytic mechanism. *Annu. Rev. Physiol.* **57,** 707–736.

Hevel, J. M., White, K. A., and Marletta, M. A. (1991). Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* **266,** 22789–22791.

Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Ewards, B. F. P., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C., and

Lipscomb, W. N. (1982). Crystal and molecular structures of native and CTP-liganded aspartate carbamoyltransferase from *Escherichia coli*. *J. Mol. Biol.* **160,** 219–263.

Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A. (1999). Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. *Biochemistry* **38,** 15689–15696.

Huse, M., Eck, M. J., and Harrison, S. C. (1998). A Zn^{2+} ion links the cytoplasmic tail of DC4 and the N-terminal region of Lck. *J. Biol. Chem.* **273,** 18729–18733.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Klatt, P., Schmidt, K., and Mayer, B. (1992). Brain nitric oxide synthase is a haemoprotein. *Biochem. J.* **288,** 15–17.

Klatt, P., Schmidt, K., Uray, G., and Mayer, B. (1993). Multiple catalytic functions of brain nitric oxide synthase. Biochemical characterization, cofactor-requirement, and the role of N^{ω}-hydroxy-L-arginine as an intermediate. *J. Biol. Chem.* **268,** 14781–14787.

Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, E. R., and Mayer, B. (1994). The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. *J. Biol. Chem.* **269,** 13861–13866.

Korth, H. G., Sustmann, R., Thater, C., Butler, A. R., and Ingold, K. U. (1994). On the mechanism of the nitric oxide synthase-catalyzed conversion of *N*-omega-hydroxyl-L-arginine to citrulline and nitric oxide. *J. Biol. Chem.* **269,** 17776–17779.

Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* **264,** 20496–20501.

Leaf, C. D., Wishnok, J. S., Hurley, J. P., Rosenblad, W. D., Fox, J. G., and Tannenbaum, S. R. (1990). Nitrate biosynthesis in rats, ferrets, and humans. Precursor studies with L-arginine. *Carcinogenesis* **11,** 855–858.

Leber, A., Hemmens, B., Klösch, B., Goessler, W., Raber, G., Mayer, B., and Schmidt, K. (1999). Characterization of recombinant human endothelial nitric-oxide synthase purified from the yeast *Pichia pestoris*. *J. Biol. Chem.* **274,** 37658–37664.

Lee, S. J., and Stull, J. T. (1998). Calmodulin-dependent regulation of inducible and neuronal nitric-oxide synthase. *J. Biol. Chem.* **273,** 27430–27437.

Lewis, D. F. V. (1996). *In* ''Cytochromes P450: Structure, Function and Mechanism,'' pp. 14–19. Taylor & Francis, Bristol, Pennsylvania.

Li, H., Raman, C. S., Glaser, C. B., Blasko, E., Young, T. A., Parkinson, J. F., Whitlow, M., and Poulos, T. L. (1999). Crystal structures of zinc-free and −bound heme domain of human inducible nitric-oxide synthase. Implications for dimer stability and comparison with endothelial nitric-oxide synthase. *J. Biol. Chem.* **274,** 21276–21284.

Lin, R. S., Rodriguez, C., Veillette, A., and Lodish, H. F. (1998). Zinc is essential for binding of $p56^{lck}$ to CD4 and CD8α. *J. Biol. Chem.* **273,** 32878–32882.

Lipinski, P., and Drapier, J. (1997). Interplay between ferritin metabolism, reactive oxygen species and nitric oxide. *J. Biol. Inorg. Chem.* **2,** 559–566.

McMillan, K., and Masters, B. S. S. (1993). Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme–substrate interaction. *Biochemistry* **32,** 9875–9880.

McMillan, K., and Masters, B. S. S. (1995). Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: Identification of the heme-binding proximal thiolate ligand as cysteine-415. *Biochemistry* **34,** 3686–3693.

McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. S. (1992). Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11141–11145.

Marletta, M. A. (1993). Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268,** 12231–12234.

Martásek, P., Liu, J., Roman, L. J., Gross, S. S., Sessa, W. C., and Masters, B. S. S. (1996). Characterization of bovine endothelial nitric oxide synthase expressed in *E. coli*. *Biochem. Biophys. Res. Commun.* **219,** 359–365.

Martásek, P., Miller, R. T., Lieu, Q., Roman, L. J., Salerno, J. C., Migita, C. T., Raman, C. S., Gross, S. S., Ikeda-Saito, M., and Masters, B. S. (1998). The C331A mutant of neuronal nitric-oxide synthase is defective in arginine binding. *J. Biol. Chem.* **273,** 34799–34805.

Martásek, P., Miller, R. T., Roman, L. J., Shea, T., and Masters, B. S. S. (1999). Assay of isoforms of *Escherichi coli*-expressed nitric oxide synthase. *Methods Enzymol.* **301,** 70–78.

Masters, B. S. S. (1994). Nitric oxide synthases: Why so complex? *Annu. Rev. Nutr.* **14,** 131–145.

Masters, B. S. S., Kamin, H., Gibson, Q. H., and Williams, C. H. (1965a). Studies on the mechanism of microsomal triophosphopyridine neucleotide-cytochrome *c* reductase. *J. Biol. Chem.* **240,** 921–931.

Masters, B. S. S., Bilimoria, M. H., Kamin, H., and Gibson, Q. H. (1965b). The mechanism of 1- and 2-electron transfers catalyzed by reduced triphosphopyridine nucleotide-cytochrome *c* reductase. *J. Biol. Chem.* **240,** 4081–4088.

Mayer, B., John, M., and Böhme, E. (1990). Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum: Cofactor role of tetrahydrobioprotein. *FEBS Lett.* **277,** 215–219.

Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G., and Böhme, E. (1991). Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett.* **288,** 187–191.

Miller, R. T., Martásek, P., Raman, C. S., and Masters, B. S. S. (1999). Zinc content of *Escherichia coli*-expressed constitutive isoforms of nitric-oxide synthase. *J. Biol. Chem.* **274,** 14537–14540.

Narayanasami, R., Nishimura, J. S., McMillan, K., Roman, L. J., Shea, T. M., Robida, A. M., Horowitz, P. M., and Masters, B. S. S. (1997). The influence of chaotropic reagents on neuronal nitric oxide synthase and its flavoprotein module. Urea and guanidine hydrochloride stimulate NADPH-cytochrome *c* reductase activity of both proteins. *Nitric Oxide: Biol. Chem.* **1,** 39–49.

Narhi, L. O., and Fulco, A. J. (1987). Identification and characterization of two functional domains in cytochrome $P\text{-}450_{BM\text{-}3}$, a catalytically self-sufficient monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **262,** 6683–6690.

Nishida, C. R., and Ortiz de Montellano, P. R. (1998). Electron transfer and catalytic activity of nitric oxide synthases. Chimeric constructs of the neuronal, inducible, and endothelial isoforms. *J. Biol. Chem.* **273,** 5566–5571.

Ostermeier, C., and Brunger, A. T. (1999). Structural basis of Rab effector specificity: Crystal structure of the small G protein Rab3A complexed with the effector domain of rabphilin-3A. *Cell* **96,** 363–374.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Perry, J., and Marletta, M. (1998). Effects of transition metals on nitric oxide synthase catalysis. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 11101–11106.

Piedrafita, D., and Liew, F. (1998). Nitric oxide: A protective or pathogenic molecule? *Rev. Med. Microbiol.* **9,** 179–189.

Pirskanen, A., Kaimio, A.-M., Myllylä, R., and Kivirikko, K. I. (1996). Site-directed mutagenesis of human lysyl hydroxylase expressed in insect cells. Identification of histidine residues and an aspartic acid residue critical for catalytic activity. *J. Biol. Chem.* **271,** 9398–9402.

Porter, T. D., and Kasper, C. B. (1986). Coding necleotide sequence of rat NADPH-cytochrome P450 oxidoreductase cDNA and identification of flavin-binding domains. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 973–977.

Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., and Kraut, J. (1985). The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem.* **260,** 16122–16130.

Pufahl, R. A., and Marletta, M. A. (1993). Oxidation of N^{G}-hydroxy-L-arginine by nitric oxide synthase: Evidence for the involvement of the heme in catalysis. *Biochem. Biophys. Res. Commun.* **193,** 963–970.

Raman, C. S., Li, H., Martásek, P., Král, V., Masters, B. S. S., and Poulos, T. L. (1998). Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell* **95,** 939–950.

Raman, C. S., Martásek, P., and Masters, B. S. S. (2000). Structural themes determining function in nitric oxide synthase. *In* ''The Porphyrin Handbook'' (K. M. Kadish, K. M. Smith, and R. Guilard, eds.), Vol. 4, pp. 293–339. Academic Press, San Diego.

Reithmuller, C., Gorren, A. C., Pitters, E., Hemmens, B., Habisch, H. J., Heales, S. J., Schmidt, K., Werner, E. R., and Mayer, B. (1999). Activation of neuronal nitric-oxide synthase by the 5-methyl analog of tetrahydrobiopterin. Functional evidence against reductive oxygen activation by the pterin cofactor. *J. Biol. Chem.* **274,** 16047–16051.

Richards, M. K., and Marletta, M. A. (1994). Characterization of neuronal nitric oxide synthase and a C415H mutant, purified from a baculovirus overexpression system. *Biochemistry* **33,** 14723–14732.

Rodríguez-Crespo, I., Nishida, C. R., Knudsen, G. M., and Ortiz de Montellano, P. R. (1999). Mutation of the five conserved histidines in the endothelial nitric-oxide synthase hemoprotein domain. No evidence for a non-heme metal requirement for catalysis. *J. Biol. Chem.* **274,** 21617–21624.

Roman, L. J., Sheta, E. A., Martásek, P., Gross, S. S., Liu, Q., and Masters, B. S. S. (1995). High level expression of functional rat neuronal nitric oxide synthase in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **92,** 8428–8432.

Ruan, J., Xie, Q. W., Hutchinson, N., Cho, H., Wolfe, G. C., and Nathan, C. (1996). Inducible nitric oxide synthase requires both the canonical calmodulin-binding domain and additional sequences in order to bind calmodulin and produce nitric oxide in the absence of free Ca^{2+}. *J. Biol. Chem.* **271,** 22679–22686.

Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M. E., Martásek, P., Roman, L. J., Masters, B. S. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997). An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J. Biol. Chem.* **272,** 29769–29777.

Schenkman, J. B., Sligar, S. G., and Ciniti, D. L. (1981). Substrate interactions with cytochrome P450. *Pharmacol. Ther.* **12,** 43–71.

Schmidt, H. H. H. W., Smith, R. M., Nakane, M., and Murad, F. (1992). Ca^{2+}/calmodulin-dependent NO synthase type I: A biopteroflavoprotein with Ca^{2+}/calmodulin-independent diaphorase and reductase activities. *Biochemistry* **31,** 3243–3249.

Schultz, G. E., Schirmer, R. H., Sachsenheimer, W., and Pai, E. F. (1978). The structure of the flavoenzyme glutathione reductase. *Nature* **273,** 120–124.

Sheta, E. A., McMillan, K., and Masters, B. S. S. (1994). Evidence for a bidomain structure of constitutive cerebellar nitric oxide. *J. Biol. Chem.* **269,** 15147–15153.

Siegel, L. M., and Kamin, H. (1968). *E. coli* TPNH-sulfite reductase. *In* ''Flavins and Flavoproteins, Second International Conference'' (K. Yagi, ed.), pp. 15–40. Univ. Park Press, Tokyo.

Stuehr, D. J., and Ikeda-Saito, M. (1992). Spectral characterization of brain macrophage nitric oxide synthases. Cytochrome P-450-like hemeproteins that contain a flavin semiquinone radical. *J. Biol. Chem.* **267,** 20547–20550.

Stuehr, D. J., Cho, J. J., Kwon, N. S., Weise, M. F., and Nathan, C. F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: An FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. USA* **88,** 7773–7777.

Tayeh, M. A., and Marletta, M. A. (1989). Macrophage oxidataion of L-arginine to nitric oxide, nitrate, and nitrite. Tetrahydrobiopterin is required as a cofactor. *J. Biol. Chem.* **264,** 19654–19658.

Tierney, D. L., Martásek, P., Doan, P. E., Masters, B. S. S., and Hoffman, B. M. (1998). Location of guanidine nitrogen of L-arginine substrate bound to neuronal nitric oxide synthase (nNOS): Determination by Q-band pulsed ENDOR spectroscopy. *J. Am. Chem. Soc.* **120,** 2983–2984.

Tierney, D. L, Huang, H., Martásek, P., Masters, B. S. S., Silverman, R. B., and Hoffman, B. M. (1999). ENDOR spectroscopic evidence for the position and structure of N^G-hydroxy-*L*-arginine bound to holo-neuronal nitric oxide synthase. *Biochemistry* **38,** 3704–3710.

Vásquez-Vivar, J., Kalyanaraman, B., Martásek, P., Hogg, N., Masters, B. S. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998). Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9220–9225.

Vásquez-Vivar, J., Hogg, N., Martásek, P., Karoui, H., Pritchard, K. A., Jr., and Kalyanaraman, B. (1999). Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. *J. Biol. Chem.* **274,** 26736–26742.

Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J.-J. P. (1997). Three-dimensional structure of NADPH-cytochrome P450 reductase: Prototype for FMN- and FAD-containing enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 8411–8416.

White, K. A., and Marletta, M. A. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* **31,** 6627–6631.

Wink, D., and Mitchell, J. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic and cytoprotective mechanisms of nitric oxide. *Free Radicals Biol. Med.* **25,** 434–456.

Witteveen, C. F. B., Giovanelli, J., and Kaufman, S. (1996). Reduction of quinonoid dihydrobiopterin to tetrahydrobiopterin by nitric oxide synthase. *J. Biol. Chem.* **271,** 4143–4147.

Witteveen, C. F. B., Giovanelli, J., and Kaufman, S. (1999). Reactivity of tetrahydrobiopterin bound to nitric-oxide synthase. *J. Biol. Chem.* **274,** 29755–29762.

Yu, H., and Schreiber, S. L. (1995). Structure of guanine-nucleotide-exchange factor human Mss4 and identification of its Rab-interacting surface. *Nature* **376,** 788–791.

CHAPTER 7

Regulation of the Expression of Nitric Oxide Synthase Isoforms

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THERE IS A LARGE ARRAY OF REGULATORY MECHANISMS FOR THE EXPRESSION OF THE DIFFERENT NOS ISOFORMS. THE HIGH-OUTPUT NOS II IS NOT ONLY TURNED ON TRANSCRIPTIONALLY, BUT THE STABILITY OF THE TRANSCRIPTS AND THEIR TRANSLATION CAN BE REGULATED DYNAMICALLY. IN ADDITION, THE EXPRESSIONAL LEVELS OF THE SERVOREGULATORY, LOW-OUTPUT ENZYMES NOS I AND NOS III CAN ALSO BE ADJUSTED TO MEET LOCAL DEMAND. THE ORIGINAL PARADIGM THAT NO IS SYNTHESIZED EITHER BY CONSTITUTIVE NO SYNTHASES (NOS I AND NOS III) OR BY THE INDUCIBLE NOS II IS NO LONGER VALID. THIS ADDS TO THE DIVERSITY OF MECHANISMS CONTROLLING NO PRODUCTION IN DIFFERENT CELLS AND TISSUES.

Cellular Expression of the Nitric Oxide Synthase (NOS) Isoforms

The prototypical nitric oxide synthase (NOS) enzyme, NOS I [NOS1, neuronal NOS (nNOS)], is found in neurons. It is a low output NOS whose activity is regulated by Ca^{2+} and calmodulin. NOS I was first purified from rat and porcine cerebellum (Bredt and Snyder, 1990; Mayer *et al.,* 1990; Schmidt *et al.,* 1991). NOS I has a widespread distribution in the central and peripheral nervous systems (Vincent and Hope, 1992). In addition, NOS I mRNA transcripts and/or protein have also been detected in nonneuronal cell types, such as rhabdomyocytes, epithelial cells, mast cells, and neutrophils (for review, see Förstermann *et al.,* 1998). NOS I is likely to play an important role in physiological neuronal functions such as neurotransmitter release, neural development, regeneration, synaptic plasticity, and regulation of gene expression but also in a variety of neurological disorders in which excessive production of nitric oxide (NO) leads to neural injury (Yun *et al.,* 1997).

NOS II [NOS2, inducible NOS (iNOS)] is normally absent from untreated cells, but its expression can be induced in almost any cell type by bacterial lipopolysaccharide (LPS), cytokines, and other agents. There are also some reports describing constitutive expression of NOS II in certain cells (Casado *et al.,* 1997; Di Cesare *et al.,* 1998; Gath *et al.,* 1996; Park *et al.,* 1996; Sherman *et al.,* 1999). NOS II is a high-output isoform; after induction the translated enzyme produces large amounts of NO in a Ca^{2+}-independent fashion. These high concentrations of NO can inhibit a number of key enzymes that contain iron in their catalytic centers. These include ribonucleotide reductase (rate-limiting in DNA replication), iron–sulfur cluster-dependent enzymes (complex I and II, involved in mitochondrial electron transport), and *cis*-aconitase (in the citric acid cycle) (Nathan and Hibbs, 1991). In addition, high concentrations of NO can directly interfere with the DNA of target cells and cause strand breaks and fragmentation (Fehsel *et al.,* 1995; Wink *et al.,* 1991). A combination of these effects is likely to form the basis of the cytostatic and cytotoxic effect of NO on parasitic microorganisms and tumor cells. Therefore, NO is an essential component of the nonspecific immune defense system (MacMicking *et al.,* 1997). NO produced by NOS II may also interfere with the immune response by regulating

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the Th1/Th2 balance (Kolb and Kolb-Bachofen, 1998). In addition to its important role in the immune system, NOS II-derived NO seems to be hepatoprotective (Taylor *et al.,* 1998a). Recent evidence suggests that NOS II also plays an important role in wound healing by enhancing the synthesis of vascular endothelial growth factor and collagen (Frank *et al.,* 1998; Thornton *et al.,* 1998). The impaired wound repair seen in NOS II knockout mice could be reversed by adenoviral-mediated transfer of NOS II (Yamasaki *et al.,* 1998).

However, NOS II is also involved in several types of pathophysiology (Kröncke *et al.,* 1998). For example, NOS II plays an important role in pancreatic β-cell destruction in type I diabetes (Kolb-Bachofen, 1996). In rheumatoid arthritis, NO synthesized by NOS II promotes inflammation and tissue damage (Stichtenoth and Frölich, 1998). High NOS II expression has also been described in patients with multiple sclerosis (Bagasra *et al.,* 1995). The marked fall in blood pressure seen in septic shock is mainly caused by NOS II-derived NO (MacMicking *et al.,* 1995). In inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, intestinal epithelial cells show a marked expression of NOS II (Singer *et al.,* 1996). High NOS II levels are also being found in bronchial epithelial cells in asthma patients, and the NOS II expression seems to correlate with the severity of the disease (Barnes, 1996). Finally, NO produced by NOS II may play a role in cancer development. However, it is not clear at this time whether NO represents a defense mechanism against tumor cell growth or a stimulator of tumor vascularization, perfusion, and thus proliferation (Chinje and Stratford, 1997). High NOS II-mediated NO production has been correlated with decreased metastasis of reticulum cell sarcoma (Xie *et al.,* 1995), whereas in human breast cancer, a correlation has been seen between the expression of NOS II and increased axillary lymph node metastasis (Duenas-Gonzalez *et al.,* 1997). Also, human cancer cells with mutated p53 expressing NOS II showed accelerated tumor growth associated with increased vascular endothelial growth factor expression and neovascularization. This suggests that tumor-associated NO production may promote cancer progression by providing a selective growth advantage to tumor cells with mutant p53 (Ambs *et al.,* 1998a). Similarly, Thomsen *et al.* (1997) described an inhibition of EMT6 murine mammary adenocarcinoma cell growth by 1400W, a specific NOS II inhibitor.

The prototypical NOS III [NOS3, endothelial (eNOS)] enzyme is being found in endothelial cells of many tissues (Pollock *et al.,* 1993). Like NOS I, NOS III is a low-output NOS whose activity is regulated by Ca^{2+} and calmodulin. Endothelium-derived NO is a physiologically significant vasodilator and inhibitor of platelet aggregation and adhesion. In addition, vascular NO can prevent leukocyte adhesion to the endothelium by downregulating the leukocyte adhesion glycoprotein complex CD11/CD18. Finally, NO has also been shown to inhibit the proliferation of vascular smooth muscle cells (for review, see Förstermann *et al.,* 1993, 1994). Therefore, endothelial NO is likely to represent a protective antiatherogenic principle. NOS III expression has also been demonstrated in several nonendothelial cell types such as neurons of the rat hippocampus and other rat brain regions, some epithelial cells, cardiomyocytes, megacaryocytes and platelets, T cells, and others (for review, see Förstermann *et al.,* 1998).

Whereas transcriptional regulation of NOS II has been established since about 1990, no expressional regulation was originally known for the other two isoforms. More recent evidence suggests, however, that the expression of NOS I and NOS III can also be regulated under various conditions. Therefore, this chapter describes mechanisms of expressional regulation of all three NOS isoforms.

Expressional Regulation of NOS I

Regulation of NOS I Expression by Neurotransmitters

NOS I expression appears to be regulated by changes in neuronal activity (Tascedda *et al.,* 1996). In cerebellar granule cells, the inhibition of the glutamatergic transmission drastically increased NOS I expression (Baader and Schilling, 1996). In neurons of the central nervous system, NOS I is often colocalized with *N*-methyl-D-aspartate receptors (Aoki *et al.,* 1997; Bhat *et al.,* 1995), which are known to mediate effectively Ca^{2+} influx. In addition, NOS I is upregulated in rat cerebellum following phenobarbital treatment (Thompson *et al.,* 1997).

Regulation of NOS I Expression by Steroid Hormones

NOS I expression can also be triggered by sex hormones. It has been demonstrated that estradiol and pregnancy can induce NOS I expression in several tissues of rat and guinea pig (Ceccatelli *et al.,* 1996; Weiner *et al.,* 1994a; Xu *et al.,* 1996). However, in pregnancy, an increase in NOS I mRNA was not apparent in the lamina terminalis and the hypothalamo–neurohypophysial system of the rat (Luckman *et al.,* 1997). In male rats, testosterone treatment has been described to stimulate the expression of the neuronal isoform in the penis (Reilly *et al.,* 1997).

Corticosterone treatment results in an upregulation of heme oxygenase-2 and a concomitant decrease of NOS I transcription in rat brain (Weber *et al.,* 1994). Lithium and tacrine, a cholinesterase inhibitor currently used in the treatment of the symptoms of Alzheimer's disease, increase the expression of NOS I synergistically in the hippocampus of the rat. This effect could be inhibited by corticosterone (Bagetta *et al.,* 1993). In murine N1E-115 neuroblastoma cells, glucocorticoids inhibit NOS I expression by reducing transcription of the NOS I gene (Schwarz *et al.,* 1998).

Regulation of NOS I Expression by Bacterial LPS and Cytokines

A downregulation of NOS I expression has been documented in guinea pig skeletal muscle and rat brain after *in*

vivo treatment with LPS (Gath *et al.,* 1997; Liu *et al.,* 1996). Treatment of rats with LPS or interferon-γ (IFN-γ) also decreased the expression of NOS I in brain, stomach, rectum, and spleen (Bandyopadhyay *et al.,* 1997). In the human T67 astrocytoma cell line, the combination of LPS and IFN-γ did not affect expression of NOS I mRNA (Colasanti *et al.,* 1999). However, an increase in tyrosine phosphorylation of the protein was observed, concomitant with inhibition of NOS I enzyme activity (Colasanti *et al.,* 1999). A transient increase in NOS I mRNA has been reported in the paraventricular nucleus of LPS-treated rats (Lee *et al.,* 1995). NOS I protein expression increased in olfactory bulb neurons during infections with vesicular stomatitis virus (Komatsu and Reiss, 1997). A similar increase was seen when NB41A3 neuroblastoma cells were treated with IFN-γ or interleukin-12 (IL-12) (Komatsu *et al.,* 1996; Komatsu and Reiss, 1997).

Physical Stimuli Regulating NOS I Expression

Several *in vivo* studies in rat suggested a time-dependent increase in NOS I mRNA following hypoxia (Guo *et al.,* 1997; Prabhakar *et al.,* 1996; Shaul *et al.,* 1995). This upregulation may be due to two distinct mechanisms: a general cellular stress response (see below) or a direct activation of the NOS I gene transcription through binding of hypoxia-induced factors to specific *cis*-acting elements, as seen in erythropoietin and several other hypoxia-induced genes (Kvietikova *et al.,* 1995). Putative ''BACGTSSK'' binding sites for the hypoxia-inducible factor-1 (HIF-1) can be detected along the NOS I genomic sequence, but the functionality of these motifs has not yet been determined. In neurons that constitute the osmoresponsive circuit of the rat hypothalamo–hypophysal system, expression of the NOS I gene was upregulated by changes in plasma osmolarity occurring, for instance, during chronic salt loading (Kadowaki *et al.,* 1994) or water deprivation (O'Shea and Gundlach, 1996). In contrast, in rat kidney, a low-salt diet resulted in an increase in NOS I mRNA with a coordinate upregulation of renal expression of renin and angiotensinogen (Singh *et al.,* 1996a; Tojo *et al.,* 1995). In addition, angiotensinogen gene-knockout mice displayed high level of NOS I in the macula densa of the kidney (Kihara *et al.,* 1997). Also, environmental lighting conditions have been shown to upregulate NOS I in the photoneuronally regulated pineal gland (Spessert *et al.,* 1995).

Regulation of NOS I Expression by Stress in Response to Various Stimuli

NOS I mRNA upregulation has also been observed in neuronal cells in response to several nonspecific stimuli such as heat (Sharma *et al.,* 1997), electrical stimulation (Reiser *et al.,* 1997), light exposure (Goldstein *et al.,* 1997; Spessert *et al.,* 1995), colchicine (Lumme *et al.,* 1997), formalin (Lam *et al.,* 1996), phenobarbital (Thompson *et al.,* 1997), and allergic substances (Calza *et al.,* 1997). A similar response is observed in the rat paraventricular nucleus and adrenal cortex during immobilization stress (Calza *et al.,* 1993; Tsuchiya *et al.,* 1996) and after mechanical or pathological lesions including spinal cord, axonal, or nerve injuries (Herdegen *et al.,* 1993; Lin *et al.,* 1997; Vizzard 1997), hypophysectomie (Villar *et al.,* 1994), or arterial occlusion leading to local ischemia (Samdani *et al.,* 1997; Zhang *et al.,* 1994). Cellular stress is known to trigger the expression of a number of genes resulting in cellular damage and apoptosis. The observed NOS I upregulation may, in most cases, represent only one component of the normal cellular stress response. Enhanced NOS I expression is often associated with co-induction of transcription factors such as c-jun (Herdegen *et al.,* 1993; Wu *et al.,* 1994) and c-fos (Hatakeyama *et al.,* 1996; Torres and Rivier, 1994).

Developmental Regulation of NOS I Expression

Spatial and temporal NOS I expression patterns occur during the development of the nervous system (Bredt and Snyder, 1994; Keilhoff *et al.,* 1996; Northington *et al.,* 1997) and the lung (North *et al.,* 1994; Xue *et al.,* 1996). These changes seem to correlate with differential susceptibility of the cells at particular developmental stages toward specific inductors, such as nerve growth factor (Holtzman *et al.,* 1996), estrogens (Lizasoain *et al.,* 1996), neurotransmitters, and neurotrophins (Baader *et al.,* 1997). Ogura *et al.* (1996) showed that the NOS I mRNA level was increased in a human neuroblastoma cell line following *trans*-retinoic acid-induced neuronal differentiation. In skeletal muscle, a developmental switch from NOS I to the alternative transcript μNOS I has been reported during myotube fusion (Silvagno *et al.,* 1996). Moreover, an enhanced expression of NOS I is generally observed with postnatal development and aging (Kanda, 1996; Ma *et al.,* 1997).

Characterization of the 5'-Flanking Sequences of NOS I

The usage of various alternative promoters seems to be the main regulatory mechanism involved in NOS I gene expression (for review, see Boissel *et al.,* 1998). Two different transcriptional clusters have been characterized. A genomic DNA fragment (2370 bp) of the human neuronal NOS I transcriptional cluster has been isolated (Hall *et al.,* 1994) (GenBank U 15666). In its 3' part, this DNA fragment contains potential first exons. This region also contains a TATA box and two inverted CAAT boxes. Potential binding sites for transcription factors [such as activator protein-2 (AP-2), transcriptional enhancer factor-1 (TEF-1), M-CAT binding factor (MCBF), cAMP-responsive element binding protein (CREB)/activating transcription factor (ATF)/cFOS, human analogs to avian acute leukemia virus E26 oncogene (ETS), nuclear factor 1 (NF-1), and nuclear factor-κB (NF-κB)-like sequences] are found. In the human NOS I testis transcrip-

tional cluster, located between exons 3 and 4, analyses of 1800 bp upstream of the Tex 1 exon (GenBank U 66360) and 1705 bp upstream of the Tex 1b exon (GenBank U 66361) indicate that the two genomic regions lack a typical TATA box (Wang *et al.,* 1997). A CAAT box is located 330 bp upstream from exon Tex 1b. Numerous potential *cis*-acting DNA elements have been detected: Sp-1, ATF/CRE-like sequences, NF-kB, AP-1, and AP-2. In addition, a variety of *cis*-regulatory elements implicated in testis-specific transcription are evident, including GATA and GATA-like sites, p53 half-element, ETS binding site, Pu box, PEA3 sequences, MEF-2 motif, and an insulin response element (IRE) site. However, the NOS I transcriptional regulatory regions have not been characterized functionally.

Expressional Regulation of NOS II

Regulation of NOS II Transcription and mRNA Stability

Control of expression seems to be the main regulatory mechanism of NOS II activity. NOS II protein can synthesize NO continuously until the enzyme becomes degraded (Geller and Billiar, 1998; MacMicking *et al.,* 1997). Regulation of the transcription of the NOS II gene is believed to be the most important control mechanism for NOS II expression. The induction level seen in transient transfection experiments using the murine or rat NOS II promoters equals the induction levels seen for the endogenous NOS II mRNA (Kinugawa *et al.,* 1997; Lowenstein *et al.,* 1993; Xie *et al.,* 1993). However, in the human system, there are marked differences between promoter activity and NOS II mRNA. In noninduced AKN hepatocytes, no NOS II mRNA could be detected. Induction with cytokines then resulted in a marked expression of NOS II mRNA. In contrast, nuclear run-on experiments demonstrated a significant basal activity of the human NOS II promoter in AKN and DLD-1 cells that was only enhanced two- to fivefold by cytokine induction (de Vera *et al.,* 1996a; Linn *et al.,* 1997). This paralleled the moderate inducibility seen in transfection experiments with 11-kb or 16-kb human NOS II promoter fragments (de Vera *et al.,* 1996a; Linn *et al.,* 1997; Kleinert, 1999, unpublished results).

These findings suggest that—especially in human cells—regulation of NOS II mRNA stability plays an important role for NOS II induction. Sequence analysis of the human NOS II mRNA (Geller *et al.,* 1993) reveals four sequence motifs (AUUUA) in the 3'-untranslated region (3'-UTR), which have been shown to confer mRNA destabilization to cytokine- and oncogene mRNAs (Caput *et al.,* 1986). The same sequence motifs are found twice in the 3'-UTR of the murine NOS II mRNA (Lyons *et al.,* 1992), and four times in the rat NOS II mRNA (Galea *et al.,* 1994). In murine RAW264.7 macrophages, a cycloheximide-sensitive degradation pathway has been described for NOS II mRNA; the half-life of the IFN-γ-induced mRNA increased from 1–1.5 hours in the absence of cycloheximide to 4–6 hours in the presence of the inhibitor (Evans *et al.,* 1994). Also the potentiating effect of IFN-γ on LPS-induced NOS II mRNA expression has been attributed to an IFN-γ-mediated stabilization of the NOS II mRNA (Weisz *et al.,* 1994).

In transfection experiments with human A549 or DLD-1 cells, the 3'-UTR of the human NOS II mRNA destabilized the mRNA of a luciferase reporter gene (Rodriguez-Pascual *et al.,* 1999). Also, Nunokawa *et al.* (1997) described an important function of the genomic 3'-flanking sequence of the human NOS II gene for NOS II expression. These authors used luciferase reporter gene constructs containing a human 1.2-kb NOS II promoter in front of luciferase. Behind the luciferase gene they cloned either a 1-kb fragment of human NOS II genomic sequence (composed of the 3'-UTR, the poly-A signal of the human NOS II gene, and 500 bp of the 3'-flanking sequence) or an SV40 poly-A signal. In transient transfection experiments with human A549 cells, the constructs containing the human NOS II 3'-UTR/poly-A/3'-flanking sequence displayed much higher inducibility (11-fold) than those containing only the SV40 poly-A sequence (1.5-fold). These data suggest that the 3'-flanking sequence of the human NOS II gene cooperates with the 5'-flanking promoter sequence in the induction of the human NOS II gene (Nunokawa *et al.,* 1997).

Regulation of NOS II mRNA Translation and Protein Stability

Regulation of the NOS II mRNA translation and protein stability has also been described. Human primary cardiomyocytes express NOS II mRNA, but no NOS II protein in cell culture (Lüss *et al.,* 1997). However, transfection of COS cells with eucaryotic expression vectors containing the human NOS II cDNA (isolated from cardiomyocytes) resulted in NOS II protein expression. Similarly, infection of primary human cardiomyocytes with a retroviral vector containing only the NOS II coding region (no 5'- and 3'-UTR) produced a marked NOS II protein expression. These data suggest that human cardiomyocytes express (protein) factors that inhibit NOS II mRNA translation by interacting with the 5'- and/or 3'-UTR sequences of the NOS II mRNA (Lüss *et al.,* 1997). The inhibition of NOS II expression by different agents, for example, by transforming growth factor-β1 (TGF-β1) in primary murine macrophages (Vodovotz *et al.,* 1993) and dexamethasone in rat mesangial cells (Kunz *et al.,* 1996) had been described to result from NOS II mRNA and protein destabilization.

Chu *et al.* (1995) showed that human macrophages and epithelial cells express small amounts (~6%) of cytokine-induced NOS II mRNAs with truncated 5' ends. These minor transcripts seem to result from transcription reactions initiated at different minor start sites. Alternative splicing of the NOS II mRNA had been described resulting in different NOS II mRNA isoforms (Eissa *et al.,* 1998). One mRNA codes for a NOS II protein lacking exons 8 and 9, which are important for NOS II dimerization (Eissa *et al.,* 1998).

Translation and potential function of these NOS II variants remain to be shown.

NOS II Promoter Sequences

The published sequences of the human, murine, and rat NOS II promoters exhibit homologies to numerous binding sites for transcription factors such as AP-1, CCAAT enhancer-binding protein (C/EBP), c-ETS-1, CREB, GATA, (hypoxia-inducible factor (HIF), heat shock factor (HSF), interferon-γ regulatory factor-1 (IRF-1), NF-1, nuclear factor of activated T cells (NFAT), NF-κB, nuclear factor IL6 (NF-IL6), octamer binding factor-1 (Oct-1), polyomavirus enhancer activator 3 (PEA3), p53, SV40 virus promoter specific transcription protein 1 (Sp1), serum response factor (SRF), and signal transducer and activator of transcription 1α (STAT1α) (Chu *et al.,* 1998; Lowenstein *et al.,* 1993; Xie *et al.,* 1993; Zhang *et al.,* 1998) (GenBank AC005697). A comparison of the first 1000 bp of the three mammalian NOS II promoters shows a much greater homology between the rodent promoters (73% identity) than between rodent and human promoters (mouse/human 55%, rat/human 59%). However, with respect to the positions of the putative transcription factor binding sites, all three mammalian promoter sequences are relatively similar (Fig. 1). All three promoters contain a TATA box roughly 30 bp from the transcription start site. Near the TATA box, all three promoters contain binding sites for the transcription factors NF-κB, NF-IL6, and octamer factors and for transcription factors induced by tumor necrosis factor-α (TNF-α). At position −900 bp, all three promoters display binding sites for transcription factors induced by IFN-γ [γ-interferon-activated site (GAS), γ-interferon-responsive element (γIRE), interferon-stimulated regulatory elements (ISRE)]. The murine and rat promoters also contain an NF-κB site at this position.

In transfection experiments, both rodent 1000-bp NOS II promoter fragments showed full promoter functionality in homologous (Lowenstein *et al.,* 1993; Xie *et al.,* 1993) and heterologous (Kleinert *et al.,* 1996a) cells. In contrast, the human 1000-bp NOS II promoter fragment showed low, but significant basal promoter activity, but no induction with cytokines (Chu *et al.,* 1998; de Vera *et al.,* 1996b; Linn *et al.,* 1997). When the same human promoter fragment was transfected into murine RAW264.7 macrophages, it became markedly inducible with LPS, IFN-γ, and IL-1β (Kolyada *et al.,* 1996). In human AKN, A549, or DLD-1 cells only NOS II promoter fragments larger than 3.8 kb showed significant induction with cytokines (Chu *et al.,* 1998; de Vera *et al.,* 1996b; Linn *et al.,* 1997). Thus, transcription factor binding sites relevant for cytokine induction of the human NOS II promoter seem to be located upstream of −3.8 kb of the 5'-flanking sequence of the human NOS II gene. Data by de Vera *et al.* (1996b) showed maximal induction (10-fold) with a 16-kb human NOS II promoter fragment. A549/8 cells

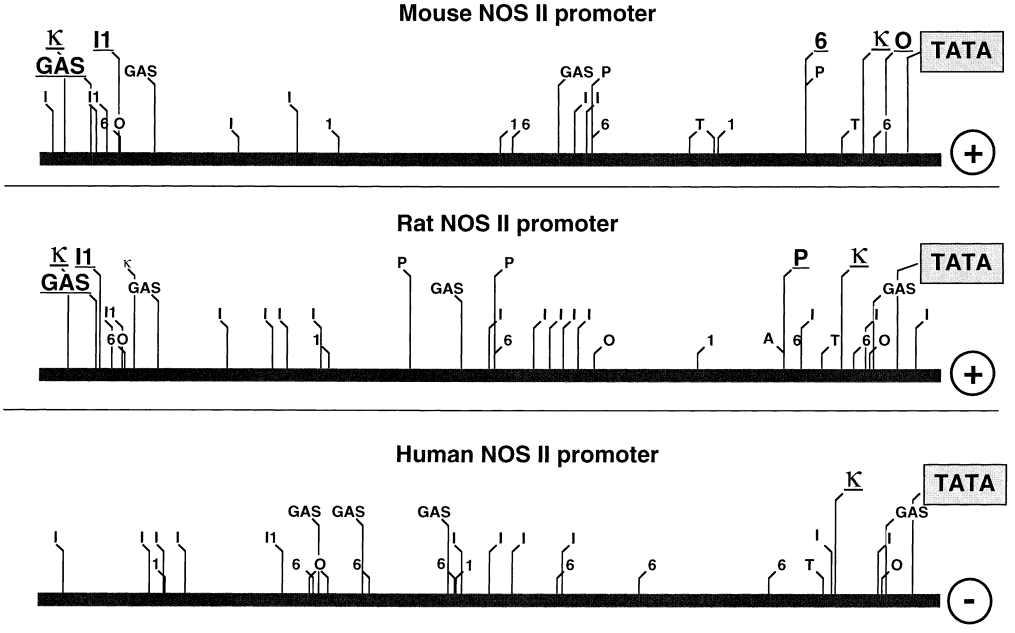


Figure 1 Comparison of the transcription factor binding sites in the first 1000 bp of the murine, rat, and human NOS II promoters (adapted from Kinugawa *et al.,* 1997; Nunokawa *et al.,* 1994; Xie *et al.,* 1993). The figure shows the TATA box (TATA) and putative binding sites for the transcription factors AP-1 (1), C/EBP (P), octamer factors (O), NF-κB (κ), as well as interleukin-6 (6) and tumor necrosis factor-α (T) response elements. Also shown are the binding sites for interferon-γ-induced transcription factors: γIRE (I), I1, for the binding of transcription factor IRF-1, and GAS, for the binding of transcription factor STAT-1α. Large underlined letters indicate an established function of the DNA binding site as demonstrated in transfection or gelshift experiments. In homologous systems, the murine and rat 1000-bp promoter fragments are inducible with cytokines (+), whereas the 1000-bp human promoter fragment is not (−).

stably transfected with this fragment cloned before luciferase showed basal NOS II promoter activity that was enhanced five- to eightfold by cytokines (Kleinert *et al.,* 1999). A construct containing a NOS II promoter fragment ranging from positions −7.2 to −16 kb cloned before a minimal thymidine kinase promoter was still inducible five- to sixfold with cytokines in transiently transfected AKN cells (de Vera *et al.,* 1996b). This suggests that transcription factor binding sites important for the cytokine induction of the human NOS II promoter activity are located upstream of position −7.2 kb. Interestingly, data by another group (Chu *et al.,* 1998; Marks-Konczalik *et al.,* 1998) showed much higher inducibility (up to 50-fold) of a human 8.3-kb NOS II promoter fragment in human A549 cells. The reason of this marked difference in promoter inducibility is not clear at this time.

Signaling Pathways and Transcription Factors Regulating the NOS II Promoter(s)

The regulation of NOS II expression seems to be cell- and species-specific. Analyses of the signal transduction pathways involved in NOS II expression show a marked heterogeneity (Table I). Murine cells generally express NOS II in response to LPS, stimulatory cytokines such as IFN-γ, IL-1β, IL-6, TNF-α, or other compounds. In contrast, most human cells require a cytokine combination including IFN-γ, IL-1β, and TNF-α for NOS II induction (Geller and Billiar, 1998). In addition, many compounds have been described that enhance (Table II) or inhibit (Table III) LPS/cytokine-induced NOS II expression in different cells and tissues.

TRANSCRIPTION FACTOR NF-κB

The transcription factor NF-κB (Ghosh *et al.,* 1998) seems to be a central target for activators or inhibitors of NOS II expression. LPS, IL-1β, TNF-α, and oxidative stress for instance have been shown to induce NOS II expression in different cell types by activating NF-κB. Also, inhibition of NOS II expression by glucocorticoids, TGF-β1, antioxidants like pyrrolidine dithiocarbamate (PDTC), and inhibitors of phosphatidyl choline-specific phospholipase C (PC-PLC) have been shown to result from inhibition of NF-κB activation. This inhibition may result from direct inhibition of NF-κB (by protein–protein interactions) (Kleinert *et al.,* 1996a; Mukaida *et al.,* 1994) or from enhancement of the expression of I-κB, the specific inhibitor of NF-κB (de Vera *et al.,* 1997). Analyses using murine (Goldring *et al.,* 1996; Lowenstein *et al.,* 1993; Xie *et al.,* 1994), rat (Eberhardt *et al.,* 1998; Spink *et al.,* 1995), and human (Marks-Konczalik *et al.,* 1998; Taylor *et al.,* 1998b) cells showed the important role of NF-κB binding sites for the induction of the NOS II promoter activity. In the murine NOS II promoter, the downstream NF-κB binding site (positions −76 to −85 bp) seems to be the most important one (Xie *et al.,* 1994), but the upstream NF-κB site (positions −974 to −960 bp) also seems to have some functionality and cooperativeness with the downstream site (Murphy *et al.,* 1996; Spink *et al.,* 1995). For the NF-κB binding sites in the human NOS II promoter, conflicting results have been published. Taylor *et al.* (1998b) reported interactions of multiple NF-κB binding sites between positions −5.2 and −6.5 kb in the induction of the NOS II promoter. In contrast, these authors attributed little functionality to the downstream NF-κB site of the human NOS II promoter (positions −115 to −106 bp, near the TATA box). Other groups, however, reported on the importance of this downstream NF-κB binding site for human NOS II promoter activity (Marks-Konczalik *et al.,* 1998; Nunokawa *et al.,* 1996; Sakitani *et al.,* 1998).

OCTAMER FACTOR-BINDING SITE

Recent data demonstrated an important function of an octamer factor-binding site (positions −54 to −62 bp) for murine NOS II promoter activity stimulated by LPS (Kim *et al.,* 1999; Pellacani *et al.,* 1999; Perrella *et al.,* 1999; Xie, 1997). Incubation of murine M1-macrophages with IL-6 induced the binding of transcription factors to this octamer site. Mutation of the octamer sequence inhibited IL-6-induced NOS II promoter activity (Sawada *et al.,* 1997). *In vivo* footprinting assays also showed binding of transcription factors to this octamer sequence (Goldring *et al.,* 1996). This octamer-binding site may be the target for the transcription factor family members Oct-1, Oct-2, Brn [brain-specific transcription factors of the POU (transcription factors containing protein sequences homologous to the mammalian *P*it-1, *O*ct-1, *O*ct-2, and *Caenorhabditis elegans U*nc-86 gene products) family]-3a, and Brn-3b (Gay *et al.,* 1998; Kim *et al.,* 1999). However, binding of members of the high mobility group-I(Y) protein family of transcription factors has also been suggested (Pellacani *et al.,* 1999; Perrella *et al.,* 1999).

BINDING SITE FOR TRANSCRIPTION FACTOR IRF-1

In murine RAW264.7 macrophages, the essential role of the IRF-1 binding site (positions −913 to −923 bp) for the induction of the murine NOS II promoter has been shown. Mutations of this binding site blocked the IFN-γ-mediated enhancement of the LPS-induced NOS II promoter activity in macrophages (Martin *et al.,* 1994; Spink and Evans, 1997). Supershift experiments showed the involvement of IRF-1 in the protein complexes bound to this binding site after IFN-γ incubation (Martin *et al.,* 1994). *In vivo* footprint experiments with LPS-incubated RAW264.7 macrophages supported this data (Goldring *et al.,* 1996). Also, nuclear extracts from rat cardiomyocytes incubated with LPS and IFN-γ contained proteins binding to the homologous ISRE on the rat NOS II promoter (Kinugawa *et al.,* 1997). The inhibitory effect of IL-4 on IFN-γ-stimulated NOS II expression in murine macrophages has been attributed to the IL-4-induced potentiation of IFN-γ-induced IRF-2 expression. Because IRF-2 competes with IRF-1 for ISRE binding (Yamamoto *et al.,* 1994), the reduced NOS II expression may result from a reduced IRF-1 binding to the ISRE on the murine NOS II promoter (Paludan *et al.,* 1999). Finally, in macrophages and glial cells from IRF-$1^{-/-}$ mice, LPS/IFN-γ incubation resulted in markedly reduced NOS II ex-

Table I Inducers of NOS II mRNA and/or Protein Expression

Inducers of NOS II	Molecular mechanism (if known)	Transcription factors involved (if known)	References
Bacterial lipopolysaccharide	Transcription ↑, mRNA stability ↑	NF-κB, Oct-2, Brn-3, IRF-1	Gay *et al.* (1998); Goldring *et al.* (1996); Xie (1997); Xie *et al.* (1994)
Cycloheximide			Gilbert and Herschman (1993a); Oguchi *et al.* (1994)
Tumor necrosis factor-α	Transcription ↑	NF-κB	Duval *et al.* (1995); Kleinert *et al.* (1996b)
Interleukin-1β	Transcription ↑, IFN-γ ↑	NF-κB	Geller *et al.* (1995); Suschek *et al.* (1993)
Interleukin-2			Juretic *et al.* (1995)
Interleukin-6	Transcription ↑	Oct-1/2, NF-IL6, IRF-1, STAT1α	Sawada *et al.* (1997)
Interleukin-12			Salvucci *et al.* (1998)
Platelet-derived growth factor	Transcription ↑	NF-κB	Gilbert and Herschman (1993b); Kleinert *et al.* (1996b)
CD23 antibody			Becherel *et al.* (1995)
CD53 antibody	PKC ↑		Bosca and Lazo (1994)
CD69 antibody			De Maria *et al.* (1994)
β-Endorphin	cAMP ↑		Aymerich *et al.* (1998)
Amyloid β-peptide		NF-κB	Rossi and Bianchini (1996)
HLTV-I $p40^{tax}$			Goto *et al.* (1997)
HIV gp 120			Mollace and Nistico (1995)
HBV pX protein	Transcription ↑	NF-κB	Majano *et al.* (1998)
Double-stranded RNA		NF-κB	Heitmeier *et al.* (1998)
cAMP-elevating compounds	Transcription ↑	CREB, C/EBP, NF-κB	Eberhardt *et al.* (1998); Kleinert *et al.* (1996b)
PKC-activating phorbol esters	Transcription ↑	NF-κB	Hortelano *et al.* (1992); Kleinert *et al.* (1996b)
Overexpression of PKC-ε	Transcription ↑	NF-κB	Diaz Guerra *et al.* (1996)
Overexpression of PKC-ζ			Miller *et al.* (1997)
Okadaic acid	Phosphatase ↓		Singh *et al.* (1996b)
Ceramide/SMase	Transcription ↑	NF-κB	Pahan *et al.* (1998a)
Inhibitors of translation			Oguchi *et al.* (1994)
Vitamin E succinate	Transcription ↑	NF-κB	Kim *et al.* (1998)
Hypoxia	Transcription ↑	HIF-1	Melillo *et al.* (1995)
UV-A/B light			Kuhn *et al.* (1998)
Oxidative stress	Transcription ↑	NF-κB	Adcock *et al.* (1994)

pression (Fujimura *et al.,* 1997; Kamijo *et al.,* 1994; Shiraishi *et al.,* 1997).

BINDING SITE FOR TRANSCRIPTION FACTOR STAT1α

In addition to IRF-1 binding sites, all mammalian NOS II promoters contain several homologies to STAT1α binding sites (GAS). Gao *et al.* (1997) reported that binding of STAT1α to the GAS of the murine NOS II promoter (positions −934 to −942 bp) is required for optimal induction of the NOS II gene by IFN-γ and LPS. Also the IFN-γ-mediated enhancement of IL-1β-induced promoter activity in rat RINm5F cells was dependent on the GAS and ISRE sites around position −900 bp of the rat NOS II promoter (Darville and Eizirik, 1998). Furthermore, incubation of rat cardiomyocytes with LPS or cytokines induced proteins with DNA binding activity for oligonucleotides containing the homologous rat GAS sequence (Kinugawa *et al.,* 1997). In contrast to macrophages, chondrocytes and hepatocytes from IRF-$1^{-/-}$ mice showed normal NOS II induction in response to LPS/IFN-γ (Shiraishi *et al.,* 1997). In these cell types, IRF-1 does not seem to be essential for NOS II induction. These data underline the cell specificity of the regulation of NOS II expression. Induction of IRF-1 activity by IFN-γ depends on protein *de novo* synthesis and on STAT1α activation (Boehm *et al.,* 1997). Induction of IRF-1 expression precedes IFN-γ-mediated potentiation of IL-1β-induced NO production in rat RINm5F cells (Flodstrom and Eizirik, 1997). Therefore, STAT1α is likely to be involved in the stimulation of NOS II induction, either directly by binding to the NOS II promoter, or indirectly by inducing IRF-1

Table II NOS II mRNA/Protein Expression-Enhancing Compounds

Stimulators of induction	Molecular mechanism (if known)	Transcription factors involved (if known)	References
Interleukin-8			Bruch-Gerharz *et al.* (1996)
Interleukin-10			Corradin *et al.* (1993)
Ciliary neurotrophic factor			Wadt *et al.* (1998)
Transforming growth factor-β			Gilbert and Herschman (1993b)
Fibroblast growth factor	Transcription ↑		Kunz *et al.* (1997)
Granulocyte–macrophage colony-stimulating factor			Jorens *et al.* (1993)
Arginine vasopressin	PKC ↑		Yamamoto *et al.* (1997)
Muramyl dipeptide			Jorens *et al.* (1993)
Migration inhibitory factor			Bernhagen *et al.* (1994)
Endothelin	PKC ↑, TNF-α ↑		Oda *et al.* (1997)
Prostaglandin E_2	cAMP ↑		Benbernou *et al.* (1997)
Lovastatin, atorvastatin (HMG-CoA reductase inhibitors); geranylgeranyl transferase inhibitors; *Clostridium difficile* toxin B	Farnesylation and geranyl-geranylation ↓, rho protein activity ↓		Finder *et al.* (1997); Hausding *et al.* (1999)
SC68376	p38 MAP-kinase ↓		Guan *et al.* (1997)
Wortmannin, LY294002	PI-3 kinase ↓		Pahan *et al.* (1999)
Superoxide	Transcription ↑		Beck *et al.* (1998)
Glucose	Transcription ↑, PKC ↑		Sharma *et al.* (1995)
Ethanol			Naassila *et al.* (1996)
Taxol	TNF-α ↑, PKC ↑		Jun *et al.* (1995)
Phosphatase inhibitors			Pahan *et al.* (1998b)
Herpes simplex virus type 2 infection	TNF-α ↑	NF-κB	Paludan *et al.* (1998)
Heat shock			Bernard *et al.* (1994)

activity. In addition, positive cooperativeness between STAT1α and IRF-1 is likely to occur for the induction of the NOS II promoter (Darville and Eizirik, 1998). Furthermore, NOS II induction was blocked in macrophages from mice with a disrupted STAT1α gene (Meraz *et al.,* 1996). In human DLD-1 cells, inhibition of the IFN-γ-activated tyrosine kinase JAK2 (janus kinase 2) by tyrphostin B42 (AG 490) reduced STAT1α DNA-binding activity and NOS II expression (Kleinert *et al.,* 1998a).

cAMP-Induced Transcription Factors

In murine 3T3 fibroblasts, the induction of NOS II mRNA by cyclic AMP-elevating agents such as forskolin and 8-bromo-cAMP is mediated by the activation of transcription factor NF-κB (Kleinert *et al.,* 1996b). However, induction of NOS II expression in rat mesangial cells by cAMP-elevating compounds is not inhibited by inhibitors of NF-kB activation (Eberhardt *et al.,* 1994). Therefore, in these cells, the cAMP-mediated induction may depend on cAMP-induced transcription factors (CREB, C/EBP) (Eberhardt *et al.,* 1998; Kinugawa *et al.,* 1997). Eberhardt *et al.* (1998) reported that a CCAAT box (C/EBP binding site, positions −155 to −163 bp) is essential for cAMP-mediated (but not IL-1β-mediated) induction of the rat NOS II promoter. Supershift experiments showed the involvement of C/EBPβ and C/EBPδ in the cAMP regulation of the rat NOS II promoter. In a heterologous transfection system using primary rat hepatocytes and fragments of the human NOS II promoter, Sakitani *et al.* (1998) showed inducibility of a human NOS II promoter fragment corresponding to the sequence from position −365 bp to the start site. When a C/EBP-binding site (or an NF-κB binding site) within this region was mutated, promoter activity was markedly reduced. Gelshift/supershift experiments demonstrated the existence of C/EBPb in the DNA–protein complexes which may stimulate NOS II gene expression synergistically with NF-κB in rat hepatocytes (Sakitani *et al.,* 1998).

Transcription Factor HIF-1

In transient transfection experiments using murine macrophages and bovine pulmonary endothelial cells, the hypoxia-mediated induction of the murine NOS II promoter

Table III Inhibitors of NOS II mRNA/Protein Expression

Inhibitor	Molecular mechanism (if known)	Transcription factors involved (if known)	References
NO	Transcription ↓	NF-κB	Colasanti *et al.* (1995); Lüss *et al.* (1994)
Cycloheximide	Protein synthesis ↓, transcription ↓		Kanno *et al.* (1993); Radomski *et al.* (1990)
Glucocorticoids	Transcription ↓, translation ↓, mRNA stability ↓, protein stability ↓	NF-κB	de Vera *et al.* (1997); Kleinert *et al.* (1996a); Kunz *et al.* (1996); Radomski *et al.* (1990)
Nonsteroidal anti-inflammatory drugs	Transcription ↓, translation ↓, TNF-α ↓		Aeberhard *et al.* (1995); Farivar and Brecher (1996)
Interleukin-4	PKC-ϵ ↓, transcription ↓, mRNA stability ↓, IRF-1 binding ↓, IRF-2 binding ↑	IRF-1/2	Bogdan *et al.* (1994); Paludan *et al.* (1999)
Interleukin-10	TNF-α ↓		Cunha *et al.* (1992)
Interleukin-13	PI-3 kinase ↓, mRNA stability ↓, translation ↓		Bogdan *et al.* (1997); Wright *et al.* (1997)
Interferon-α/β	Transcription ↓	IRF-1	Faure *et al.* (1997)
Transforming growth factor-β1	Transcription ↓, translation ↓, protein stability ↓		Perrella *et al.* (1994); Vodovotz *et al.* (1993)
Platelet derived growth factor	PKC ↑, transcription ↓		Kunz *et al.* (1997)
Fibroblast-derived growth factor	Transcription ↓		Goureau *et al.* (1995)
Insulin			Bedard *et al.* (1998)
Angiotensin II	PKC ↑		Nakayama *et al.* (1994)
Serotonin	PKC ↑		Shimpo *et al.* (1997)
Endothelin	Transcription ↓		Beck *et al.* (1995); Beck and Sterzel (1996)
Tumor suppressor p53	Transcription ↓		Forrester *et al.* (1996)
Melatonin	Transcription ↓	NF-κB	Gilad *et al.* (1998)
Antioxidants, pyrrollidine dithiocarbamate, dichloroisocoumarine, chrysine, *N*-acetylserotonin	Transcription ↓	NF-κB, IRF-1	Eberhardt *et al.* (1994); Hecker *et al.* (1996); Saura *et al.* (1995); Sherman *et al.* (1993)
cAMP-elevating agents (forskolin, 8-Br-cAMP)			Messmer and Brüne (1994); Pang and Hoult (1997)
Prostaglandins E_2 and D_2	cAMP ↑, Transcription ↓	PPAR-γ	Harbrecht *et al.* (1996); Ricote *et al.* (1998)
PKC-stimulating phorbol ester	PKC ↑		Mühl and Pfeilschifter (1994)
PKC inhibitor bisindolylmaleimide			Singh *et al.* (1996b)
Tyrosine kinase inhibitors	JAK2 ↓, Transcription ↓	NF-κB, STAT1α	Dong *et al.* (1993); Kitamura *et al.* (1996); Kleinert *et al.* (1998b); Lee *et al.* (1997)
Phosphatase inhibitors	Transcription ↓	AP-1	Dong *et al.* (1995); Kleinert *et al.* (1998a)
PD98059	Transcription ↓, ERK ↓		Singh *et al.* (1996b)
SB 203580	Transcription ↓, p38 MAP-kinase ↓		Badger *et al.* (1998)
D609, U73122	PC-PLC ↓, Transcription ↓	NF-κB	Spitsin *et al.* (1997)
Farnesyltransferase inhibitors	Farnesyl transferase ↓, ras ↓		Pahan *et al.* (1997)
HMG-CoA reductase inhibitors	Prenylation ↓	NF-κB	Pahan *et al.* (1997)
26S proteasome complex inhibitors, serin protease inhibitors	I-κB ↑	NF-κB	Kwon *et al.* (1998)
Retinoids			Hirokawa *et al.* (1994)
Glucagon	cAMP ↓		Smith *et al.* (1997)
Ethanol		NF-κB, AP-1	Greenberg *et al.* (1995); Spolarics *et al.* (1993)
Nocodazole, colchicine	Depolymerization of microtubules		Marczin *et al.* (1996)
Cyclosporin A, tacrolimus	Transcription ↓	NF-κB	Kunz *et al.* (1995); Schaffer *et al.* (1998)
Glucose	PKC ↑		Muniyappa *et al.* (1998); Nishio and Watanabe (1996)
ADP-ribosylation inhibitors	Transcription ↓	NF-κB	Le Page *et al.* (1998); Szabo *et al.* (1997)
Mechanical strain	PKC ↑		Yamamoto *et al.* (1998)
Heat shock	Transcription ↓	hsp 70, NF-κB	de Vera *et al.* (1996c); Wong *et al.* (1995)

has been shown to be regulated by the transcription factor HIF-1 (Melillo *et al.,* 1995, 1996; Palmer *et al.,* 1998). Site-directed mutation of the HIF-1 binding site (positions −212 to −226 bp) blocked hypoxia-mediated induction of the murine NOS II promoter. Nuclear extracts from hypoxia-treated cells contained high levels of DNA-binding activity specific for the HIF-1 site of the murine NOS II promoter. Supershift experiments showed the presence of HIF-1 in the DNA–protein complexes (Palmer *et al.,* 1998). Also the induction of the NOS II promoter activity by picolinic acid and desferrioxamine seems to depend on the HIF-1 binding site (Melillo *et al.,* 1995, 1997).

TRANSCRIPTION FACTOR AP-1

The role of the transcription factor AP-1 in the regulation of NOS II expression is controversial. In RAW264.7 macrophages, a truncated fragment of the murine NOS II promoter lacking the upstream AP-1 binding site (at position −1125 bp) exhibited slightly higher promoter activity than a longer promoter fragment including this binding site (Lowenstein *et al.,* 1993). In human DLD-1- and A549/8 cells, overexpression of AP-1 by cotransfection of c-jun and c-fos expression vectors inhibited the promoter activity of a 7-kb human NOS II promoter fragment (Kleinert *et al.,* 1998a). Similarly, agents such as calyculin A, ocadaic acid, phenylarsine oxide, and anisomycin, which markedly enhance c-jun and c-fos mRNA expression and AP-1 binding activity, inhibited cytokine-induced NOS II expression in human DLD-1- and A549/8 cells (Kleinert *et al.,* 1998a, 1999). In stably transfected A549/8 cells containing a 16-kb NOS II promoter-luciferase reporter gene construct, all of these compounds also reduced the cytokine-induced NOS II promoter activity (Kleinert *et al.,* 1999). In contrast, Marks-Konczalik et al. (1998) described a marked inhibition (90%) of cytokine-induced activity of an 8.3-kb NOS II promoter fragment transfected into A549 cells after site-directed mutagenesis of an AP-1 binding sequence located 5301 bp upstream of the transcription start site. Site-directed mutagenesis of another AP-1 site located 5115 bp upstream of the transcription start site reduced NOS II promoter activity by 45%. In supershift experiments, these authors detected Jun D and Fra-2 as components of the cytokine-induced AP-1–DNA–protein complexes (Marks-Konczalik *et al.,* 1998). The discrepancies between these findings may result from different AP-1 expression levels. The marked overexpression of AP-1 induced by calyculin A, ocadaic acid, phenylarsine oxide, and anisomycin may result in squelching of transcription cofactors such as CBP/p300 which may be essential for STAT1α- or NF-κB-mediated transcription.

TUMOR SUPPRESSOR P53

Overexpression of the tumor suppressor p53 inhibited the activity of the human NOS II promoter (Forrester *et al.,* 1996), and mice with a disrupted p53 gene showed a higher NOS II expression than wild-type mice (Ambs *et al.,* 1998b).

Expressional Regulation of NOS III

Shear Stress

Shear stress produced in blood vessels by the flowing blood upregulates NOS III expression (Nishida *et al.,* 1992; Sessa *et al.,* 1994; Xiao *et al.,* 1997) (Table IV). The enhancement of NOS III mRNA expression in bovine aortic endothelial cells (BAEC) by shear stress was not inhibited by dexamethasone, inhibitors of tyrosine kinases, or inhibition of G-protein signaling. In contrast, chelation of intracellular calcium in BAEC reduced shear stress induction of NOS III mRNA by almost 70% (Xiao *et al.,* 1997). Pertussis toxin also inhibited the shear-induced increase in NOS III mRNA, implicating a pertussis toxin-sensitive G-protein in this response (Malek *et al.,* 1999). Shear-induced upregulation of NOS III mRNA was potentiated by the PI (phosphatidylinositol) 3-kinase inhibitors wortmannin and LY294002, suggesting that PI 3-kinase inhibits the shear response (Malek *et al.,* 1999). A putative shear stress-responsive element (6-bp core sequence 5'-GAGACC-3') has been described in the promoter sequence of the human and bovine NOS III gene (see below) (Marsden *et al.,* 1993; Venema *et al.,* 1994). However, the functionality of this element has not been proven. In BAEC, shear responsiveness was conferred on a luciferase reporter gene by a portion of the bovine NOS III gene promoter (positions −779 to −1600 bp) (Malek *et al.,* 1999).

Oxygen Tension and Hypoxia

Hypoxia has been shown consistently to downregulate NOS III expression. In human primary pulmonary artery endothelial cells (Ziesche *et al.,* 1996), cultured porcine pulmonary artery endothelial cells (Dai *et al.,* 1995), and bovine pulmonary artery endothelial cells (Liao *et al.,* 1995b), hypoxia reduced NOS III mRNA and/or protein. At least in the bovine species this was attributed to both a decreased rate of transcription and a destabilization of the NOS III mRNA (Liao *et al.,* 1995b). The downregulation of NOS III may be implicated in pulmonary ventilation–perfusion coupling (with poorly ventilated areas of the lung being poorly perfused).

In nonpulmonary endothelial cells, the findings are more controversial. In BAEC, Arnet *et al.* (1996) saw an upregulation of NOS III mRNA and protein expression in cells incubated at low oxygen tension (1%). In this study, hypoxia did not change the stability of NOS III mRNA. On the other hand, the promoter activity of a 1.6-kb DNA fragment of the 5'-flanking sequence of the human NOS III gene was enhanced by hypoxia (Arnet *et al.,* 1996). Therefore, enhanced NOS III expression in response to hypoxia is likely to result from enhanced promoter activity (Arnet *et al.,* 1996). However, the human NOS III promoter contains no homology to the published binding sequence of the hypoxia-induced transcription factor HIF. Zhang *et al.* (1993) reported upregulation of endothelial NOS III immunoreactivity in cerebral

blood vessels during cerebral ischemia. Other reports, however, demonstrated reductions of NOS III expression in human umbilical vein endothelial cells (HUVEC) and BAEC exposed to low oxygen tension (McQuillan *et al.,* 1994; Phelan and Faller 1996) (Table IV). This reduction in NOS III expression was reported to result from decreased stability of NOS III mRNA and reduced NOS III promoter activity (McQuillan *et al.,* 1994).

Proliferation and Growth Status

It has been shown that NOS III mRNA and protein is increased in growing versus resting BAEC (Arnal *et al.,* 1994) (Table IV). This enhanced NOS III expression was found to be the result of a greater stability of the NOS III mRNA in proliferating compared with confluent BAEC (Arnal *et al.,* 1994). Growth-arrested BAEC showed enhanced expression of a protein that interacts with the 3'-UTR of the bovine NOS III mRNA and destabilized the mRNA (Harrison, 1997). In contrast to the above results, Flowers *et al.* (1995) reported a reduction of NOS III mRNA and protein in asynchronously proliferating cultures and wounded endothelial cell monolayers compared with quiescent nonproliferating cells (Table IV). Because run-on experiments showed no change in transcription rates, the reduction of NOS III mRNA in proliferating cells was attributed to NOS III mRNA destabilization (Flowers *et al.,* 1995). The reason for these discrepant findings between the two groups is not clear at this time.

Cytokines and Bacterial LPS

TNF-α downregulates NOS III mRNA, protein, and activity in BAEC and HUVEC (Lamas *et al.,* 1992; Nishida *et al.,* 1992). The downregulation of NOS III mRNA expression by TNF-α in HUVEC has been ascribed to a destabilization of NOS III mRNA with no effect on transcription (Yoshizumi *et al.,* 1993). In bovine endothelial cells, this destabilization seems to result from a specific interaction of a TNF-α-induced protein with the 3'-UTR-sequence of the NOS III mRNA (Alonso *et al.,* 1997). In contrast, in resident astrocytes of the central nervous system, Barna *et al.* (1996) described an enhancement of NOS III immunoreactivity when mice were treated intraperitoneally with IL-12, a potent activator of IFN-γ and TNF-α production. These authors also found a cytokine-mediated activation of NOS III expression following exposure of C6 glioma cells to IL-12, IFN-γ, and TNF-α (Barna *et al.,* 1996). C6 glioma cells express NOS III constitutively. Kaku *et al.* (1997) described an upregulation of NOS III mRNA expression and NOS III promoter activity by treatment of BAEC with IFN-α/β and LPS. Similarly, Bucher *et al.* (1997) found an upregulation of NOS III mRNA expression in the liver of LPS- or lipoteichoic acid-treated rats. They concluded that NOS III might be an even more important source of NO than NOS II in the liver after stimulation with LPS or lipoteichoic acid (Bucher *et al.,* 1997). In contrast, LPS injection into rats has been shown to reduce NOS III mRNA expression (along with NOS I mRNA expression) in aorta, heart, and lung (Liu *et al.,* 1996). Another study demonstrated a downregulation of NOS III in gastrointestinal mucosa following LPS treatment of rats (Chen *et al.,* 1997). Therefore, cytokines and LPS seem to regulate NOS III expression in different ways depending on the cytokine combination, the species, and the cell type analyzed.

Estrogens and Other Sex Steroids

Both pregnancy and estradiol treatment enhanced NOS III mRNA (along with NOS I mRNA) in guinea pig skeletal muscle (Weiner *et al.,* 1994b). An increase in NOS III mRNA has also been seen in the aortae of pregnant or estrogen-treated, but not progesterone- or testosterone-treated, rats (Goetz *et al.,* 1994). Also, the kidneys from female rats contained more NOS III protein than those of male or oophorectomized female rats (Neugarten *et al.,* 1997). Estrogen replacement therapy increased medullary NOS III levels in oophorectomized animals (Neugarten *et al.,* 1997). On the other hand, in *in vitro* studies with ovine fetal pulmonary artery endothelial cells, estrogens enhanced NOS III activity but did not affect NOS III mRNA expression (Lantin-Hermoso *et al.,* 1997). A study on bovine endothelial cells claimed that 17α-ethinyl estradiol did not enhance the expression of NOS III but increased the release of bioactive NO by inhibiting superoxide anion production (Arnal *et al.,* 1996). Studies performed in our own laboratory (Kleinert *et al.,* 1998b) demonstrated that estrogens did enhance NOS III mRNA and protein expression in EA.hy 926 human endothelial cells. The increased NOS III expression resulted from an increased NOS III promoter activity with unchanged mRNA stability. In the absence of a bona fide estrogen responsive element in the human NOS III promoter (see below), the increased NOS III promoter activity may result from an enhanced binding activity of transcription factor Sp1 (which is essential for the human NOS III promoter, see below) (Kleinert *et al.,* 1998b).

Growth Factors

Recent evidence indicates that an incubation of endothelial cells with certain growth factors may upregulate NOS III expression. Incubation of BAEC with TGF-β1 produced a modest upregulation of NOS III mRNA and protein (Inoue *et al.,* 1995). This upregulation of NOS III was reported to result from enhanced promoter activity. Deletion of a putative NF-1 binding site in the bovine NOS III promoter abolished the TGF-β1-enhanced promoter activity (Inoue *et al.,* 1995). Similarly, incubation of BAEC with basic fibroblast growth factor enhanced NOS III mRNA, protein, and activity (Kostyk *et al.,* 1995). In addition, vascular endothelial growth factor had been described to upregulate NOS III

Table IV Conditions or Compounds That Modify NOS III Expression

Condition or compound	Cell, tissue	Species	Regulation	Method of detection	References
Shear stress	Aortic endothelial cells	Bovine, porcine	Upregulation	Northern blot	Nishida *et al.* (1992); Woodman *et al.* (1999)
Exercise training	Aortic endothelial cells, coronary arteries	Canine	Upregulation	Northern blot, nitrite assay	Sessa *et al.* (1994)
Hypoxia[a]	Aortic endothelial cells	Bovine	Upregulation	Northern blot, Western blot, L-arginine to L-citrulline conversion assay, reporter gene assay	Arnet *et al.* (1996)
Proliferation[b]	Aortic endothelial cells	Bovine	Upregulation	Northern blot, Western blot, L-arginine to L-citrulline conversion assay	Arnal *et al.* (1994)
Interferon-α/β and lipopolysaccharide	Aortic endothelial cells	Bovine	Upregulation	RT–PCR, Ca^{2+}-dependent NOS activity	Kaku *et al.* (1997)
Lipopolysaccharide, lipoteichoic acid	Liver	Rat	Upregulation	RNase protection assay	Bucher *et al.* (1997)
Estrogens[c]	Aorta, other tissues, EA.hy 926 endothelial cells	Guinea pig, rat, human	Upregulation	Northern blot, RNase protection assay, Western blot, reporter gene assay	Goetz *et al.* (1994); Kleinert *et al.* (1998b); Weiner *et al.* (1994b)
Transforming growth factor-β1, basic fibroblast growth factor	Aortic endothelial cells	Bovine	Upregulation	Northern blot, NOS III promoter analysis, Western blot, NO bioassay	Inoue *et al.* (1995); Kostyk *et al.* (1995)
Vascular endothelial growth factor	Umbilical vein endothelial cells	Human	Upregulation	Northern blot, Western blot, NO measurement	Hood *et al.* (1998); Kroll and Waltenberger (1998)
Oxidized low-density lipoprotein (low conc.)	Aortic endothelial cells, saphenous vein endothelial cells	Bovine, human	Upregulation	RNase protection assay, Northern blot, nuclear run-on analysis, Western blot, L-arginine to L-citrulline conversion assay	Hirata *et al.* (1995); Liao *et al.* (1995a)
Lysophosphatidylcholine	Aortic endothelial cells, umbilical vein endothelial cells	Bovine, human	Upregulation	RNase protection assay, nuclear run-on analysis, Western blot	Hirata *et al.* (1995); Zembowicz *et al.* (1995)
Phorbol esters[d]	Aortic endothelial cells, EA.hy 926 endothelial cells	Bovine, human	Upregulation	Northern blot, RNase protection assay, Western blot	Li *et al.* (1998); Ohara *et al.* (1995)
Amyotrophic lateral sclerosis	Motor neurons	Human	Upregulation	Immunohistochemistry	Abe *et al.* (1997)
Atherosclerosis	Thoracic aorta	Rabbit	Upregulation	Immunohistochemistry, *in situ* hybridization	Kanazawa *et al.* (1996)
Increase in cGMP	Pulmonary endothelial cells	Bovine	Upregulation	Northern blot, Western blot	Ravichandran and Johns (1995)
HMG-CoA reductase inhibitors	Umbilical vein endothelial cells	Human	Upregulation	Northern blot, Western blot	Laufs *et al.* (1998)
Cyclosporin A, FK 506	Aortic endothelial cells	Bovine	Upregulation	Northern blot	Navarro-Antolin *et al.* (1998)
Glucose	Pancreatic islets, aortic endothelial cells	Rat, human	Upregulation	Western blot, RT–PCR	Cosentino *et al.* (1997); Suschek *et al.* (1994)

Chorion gonadotropin	Ovary	Rat	Upregulation	Immunohistochemistry, Western blot	Jablonka Shariff and Olson (1997)
Angiotensin II	Pulmonary artery endothelial cells	Bovine	Upregulation	Northern blot, Western blot	Olson *et al.* (1997)
Hyperthyroidism (Graves' disease)	Thyroid follicular cells and endothelial cells	Human	Upregulation	Immunohistochemistry, RT–PCR	Colin *et al.* (1997)
Liver cirrhosis	Aorta and mesenteric artery	Rat	Upregulation	Western blot	Martin *et al.* (1996)
Hypoxia	Pulmonary artery endothelial cells	Human, porcine, bovine	Downregulation	Northern blot, DNA–RNA dot blot, Western blot, NADPH-diaphorase cytochemistry, nitrite determination	Dai *et al.* (1995); Liao *et al.* (1995b); Ziesche *et al.* (1996)
Hypoxia[a]	Umbilical vein endothelial cells, aortic endothelial cells	Human, bovine	Downregulation	Northern blot, Western blot, NO bioassay	McQuillan *et al.* (1994); Phelan and Faller (1996)
Proliferation[b]	Aortic endothelial cells	Bovine	Downregulation	Northern blot, nuclear run-on analysis, Western blot	Flowers *et al.* (1995)
Tumor necrosis factor-α	Umbilical vein endothelial cells, aortic endothelial cells	Human, bovine	Downregulation	Northern blot, nuclear run-on analysis, analysis of the 3'-UTR of the bovine NOS III mRNA	Alonso *et al.* (1997); Lamas *et al.* (1992); Nishida *et al.* (1992); Yoshizumi *et al.* (1993)
Oxidized low-density lipoprotein (high conc.)	Aortic endothelial cells, saphenous vein endothelial cells	Bovine, human	Downregulation	RNase protection assay, Northern blot, nuclear run-on analysis, Western blot, L-arginine to L-citrullin conversion assay	Hirata *et al.* (1995); Liao *et al.* (1995a)
Lipopolysaccharide	Aorta, heart, lung, gastrointestinal mucosa	Rat	Downregulation	Northern blot	Chen *et al.* (1997); Liu *et al.* (1996)
Hypothyreosis	Thyroid follicular cells and endothelial cells	Human	Downregulation	Immunohistochemistry, RT–PCR	Colin *et al.* (1997)
Hypertension	Endothelial cells	Rat	Downregulation	Immunohistochemistry	Crabos *et al.* (1997)
Pulmonary hypertension	Lung	Human	Downregulation	Northern blot, *in situ* hybridization, immunohistochemistry	Giaid and Saleh (1995)
Secondary biliary fibrosis	Hepatocytes	Rat	Downregulation	Immunohistochemistry	Zimmermann *et al.* (1996)
Increase in cAMP	Cardiomyocytes	Rat	Downregulation	Western blot, Northern blot	Belhassen *et al.* (1996)

[a] Discrepant findings in nonpulmonary endothelial cells; both upregulation (Arnet *et al.,* 1996; Zhang *et al.,* 1993) and downregulation (McQuillan *et al.,* 1994; Phelan and Faller, 1996) of NOS III have been reported.

[b] Discrepant findings; both upregulation (Arnal *et al.,* 1994) and downregulation (Flowers *et al.,* 1995) of NOS III have been reported.

[c] The NOS III upregulation has not been seen in some other models (Arnal *et al.,* 1996; Lantin-Hermoso *et al.,* 1997).

[d] Causing downregulation (Ohara *et al.,* 1995) or stimulation (Li *et al.,* 1998) of protein kinase C.

expression in HUVEC (Hood *et al.,* 1998; Kroll and Waltenberger, 1998).

Oxidized Low-Density Lipoproteins and Lysophosphatidylcholine

The effects of oxidized low-density lipoprotein (ox-LDL) on NOS III expression are complex. In human saphenous vein endothelial cells, ox-LDL (50 μg/ml) has been reported to reduce NOS III mRNA levels by decreasing the half-life of the mRNA (Liao *et al.,* 1995a). The effect of ox-LDL (50 μg/ml) on NOS III promoter activity was biphasic as measured in nuclear run-on experiments. Treatment with ox-LDL decreased NOS III promoter activity by about 25% in the first 6 hours followed by 1.8- and 2.2-fold increases at 12 and 24 hours, respectively (Liao *et al.,* 1995a) (Table IV). Hirata *et al.* (1995) found an upregulation of NOS III mRNA in BAEC incubated with low concentrations of ox-LDL (10 μg/ml), whereas high concentrations (100 μg/ml) reduced mRNA levels after 24 hours. Similar responses were seen with lysophosphatidylcholine, another component of atherogenic lipoprotein (Table IV). In HUVEC, lysophosphatidylcholine upregulated NOS III expression (Zembowicz *et al.,* 1995) (Table IV). Using nuclear run-on assays and reporter gene analyses, this enhanced NOS III expression was shown to result from lysophosphatidylcholine-stimulated NOS III promoter activity (Wu *et al.,* 1997).

Modulation of Protein Kinase C Activity

Incubation of BAEC (Ohara *et al.,* 1995) or human EA.hy 926 endothelial cells (Li *et al.,* 1998) with phorbol esters enhanced NOS III expression. Ohara *et al.* (1995) concluded from their results that downregulation of protein kinase C (PKC) by long-term incubation with phorbol esters (or PKC inhibition with staurosporine) enhances NOS III expression. In contrast to these results, in our own experiments with human EA.hy 926 endothelial cells, the time course of phorbol ester-induced enhancement of NOS III expression paralleled PKC activation rather than inhibition (Li *et al.,* 1998) (Table IV). Also, specific PKC inhibitors such as bisindolylmaleimide I, Gö 6976, Ro-31-8220, and chelerythrine prevented the phorbol ester-induced enhancement of NOS III expression. Based on transfection experiments with a 3.5-kb human NOS III promoter fragment, the phorbol ester-stimulated enhancement of NOS III expression seems to be a transcriptional event (Li *et al.,* 1998). Additional conditions and compounds that have been described to up- or downregulate NOS III expression are summarized in Table IV.

Characterization of the 5'-Flanking Sequences of the NOS III Gene

Analyses of the 5'-flanking region of human NOS III demonstrated that the promoter is ''TATA-less.'' It exhibits proximal promoter elements consistent with a constitutively expressed gene such as Sp1 and GATA motifs (Karantzoulis-Fegaras *et al.,* 1999; Zhang *et al.,* 1995) (Fig. 2). Furthermore, the human NOS III promoter contains consensus sequences for the binding of the transcription factors AP-1, AP-2, ETS, myc-associated zinc finger protein (MAZ), NF-1, NF-IL6, NF-κB, PEA3, and Ying Yang (YY1), as well as CACCC-, CCAAT-, heavy metal-, acute-phase response-, shear stress-, cAMP-response-, retinoblastoma control-, IFN-γ-response-, and sterol-regulatory *cis* elements (Fig. 2). The promoter sequence also contains several half sites of the estrogen-responsive element (ERE). However, no bona fide EREs are found (Fig. 2). A functional relevance has been demonstrated for only a few of these potential binding sites. Deletion and mutation analyses revealed an essential role of the Sp1 binding site at position −103 bp (Zhang *et al.,* 1995). Also, the stimulation of the human NOS III promoter by estrogens in human EA.hy 926 endothelial cells (Kleinert *et al.,* 1998b) and by lysophosphatidylcholine in HUVEC (Wu *et al.,* 1997) may result from an enhanced binding activity of transcription factor Sp1. Mutation of the consensus GATA site at position −230 bp reduced human NOS III promoter activity by about 30% (Zhang *et al.,* 1995). Mutation of the PEA3 binding site at position −26 bp reduced promoter activity by about 50% (Wu *et al.,* 1997). Linker scanning mutation, competitive gelshift, and cotransfection analyses showed involvement of the transcription factors Elf-1, Ets-1, MAZ, Sp3, and YY1 in the regulation of human NOS III promoter activity (Karantzoulis-Fegaras *et al.,* 1999). These transcription factors bind to two tightly clustered regions (positions −95 to −104 bp and −115 to −144 bp) of the human NOS III promoter. Elf-1, Ets-1, Sp3, and YY1 seem to positively regulate human NOS III promoter activity, whereas MAZ seems to inhibit the NOS III promoter (Karantzoulis-Fegaras *et al.,* 1999). Arnet *et al.* (1996) demonstrated an enhanced activity of the human NOS III promoter in transfected bovine endothelial cells exposed to hypoxia. However, the human NOS III promoter contains no homology to a HIF-1 consensus sequence, and none of the other consensus sequences described above are known to mediate induction of promoter activity by hypoxia. Phorbol ester incubation of human endothelial cells transfected with a human NOS III promoter–luciferase construct produced an enhancement of promoter activity (Li *et al.,* 1998). However, the transcription factors involved are not yet known.

The published 2.9-kb 5'-flanking sequence of the bovine gene (Venema *et al.,* 1994) and the 1.8-kb 5'-flanking sequence of the murine gene (Teichert *et al.,* 1998) lack a typical ''TATA box'' and contain mostly the same transcription factor binding sites as the human NOS III promoter (Fig. 2). In reporter gene assays, a bovine promoter fragment (positions −1548 to +240 bp) showed significant basal activity in transfected BAEC. Interestingly, a deletion fragment (positions −1548 to +192 bp) lacking two putative Sp1 binding sites in the 5'-UTR of the NOS III cDNA lost almost all of its promoter activity, suggesting that transcrip-

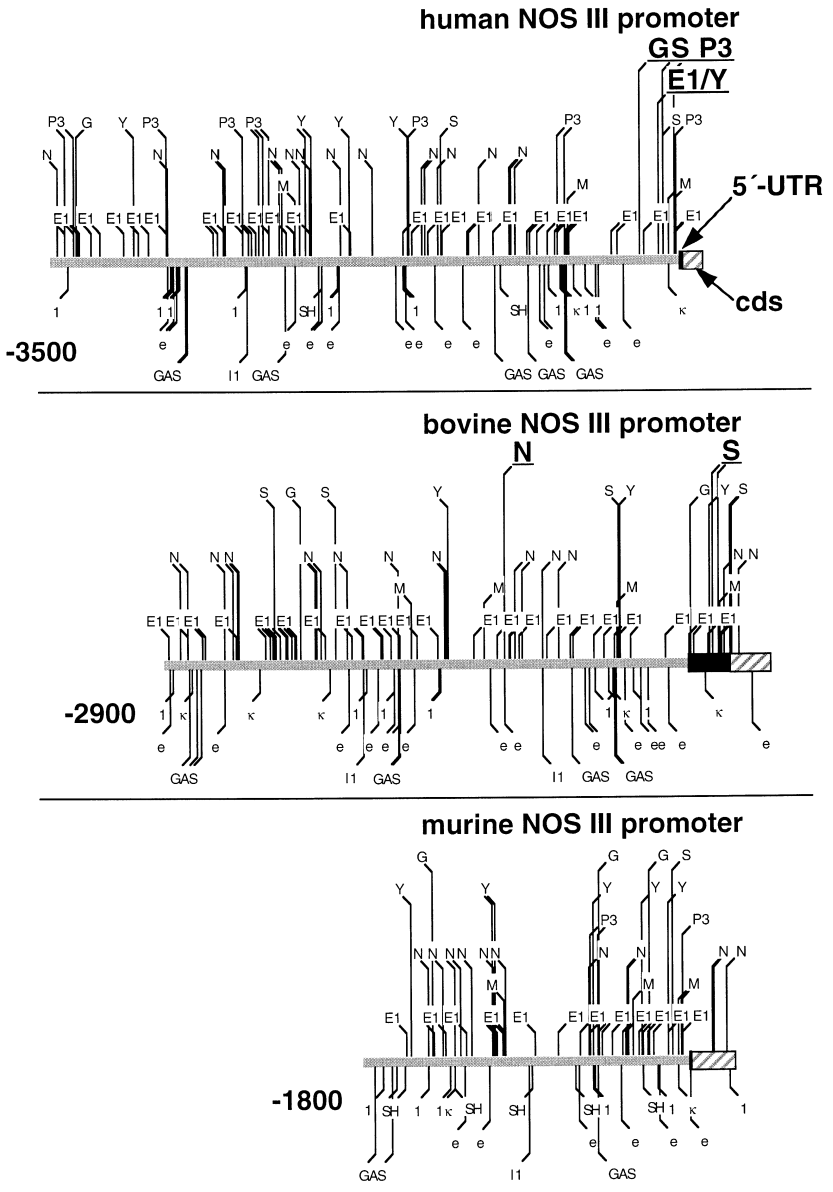


Figure 2 Comparison of the transcription factor binding sites of the human, bovine, and murine NOS III promoters (adapted from Miyahara *et al.,* 1994; Teichert *et al.,* 1998; Zhang *et al.,* 1995). The figure shows putative binding sites for the transcription factors AP-1 (1), Ets-1 (E1), GATA (G), IRF-1 (I1), MAZ (M), NF-1 (N), NF-κB (κ), PEA3 (P3), Sp1 (S), STAT-1α (GAS), YY1 (Y), as well as shear stress responsive elements (SH) and ERE half-sites (e). Large underlined letters indicate an established function of the DNA binding site as demonstrated in transfection or gelshift experiments. 5'-UTR, 5'-untranslated region of the mRNA; cds, coding sequence.

tion factor Sp1 is important for NOS III gene transcription in the bovine species as well. The modest upregulation of NOS III mRNA by TGF-β1 in BAEC is likely to be the result of enhanced activity of the bovine NOS III promoter based on increased binding of transcription factor NF-1 to its response element (Inoue *et al.,* 1995). Transfection of BAEC with a β-galactosidase reporter gene construct containing 5.2 kb of the 5'-flanking sequence of the murine NOS III gene showed significant promoter activity (Teichert *et al.,* 1998).

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References

Abe, K., Pan, L. H., Watanabe, M., Konno, H., Kato, T., and Itoyama, Y. (1997). Upregulation of protein–tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis. *Neurol. Res.* **19,** 124–128.

Adcock, I. M., Brown, C. R., Kwon, O., and Barnes, P. J. (1994). Oxidative stress induces NF-κB DNA binding and inducible NOS messenger RNA in human epithelial cells. *Biochem. Biophys. Res. Commun.* **199,** 1518–1524.

Aeberhard, E. E., Henderson, S. A., Arabolos, N. S., Griscavage, J. M., Castro, F. E., Barrett, C. T., and Ignarro, L. J. (1995). Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.* **208,** 1053–1059.

Alonso, J., Sanchez de Miguel, L., Monton, M., Casado, S., and Lopez-Farre, A. (1997). Endothelial cytosolic proteins bind to the 3' untranslated region of endothelial nitric oxide synthase mRNA: Regulation by tumor necrosis factor alpha. *Mol. Cell. Biol.* **17,** 5719–5726.

Ambs, S., Merriam, W. G., Ogunfusika, M. O., Bennett, W. P., Ishibe, N., Hussain, S. P., Tzeng, E. E., Geller, D. A., Billiar, T. R., and Harris, C. C. (1998a). p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. *Nat. Med.* **4,** 1371–1376.

Ambs, S., Ogunfusika, M. O., Merriam, W. G., Bennett, W. P., Billiar, T. R., and Harris, C. C. (1998b). Upregulation of inducible nitric oxide synthase expression in cancer-prone p53 knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8823–8828.

Aoki, C., Rhee, J., Lubin, M., and Dawson, T. M. (1997). NMDA-R1 subunit of the cerebral cortex colocalizes with neuronal nitric oxide synthase at pre- and postsynaptic sites and in spines. *Brain Res.* **750,** 25–40.

Arnal, J. F., Yamin, J., Dockery, S., and Harrison, D. G. (1994). Regulation of endothelial nitric oxide synthase mRNA, protein, and activity during cell growth. *Am. J. Physiol.* **267,** C1381–C1388.

Arnal, J. F., Clamens, S., Pechet, C., Negre Salvayre, A., Allera, C., Girolami, J. P., Salvayre, R., and Bayard, F. (1996). Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4108–4113.

Arnet, U. A., McMillan, A., Dinerman, J. L., Ballermann, B., and Lowenstein, C. J. (1996). Regulation of endothelial nitric-oxide synthase during hypoxia. *J. Biol. Chem.* **271,** 15069–15073.

Aymerich, M. S., Bengoechea-Alonso, M. T., Lopez-Zabalza, M. J., Santiago, E., and Lopez-Moratalla, N. (1998). Inducible nitric oxide synthase (iNOS) expression in human monocytes triggered by β-endorphin through an increase in cAMP. *Biochem. Biophys. Res. Commun.* **245,** 717–721.

Baader, S. L., and Schilling, K. (1996). Glutamate receptors mediate dynamic regulation of nitric oxide synthase expression in cerebellar granule cells. *J. Neurosci.* **16,** 1440–1449.

Baader, S. L., Bucher, S., and Schilling, K. (1997). The developmental expression of neuronal nitric oxide synthase in cerebellar granule cells is sensitive to GABA and neurotrophins. *Dev. Neurosci.* **19,** 283–290.

Badger, A. M., Cook, M. N., Lark, M. W., NewmanTarr, T. M., Swift, B. A., Nelson, A. H., Barone, F. C., and Kumar, S. (1998). SB 203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes. *J. Immunol.* **161,** 467–473.

Bagasra, O., Michaels, F. H., Zheng, Y. M., Bobroski, L. E., Spitsin, S. V., Fu, Z. F., Tawadros, R., and Koprowski, H. (1995). Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 12041–12045.

Bagetta, G., Corasaniti, M. T., Melino, G., Paoletti, A. M., Finazzi Agro, A., and Nistico, G. (1993). Lithium and tacrine increase the expression of nitric oxide synthase mRNA in the hippocampus of rat. *Biochem. Biophys. Res. Commun.* **197,** 1132–1139.

Bandyopadhyay, A., Chakder, S., and Rattan, S. (1997). Regulation of inducible and neuronal nitric oxide synthase gene expression by interferon-gamma and VIP. *Am. J. Physiol.* **272,** C1790–C1797.

Barna, M., Komatsu, T., and Reiss, C. S. (1996). Activation of type III nitric oxide synthase in astrocytes following a neurotropic viral infection. *Virology* **223,** 331–343.

Barnes, P. J. (1996). NO or no NO in asthma? *Thorax* **51,** 218–220.

Becherel, P. A., Legoff, L., and Arock, M. (1995). CD23 (Fc epsilon RII) activation induces the nitric oxide synthase pathway in human keratinocytes: Possible relevance in cutaneous allergic diseases. *Res. Immunol.* **146,** 703–707.

Beck, K. F., and Sterzel, R. B. (1996). Cloning and sequencing of the proximal promoter of the rat iNOS gene: Activation of NF-κB is not sufficient for transcription of the iNOS gene in rat mesangial cells. *FEBS Lett.* **394,** 263–267.

Beck, K. F., Mohaupt, M. G., Sterzel, R. B., Peters, S., and Fees, H. (1995). Endothelin-1 inhibits cytokine-stimulated transcription of inducible nitric oxide synthase in glomerular mesangial cells. *Kidney Int.* **48,** 1893–1899.

Beck, K. F., Eberhardt, W., Walpen, S., Apel, M., and Pfeilschifter, J. (1998). Potentiation of nitric oxide synthase expression by superoxide in interleukin-1β-stimulated rat mesangial cells. *FEBS Lett.* **435,** 35–38.

Bedard, S., Marcotte, B., and Marette, A. (1998). Insulin inhibits inducible nitric oxide synthase in skeletal muscle cells. *Diabetologia* **41,** 1523–1527.

Belhassen, L., Kelly, R. A., Smith, T. W., and Balligand, J. L. (1996). Nitric oxide synthase (NOS3) and contractile responsiveness to adrenergic and cholinergic agonists in the heart. Regulation of NOS3 transcription in vitro and in vivo by cyclic adenosine monophosphate in rat cardiac myocytes. *J. Clin. Invest.* **97,** 1908–1915.

Benbernou, N., Esnault, S., Shin, H. C., Fekkar, H., and Guenounou, M. (1997). Differential regulation of IFN-γ, IL-10 and inducible nitric oxide synthase in human T cells by cyclic AMP-dependent signal transduction pathway. *Immunology* **91,** 361–368.

Bernard, C., Merval, R., Esposito, B., and Tedgui, A. (1994). Elevated temperature accelerates and amplifies the induction of nitric oxide synthesis in rat macrophages. *Eur. J. Pharmacol. Environ. Toxicol.* **270,** 115–118.

Bernhagen, J., Mitchell, R. A., Calandra, T., Voelter, W., Cerami, A., and Bucala, R. (1994). Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry* **33,** 14144–14155.

Bhat, G. K., Mahesh, V. B., Lamar, C. A., Ping, L., Aguan, K., and Brann, D. W. (1995). Histochemical localization of nitric oxide neurons in the hypothalamus: Association with gonadotropin-releasing hormone neurons and colocalization with *N*-methyl-D-aspartate receptors. *Neuroendocrinology* **62,** 187–197.

Boehm, U., Klamp, T., Groot, M., and Howard, J. C. (1997). Cellular responses to interferon-γ. *Annu. Rev. Immunol.* **15,** 749–795.

Bogdan, C., Thuring, H., Dlaska, M., Rollinghoff, M., and Weiss, G. (1997). Mechanism of suppression of macrophage nitric oxide release by IL-13—Influence of the macrophage population. *J. Immunol.* **159,** 4506–4513.

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q. W., and Nathan, C. (1994). Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukocyte Biol.* **55,** 227–233.

Boissel, J.-P., Schwarz, P. M., and Förstermann, U. (1998). Neuronal-type NO synthase: Transcript diversity and expressional regulation. *Nitric Oxide Biol. Chem.* **2,** 337–349.

Bosca, L., and Lazo, P. A. (1994). Induction of nitric oxide release by MRC OX-44 (Anti-CD53) through a protein kinase C-dependent pathway in rat macrophages. *J. Exp. Med.* **179,** 1119–1126.

Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* **87,** 682–685.

Bredt, D. S., and Snyder, S. H. (1994). Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* **13,** 301–313.

Bruch-Gerharz, D., Fehsel, K., Suschek, C., Michel, G., Ruzicka, T., and

Kolb-Bachofen, V. (1996). A proinflammatory activity of interleukin 8 in human skin: Expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. *J. Exp. Med.* **184,** 2007–2012.

Bucher, M., Ittner, K. P., Zimmermann, M., Wolf, K., Hobbhahn, J., and Kurtz, A. (1997). Nitric oxide synthase isoform III gene expression in rat liver is up-regulated by lipopolysaccharide and lipoteichoic acid. *FEBS Lett.* **412,** 511–514.

Calza, L., Giardino, L., and Ceccatelli, S. (1993). NOS mRNA in the paraventricular nucleus of young and old rats after immobilization stress. *Neuroreport* **4,** 627–630.

Calza, L., Giardino, L., Pozza, M., Micera, A., and Aloe, L. (1997). Time-course changes of nerve growth factor, corticotropin-releasing hormone, and nitric oxide synthase isoforms and their possible role in the development of inflammatory response in experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3368–3373.

Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1986). Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 1670–1674.

Casado, M., Diaz-Guerra, M. J., Rodrigo, J., Fernandez, A. P., Bosca, L., and Martin Sanz, P. (1997). Expression of the calcium-independent cytokine-inducible (iNOS) isoform of nitric oxide synthase in rat placenta. *Biochem. J.* **324,** 201–207.

Ceccatelli, S., Grandison, L., Scott, R. E., Pfaff, D. W., and Kow, L. M. (1996). Estradiol regulation of nitric oxide synthase mRNAs in rat hypothalamus. *Neuroendocrinology* **64,** 357–363.

Chen, K., Inoue, M., Wasa, M., Fukuzawa, M., Kamata, S., and Okada, A. (1997). Expression of endothelial constitutive nitric oxide synthase mRNA in gastrointestinal mucosa and its downregulation by endotoxin. *Life Sci.* **61,** 1323–1329.

Chinje, E. C., and Stratford, I. J. (1997). Role of nitric oxide in growth of solid tumours: A balancing act. *Essays Biochem.* **32,** 61–72.

Chu, S. C., Wu, H. P., Banks, T. C., Eissa, N. T., and Moss, J. (1995). Structural diversity in the 5'-untranslated region of cytokine-stimulated human inducible nitric oxide synthase mRNA. *J. Biol. Chem.* **270,** 10625–10630.

Chu, S. C., Marks Konczalik, J., Wu, H. P., Banks, T. C., and Moss, J. (1998). Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: Characterization of differences between human and mouse iNOS promoters. *Biochem. Biophys. Res. Commun.* **248,** 871–878.

Colasanti, M., Persichini, T., Menegazzi, M., Mariotto, S., Giordano, E., Caldarera, C. M., Sogos, V., Lauro, G. M., and Suzuki, H. (1995). Induction of nitric oxide synthase mRNA expression. Suppression by exogenous nitric oxide. *J. Biol. Chem.* **270,** 26731–26733.

Colasanti, M., Persichini, T., Cavalieri, E., Fabrizi, C., Mariotto, S., Menegazzi, M., Lauro, G. M., and Suzuki, H. (1999). Rapid inactivation of NOS-I by lipopolysaccharide plus interferon-gamma-induced tyrosine phosphorylation. *J. Biol. Chem.* **274,** 9915–9917.

Colin, I. M., Kopp, P., Zbaren, J., Haberli, A., Grizzle, W. E., and Jameson, J. L. (1997). Expression of nitric oxide synthase III in human thyroid follicular cells: Evidence for increased expression in hyperthyroidism. *Eur. J. Endocrinol.* **136,** 649–655.

Corradin, S. B., Fasel, N., Buchmullerrouiller, Y., Ransijn, A., Smith, J., and Mauel, J. (1993). Induction of macrophage nitric oxide production by interferon-γ and tumor necrosis factor-α is enhanced by interleukin-10. *Eur. J. Immunol.* **23,** 2045–2048.

Cosentino, F., Hishikawa, K., Katusic, Z. S., and Lüscher, T. F. (1997). High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* **96,** 25–28.

Crabos, M., Coste, P., Paccalin, M., Tariosse, L., Daret, D., Besse, P., and Bonoron Adele, S. (1997). Reduced basal NO-mediated dilation and decreased endothelial NO-synthase expression in coronary vessels of spontaneously hypertensive rats. *J. Mol. Cell. Cardiol.* **29,** 55–65.

Cunha, F. Q., Moncada, S., and Liew, F. Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. *Biochem. Biophys. Res. Commun.* **182,** 1155–1159.

Dai, A., Zhang, Z., and Niu, R. (1995). The effects of hypoxia on nitric oxide synthase activity and mRNA expression of pulmonary artery endothelial cells in pigs. *Chung Hua Chieh Ho Ho Hu Hsi Tsa Chih* **18,** 164–166.

Darville, M. I., and Eizirik, D. L. (1998). Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* **41,** 1101–1108.

De Maria, R., Cifone, M. G., Trotta, R., Rippo, M. R., Festuccia, C., Santoni, A., and Testi, R. (1994). Triggering of human monocyte activation through CD69, a member of the natural killer cell gene complex family of signal transducing receptors. *J. Exp. Med.* **180,** 1999–2004.

de Vera, M. E., Shapiro, R. A., Nüssler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1996a). Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1054–1059.

de Vera, M. E., Wong, J. M., Zhou, J. Y., Tzeng, E., Wong, H. R., Billiar, T. R., and Geller, D. A. (1996b). Cytokine-induced nitric oxide synthase gene transcription is blocked by the heat shock response in human liver cells. *Surgery* **120,** 144–149.

de Vera, M. E., Kim, Y. M., Wong, H. R., Wang, Q., Billiar, T. R., and Geller, D. A. (1996c). Heat shock response inhibits cytokine-inducible nitric oxide synthase expression in rat hepatocytes. *Hepatology* **24,** 1238–1245.

de Vera, M. E., Taylor, B. S., Wang, Q., Shapiro, R. A., Billiar, T. R., and Geller, D. A. (1997). Dexamethasone suppresses iNOS gene expression by upregulating I-κB-α and inhibiting NF-κB. *Am. J. Physiol.* **273,** G1290–G1296.

Di Cesare, P. E., Carlson, C. S., Attur, M., Kale, A. A., Abramson, S. B., Della Valle, C., Steiner, G., and Amin, A. R. (1998). Upregulation of inducible nitric oxide synthase and production of nitric oxide by the Swarm rat and human chondrosarcoma. *J. Orthop. Res.* **16,** 667–674.

Diaz Guerra, M. J., Bodelon, O. G., Velasco, M., Whelan, R., Parker, P. J., and Bosca, L. (1996). Upregulation of protein kinase C-epsilon promotes the expression of cytokine-inducible nitric oxide synthase in RAW 264.7 cells. *J. Biol. Chem.* **271,** 32028–32033.

Dong, Z. Y., Qi, X. O., Xie, K. P., and Fidler, I. J. (1993). Protein tyrosine kinase inhibitors decrease induction of nitric oxide synthase activity in lipopolysaccharide-responsive and lipopolysaccharide-nonresponsive murine macrophages. *J. Immunol.* **151,** 2717–2724.

Dong, Z. Y., Yang, X. L., Xie, K. P., Juang, S. H., Llansa, N., and Fidler, I. J. (1995). Activation of inducible nitric oxide synthase gene in murine macrophages requires protein phosphatases 1 and 2A activities. *J. Leukocyte Biol.* **58,** 725–732.

Duenas-Gonzalez, A., Isales, C. M., del Mar Abad-Hernandez, M., Gonzalez-Sarmiento, R., Sangueza, O., and Rodriguez-Commes, J. (1997). Expression of inducible nitric oxide synthase in breast cancer correlates with metastatic disease. *Mod. Pathol.* **10,** 645–649.

Duval, D. L., Sieg, D. J., and Billings, R. E. (1995). Regulation of hepatic nitric oxide synthase by reactive oxygen intermediates and glutathione. *Arch. Biochem. Biophys.* **316,** 699–706.

Eberhardt, W., Kunz, D., and Pfeilschifter, J. (1994). Pyrrolidine dithiocarbamate differentially affects interleukin-1β- and cAMP-induced nitric oxide synthase expression in rat renal mesangial cells. *Biochem. Biophys. Res. Commun.* **200,** 163–170.

Eberhardt, W., Pluss, C., Hummel, R., and Pfeilschifter, J. (1998). Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J. Immunol.* **160,** 4961–4969.

Eissa, N. T., Yuan, J. W., Haggerty, C. M., Choo, E. K., Palmer, C. D., and Moss, J. (1998). Cloning and characterization of human inducible nitric oxide synthase splice variants: A domain, encoded by exons 8 and 9, is critical for dimerization. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7625–7630.

Evans, T., Carpenter, A., and Cohen, J. (1994). Inducible nitric-oxide-synthase mRNA is transiently expressed and destroyed by a cycloheximide-sensitive process. *Eur. J. Biochem.* **219,** 563–569.

Farivar, R. S., and Brecher, P. (1996). Salicylate is a transcriptional inhibitor of the inducible nitric oxide synthase in cultured cardiac fibroblasts. *J. Biol. Chem.* **271,** 31585–31592.

Faure, V., Courtois, Y., and Goureau, O. (1997). Inhibition of inducible nitric oxide synthase expression by interferons α and β in bovine retinal pigmented epithelial cells. *J. Biol. Chem.* **272,** 32169–32175.

Fehsel, K., Kröncke, K. D., Meyer, K. L., Huber, H., Wahn, V., and Kolb-Bachofen, V. (1995). Nitric oxide induces apoptosis in mouse thymocytes. *J. Immunol.* **155,** 2858–2865.

Finder, J. D., Litz, J. L., Blaskovich, M. A., McGuire, T. F., Qian, Y. M., Hamilton, A. D., Davies, P., and Sebti, S. M. (1997). Inhibition of protein geranylgeranylation causes a superinduction of nitric-oxide synthase-2 by interleukin-1β in vascular smooth muscle cells. *J. Biol. Chem.* **272,** 13484–13488.

Flodstrom, M., and Eizirik, D. L. (1997). Interferon-gamma-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. *Endocrinology* **138,** 2747–2753.

Flowers, M. A., Wang, Y., Stewart, R. J., Patel, B., and Marsden, P. A. (1995). Reciprocal regulation of endothelin-1 and endothelial constitutive NOS in proliferating endothelial cells. *Am. J. Physiol.* **269,** H1988–H1997.

Forrester, K., Ambs, S., Lupold, S. E., Kapust, R. B., Spillare, E. A., Weinberg, W. C., Felley Bosco, E., Wang, X. W., Geller, D. A., Tzeng, E., Billiar, T. R., and Harris, C. C. (1996). Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2442–2447.

Förstermann, U., Nakane, M., Tracey, W. R., and Pollock, J. S. (1993). Isoforms of nitric oxide synthase: Functions in the cardiovascular system. *Eur. Heart J.* **14** (Suppl 1), 10–15.

Förstermann, U., Closs, E. I., Pollock, J. S., Nakane, M., Schwarz, P., Gath, I., and Kleinert, H. (1994). Nitric oxide synthase isozymes: Characterization, molecular cloning and functions. *Hypertension* **23,** 1121–1131.

Förstermann, U., Boissel, J. P., and Kleinert, H. (1998). Expressional control of the ''constitutive'' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J.* **12,** 773–790.

Frank, S., Kolb, N., Werner, E. R., and Pfeilschifter, J. (1998). Coordinated induction of inducible nitric oxide synthase and GTP-cyclohydrolase I is dependent on inflammatory cytokines and interferon-gamma in HaCaT keratinocytes: Implications for the model of cutaneous wound repair. *J. Invest. Dermatol.* **111,** 1065–1071.

Fujimura, M., Tominaga, T., Kato, I., Takasawa, S., Kawase, M., Taniguchi, T., Okamoto, H., and Yoshimoto, T. (1997). Attenuation of nitric oxide synthase induction in IRF-1-deficient glial cells. *Brain Res.* **759,** 247–250.

Galea, E., Reis, D. J., and Feinstein, D. L. (1994). Cloning and expression of inducible nitric oxide synthase from rat astrocytes. *J. Neurosci. Res.* **37,** 406–414.

Gao, J., Morrison, D. C., Parmely, T. J., Russell, S. W., and Murphy, W. J. (1997). An interferon-γ-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. *J. Biol. Chem.* **272,** 1226–1230.

Gath, I., Closs, E. I., Gödtel-Armbrust, U., Schmitt, S., Nakane, M., Wessler, I., and Förstermann, U. (1996). Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: Implications for contractile function. *FASEB J.* **10,** 1614–1620.

Gath, I., Gödtel-Armbrust, U., and Förstermann, U. (1997). Expressional downregulation of neuronal-type NO synthase I in guinea pig skeletal muscle in response to bacterial lipopolysaccharide. *FEBS Lett.* **410,** 319–323.

Gay, R. D., Dawson, S. J., Murphy, W. J., Russell, S. W., and Latchman, D. S. (1998). Activation of the iNOS gene promoter by Brn-3 POU family transcription factors is dependent upon the octamer motif in the promoter. *Biochim. Biophys. Acta* **1443,** 315–322.

Geller, D. A., and Billiar, T. R. (1998). Molecular biology of nitric oxide synthases. *Cancer Metastasis Rev.* **17,** 7–23.

Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Billiar, T. R. (1993). Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3491–3495.

Geller, D. A., de Vera, M. E., Russell, D. A., Shapiro, R. A., Nussler, A. K., Simmons, R. L., and Billiar, T. R. (1995). A central role for IL-1β in the in vitro and in vivo regulation of hepatic inducible nitric oxide synthase. IL-1β induces hepatic nitric oxide synthesis. *J. Immunol.* **155,** 4890–4898.

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–260.

Giaid, A., and Saleh, D. (1995). Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* **333,** 214–221.

Gilad, E., Wong, H. R., Zingarelli, B., Virag, L., O'Connor, M., Salzman, A. L., and Szabo, C. (1998). Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: Role of inhibition of NF-κB activation. *FASEB J.* **12,** 685–693.

Gilbert, R. S., and Herschman, H. R. (1993a). ''Macrophage'' nitric oxide synthase is a glucocorticoid-inhibitable primary response gene in 3T3 cells. *J. Cell. Physiol.* **157,** 128–132.

Gilbert, R. S., and Herschman, H. R. (1993b). Transforming growth factor-β differentially modulates the inducible nitric oxide synthase gene in distinct cell types. *Biochem. Biophys. Res. Commun.* **195,** 380–384.

Goetz, R. M., Morano, I., Calovini, T., Studer, R., and Holtz, J. (1994). Increased expression of endothelial constitutive nitric oxide synthase in rat aorta during pregnancy. *Biochem. Biophys. Res. Commun.* **205,** 905–910.

Goldring, C., Reveneau, S., Algarte, M., and Jeannin, J. F. (1996). In vivo footprinting of the mouse inducible nitric oxide synthase gene: Inducible protein occupation of numerous sites including oct and NF-IL6. *Nucleic Acids Res.* **24,** 1682–1687.

Goldstein, J., LopezCosta, J. J., and Saavedra, J. P. (1997). Changes in NADPH diaphorase reactivity and neuronal nitric oxide synthase in the rat retina following constant illumination. *Neurosci. Lett.* **231,** 45–48.

Goto, H., Nakamura, T., Shirabe, S., Ueki, Y., Nishiura, Y., Furuya, T., Tsujino, A., Nakane, S., Eguchi, K., and Nagataki, S. (1997). Upregulation of iNOS mRNA expression and increased production of NO in human monoblast cell line, U937 transfected by HTLV-I tax gene. *Immunobiology* **197,** 513–521.

Goureau, O., Faure, V., and Courtois, Y. (1995). Fibroblast growth factors decrease inducible nitric oxide synthase mRNA accumulation in bovine retinal pigmented epithelial cells. *Eur. J. Biochem.* **230,** 1046–1052.

Greenberg, S., Xie, J., Kolls, J., Nelson, S., Didier, P., and Mason, C. (1995). Ethanol suppresses *Mycobacteria tuberculosis*-induced mRNA for nitric oxide synthase in alveolar macrophages, in vivo. *Alcohol Clin. Exp. Res.* **19,** 394–401.

Guan, Z., Baier, L. D., and Morrison, A. R. (1997). p38 mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E2 biosynthesis stimulated by interleukin-1β. *J. Biol. Chem.* **272,** 8083–8089.

Guo, Y., Ward, M. E., Beasjours, S., Mori, M., and Hussain, S. N. A. (1997). Regulation of cerebellar nitric oxide production in response to prolonged in vivo hypoxia. *J. Neurosci. Res.* **49,** 89–97.

Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L., and Marsden, P. A. (1994). Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J. Biol. Chem.* **269,** 33082–33090.

Harbrecht, B. G., Kim, Y. M., Wirant, E. M., Shapiro, R. A., and Billiar, T. R. (1996). PGE2 and LTB4 inhibit cytokine-stimulated nitric oxide synthase type 2 expression in isolated rat hepatocytes. *Prostaglandins* **52,** 103–116.

Harrison, D. G. (1997). Post transcriptional regulation of nitric oxide synthase. *Jpn. J. Pharmacol.* **75,** P-399.

Hatakeyama, S., Kawai, Y., Ueyama, T., and Senba, E. (1996). Nitric oxide synthase-containing magnocellular neurons of the rat hypothalamus synthesize oxytocin and vasopressin and express Fos following stress stimuli. *J. Chem. Neuroanat.* **11,** 243–256.

Hausding, M., Kleinert, H., von Eichel-Streiber, C., and Förstermann, U. (1999). Regulation of the induction of the human NO synthase II gene by small G-proteins. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **359,** R 38.

Hecker, M., Preiss, C., Schini-Kerth, V. B., and Busse, R. (1996). Antioxidants differentially affect nuclear factor-κB-mediated nitric oxide synthase expression in vascular smooth muscle cells. *FEBS Lett.* **380,** 224–228.

Heitmeier, M. R., Scarim, A. L., and Corbett, J. A. (1998). Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF-κB activation. *J. Biol. Chem.* **273,** 15301–15307.

Herdegen, T., Brecht, S., Mayer, B., Leah, J., Kummer, W., Bravo, R., and Zimmermann, M. (1993). Long-lasting expression of JUN and KROX transcription factors and nitric oxide synthase in intrinsic neurons of the rat brain following axotomy. *J. Neurosci.* **13,** 4130–4145.

Hirata, K., Miki, N., Kuroda, Y., Sakoda, T., Kawashima, S., and Yokoyama, M. (1995). Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine upregulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells. *Circ. Res.* **76,** 958–962.

Hirokawa, K., Oshaughnessy, K. M., Ramrakha, P., and Wilkins, M. R. (1994). Inhibition of nitric oxide synthesis in vascular smooth muscle by retinoids. *Br. J. Pharmacol.* **113,** 1448–1454.

Holtzman, D. M., Lee, S., Li, Y., Chua-Couzens, J., Xia, H., Bredt, D. S., and Mobley, W. C. (1996). Expression of neuronal-NOS in developing basal forebrain cholinergic neurons: Regulation by NGF. *Neurochem. Res.* **21,** 861–868.

Hood, J. D., Meininger, C. J., Ziche, M., and Granger, H. J. (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am. J. Physiol.* **274,** H1054–H1058.

Hortelano, S., Genaro, A. M., and Bosca, L. (1992). Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. Antagonism with the induction elicited by lipopolysaccharide. *J. Biol. Chem.* **267,** 24937–24940.

Inoue, N., Venema, R. C., Sayegh, H. S., Ohara, Y., Murphy, T. J., and Harrison, D. G. (1995). Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-β1. *Arterioscler. Thromb. Vasc. Biol.* **15,** 1255–1261.

Jablonka Shariff, A., and Olson, L. M. (1997). Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. *Endocrinology* **138,** 460–468.

Jorens, P. G., van Overveld, F., Bult, H., Vermeire, P. A., and Herman, A. G. (1993). Muramyldipeptide and granulocyte-macrophage colony-stimulating factor enhance interferon-γ-induced nitric oxide production by rat alveolar macrophages. *Agents Actions* **38,** 100–105.

Jun, C. D., Choi, B. M., Kim, H. M., and Chung, H. T. (1995). Involvement of protein kinase c during taxol-induced activation of murine peritoneal macrophages. *J. Immunol.* **154,** 6541–6547.

Juretic, A., Spagnoli, G. C., Horig, H., Shipman, R., Kocher, T., Samija, M., Turic, M., Eljuga, D., Harder, F., and Heberer, M. (1995). Tyrosine kinase-dependent and -independent events induced by interleukin-2 stimulation: Interleukin-2-mediated NO production required for the induction of lymphokine-activated killer cell activity in rat splenocytes is tyrosine kinase independent. *Immunology* **85,** 325–330.

Kadowaki, K., Kishimoto, J., Leng, G., and Emson, P. C. (1994). Upregulation of nitric oxide synthase (NOS) gene expression together with NOS activity in the rat hypothalamo–hypophysial system after chronic salt loading: Evidence of a neuromodulatory role of nitric oxide in arginine vasopressin and oxytocin secretion. *Endocrinology* **134,** 1011–1017.

Kaku, Y., Nanri, H., Sakimura, T., Ejima, K., Kuroiwa, A., and Ikeda, M. (1997). Differential induction of constitutive and inducible nitric oxide synthases by distinct inflammatory stimuli in bovine aortic endothelial cells. *Biochim. Biophys. Acta* **1356,** 43–52.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S. I., Kimura, T., Green, S. J., Mak, T. W., Taniguchi, T., and Vilcek, J. (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* **263,** 1612–1615.

Kanazawa, K., Kawashima, S., Mikami, S., Miwa, Y., Hirata, K., Suematsu, M., Hayashi, Y., Itoh, H., and Yokoyama, M. (1996). Endothelial constitutive nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired endothelium-dependent vascular relaxation. *Am. J. Pathol.* **148,** 1949–1956.

Kanda, K. (1996). Expression of neuronal nitric oxide synthase in spinal motoneurons in aged rats. *Neurosci. Lett.* **219,** 41–44.

Kanno, K., Hirata, Y., Imai, T., and Marumo, F. (1993). Induction of nitric oxide synthase gene by interleukin in vascular smooth muscle cells. *Hypertension* **22,** 34–39.

Karantzoulis-Fegaras, F., Antoniou, H., Lai, S. L. M., Kulkarni, G., Dabreo, C., Wong, G. K. T., Miller, T. L., Chan, Y., Atkins, J., Wang, Y., and Marsden, P. A. (1999). Characterization of the human endothelial nitric-oxide synthase promoter. *J. Biol. Chem.* **274,** 3076–3093.

Keilhoff, G., Seidel, B., Noack, H., Tischmeyer, W., Stanek, D., and Wolf, G. (1996). Patterns of nitric oxide synthase at the messenger RNA and protein levels during early rat brain development. *Neuroscience* **75,** 1193–1201.

Kihara, M., Umemura, S., Kadota, T., Yabana, M., Tamura, K., Nyuui, N., Ogawa, N., Murakami, K., Fukamizu, A., and Ishii, M. (1997). The neuronal isoform of constitutive nitric oxide synthase is upregulated in the macula densa of angiotensinogen gene-knockout mice. *Lab. Invest.* **76,** 285–294.

Kim, S. J., Bang, O. S., Lee, Y. S., and Kang, S. S. (1998). Production of inducible nitric oxide is required for monocytic differentiation of U937 cells induced by vitamin E-succinate. *J. Cell. Sci.* **111,** 435–441.

Kim, Y. M., Ko, C. B., Park, Y. P., Kim, Y. J., and Paik, S. G. (1999). Octamer motif is required for the NF-κB-mediated induction of the inducible nitric oxide synthase gene expression in RAW 264.7 macrophages. *Mol. Cells* **9,** 99–109.

Kinugawa, K., Shimizu, T., Yao, A., Kohmoto, O., Serizawa, T., and Takahashi, T. (1997). Transcriptional regulation of inducible nitric oxide synthase in cultured neonatal rat cardiac myocytes. *Circ. Res.* **81,** 911–921.

Kitamura, Y., Takahashi, H., Nomura, Y., and Taniguchi, T. (1996). Possible involvement of Janus kinase Jak2 in interferon-γ induction of nitric oxide synthase in rat glial cells. *Eur. J. Pharmacol.* **306,** 297–306.

Kleinert, H., Euchenhofer, C., Ihrig Biedert, I., and Förstermann, U. (1996a). Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Mol. Pharmacol.* **49,** 15–21.

Kleinert, H., Euchenhofer, C., Ihrig-Biedert, I., and Förstermann, U. (1996b). In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF-κB. *J. Biol. Chem.* **271,** 6039–6044.

Kleinert, H., Wallerath, T., Fritz, G., Ihrig-Biedert, I., Rodriguez-Pascual, F., Geller, D. A., and Förstermann, U. (1998a). Cytokine induction of NO Synthase II in human DLD-1 cells: Roles of the JAK-STAT, AP-1 and NF-κB-signaling pathways. *Br. J. Pharmacol.* **125,** 193–201.

Kleinert, H., Wallerath, T., Euchenhofer, C. E., Ihrig-Biedert, I., Li, H., and Förstermann, U. (1998b). Estrogens increase transcription of the human endothelial NO synthase gene: Analysis of the transcription factors involved. *Hypertension* **31,** 582–588.

Kleinert, H., Geller, D. A., and Förstermann, U. (1999). Analysis of the molecular mechanism regulating the induction of the human NO synthase II promoter in human A549/8 alveolar carcinoma cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **359,** R 37.

Kolb, H., and Kolb-Bachofen, V. (1998). Nitric oxide in autoimmune disease: Cytotoxic or regulatory mediator. *Immunol. Today* **19,** 556–561.

Kolb-Bachofen, V. (1996). Intraislet expression of inducible nitric oxide synthase and islet cell death. *Biochem. Soc. Trans.* **24,** 233–234.

Kolyada, A. Y., Savikovsky, N., and Madias, N. E. (1996). Transcriptional regulation of the human iNOS gene in vascular-smooth-muscle cells and macrophages: Evidence for tissue specificity. *Biochem. Biophys. Res. Commun.* **220,** 600–605.

Komatsu, T., and Reiss, C. S. (1997). IFN-γ is not required in the IL-12 response to vesicular stomatitis virus infection of the olfactory bulb. *J. Immunol.* **159,** 3444–3452.

Komatsu, T., Bi, Z., and Reiss, C. S. (1996). Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. *J. Neuroimmunol.* **68,** 101–108.

Kostyk, S. K., Kourembanas, S., Wheeler, E. L., Medeiros, D., Mcquillan, L. P., Damore, P. A., and Braunhut, S. J. (1995). Basic fibroblast growth factor increases nitric oxide synthase production in bovine endothelial cells. *Am. J. Physiol.* **269,** H1583–H1589.

Kroll, J., and Waltenberger, J. (1998). VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem. Biophys. Res. Commun.* **252,** 743–746.

Kröncke, K. D., Fehsel, K., and Kolb-Bachofen, V. (1998). Inducible nitric oxide synthase in human diseases. *Clin. Exp. Immunol.* **113,** 147–156.

Kuhn, A., Fehsel, K., Lehmann, P., Krutmann, J., Ruzicka, T., and Kolb-Bachofen, V. (1998). Aberrant timing in epidermal expression of inducible nitric oxide synthase after UV irradiation in cutaneous lupus erythematosus. *J. Invest. Dermatol.* **111,** 149–153.

Kunz, D., Walker, G., Eberhardt, W., Nitsch, D., and Pfeilschifter, J. (1995). Interleukin 1β-induced expression of nitric oxide synthase in rat renal mesangial cells is suppressed by cyclosporin A. *Biochem. Biophys. Res. Commun.* **216,** 438–446.

Kunz, D., Walker, G., Eberhardt, W., and Pfeilschifter, J. (1996). Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin-1β-stimulated mesangial cells: Evidence for the involvement of transcriptional and posttranscriptional regulation. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 255–259.

Kunz, D., Walker, G., Eberhardt, W., Messmer, U. K., Huwiler, A., and Pfeilschifter, J. (1997). Platelet-derived growth factor and fibroblast growth factor differentially regulate interleukin 1β- and cAMP-induced nitric oxide synthase expression in rat renal mesangial cells. *J. Clin. Invest.* **100,** 2800–2809.

Kvietikova, I., Wenger, R. H., Marti, H. H., and Gassmann, M. (1995). The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1). DNA recognition site. *Nucleic Acids Res.* **23,** 4542–4550.

Kwon, G., Corbett, J. A., Hauser, S., Hill, J. R., Turk, J., and McDaniel, M. L. (1998). Evidence for involvement of the proteasome complex (26S) and NF-κB in IL-1β-induced nitric oxide and prostaglandin production by rat islets and RINm5F cells. *Diabetes* **47,** 583–591.

Lam, H. H., Hanley, D. F., Trapp, B. D., Saito, S., Raja, S., Dawson, T. M., and Yamaguchi, H. (1996). Induction of spinal cord neuronal nitric oxide synthase (NOS) after formalin injection in the rat hind paw. *Neurosci. Lett.* **210,** 201–204.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6348–6352.

Lantin-Hermoso, R. L., Rosenfeld, C. R., Yuhanna, I. S., German, Z., Chen, Z., and Shaul, P. W. (1997). Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *Am. J. Physiol.* **273,** L119–L126.

Laufs, U., La Fata, V., Plutzky, J., and Liao, J. K. (1998). Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* **97,** 1129–1135.

Lee, B. S., Kang, H. S., Pyun, K. H., and Choi, I. (1997). Roles of tyrosine kinases in the regulation of nitric oxide synthesis in murine liver cells: Modulation of NF-κB activity by tyrosine kinases. *Hepatology* **25,** 913–919.

Lee, S., Barbanel, G., and Rivier, C. (1995). Systemic endotoxin increases steady-state gene expression of hypothalamic nitric oxide synthase: Comparison with corticotropin-releasing factor and vasopressin gene transcripts. *Brain Res.* **705,** 136–148.

Le Page, C., Sanceau, J., Drapier, J. C., and Wietzerbin, J. (1998). Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NF-κB activation. *Biochem. Biophys. Res. Commun.* **243,** 451–457.

Li, H., Oehrlein, S. A., Wallerath, T., Ihrig-Biedert, I., Wohlfart, P., Ulshofer, T., Jessen, T., Herget, T., Förstermann, U., and Kleinert, H. (1998). Activation of protein kinase C α and/or ϵ enhances transcription of the human endothelial nitric oxide synthase gene. *Mol. Pharmacol.* **53,** 630–637.

Liao, J. K., Shin, W. S., Lee, W. Y., and Clark, S. L. (1995a). Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J. Biol. Chem.* **270,** 319–324.

Liao, J. K., Zulueta, J. J., Yu, F. S., Peng, H. B., Cote, C. G., and Hassoun, P. M. (1995b). Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. *J. Clin. Invest.* **96,** 2661–2666.

Lin, L. H., Sandra, A., Boutelle, S., and Talman, W. T. (1997). Upregulation of nitric oxide synthase and its mRNA in vagal motor nuclei following axotomy in rat. *Neurosci. Lett.* **221,** 97–100.

Linn, S. C., Morelli, P. J., Edry, I., Cottongim, S. E., Szabo, C., and Salzman, A. L. (1997). Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am. J. Physiol.* **272,** G1499–G1508.

Liu, S. F., Adcock, I. M., Old, R. W., Barnes, P. J., and Evans, T. W. (1996). Differential regulation of the constitutive and inducible nitric oxide synthase mRNA by lipopolysaccharide treatment in vivo in the rat. *Crit. Care Med.* **24,** 1219–1225.

Lizasoain, I., Weiner, C. P., Knowles, R. G., and Moncada, S. (1996). The ontogeny of cerebral and cerebellar nitric oxide synthase in the guinea pig and rat. *Pediatr. Res.* **39,** 779–783.

Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., and Murphy, W. J. (1993). Macrophage nitric oxide synthase gene—two upstream regions mediate induction by interferon-γ and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9730–9734.

Luckman, S. M., Huckett, L., Bicknell, R. J., Voisin, D. L., and Herbison, A. E. (1997). Upregulation of nitric oxide synthase messenger RNA in an integrated forebrain circuit involved in oxytocin secretion. *Neuroscience* **77,** 37–48.

Lumme, A., Vanhatalo, S., Sadeniemi, M., and Soinila, S. (1997). Expression of nitric oxide synthase in hypothalamic nuclei following axonal injury or colchicine treatment. *Exp. Neurol.* **144,** 248–257.

Lüss, H., DiSilvio, M., Litton, A. L., Molina y Vedia, L., Nussler, A. K., and Billiar, T. R. (1994). Inhibition of nitric oxide synthesis enhances the expression of inducible nitric oxide synthase mRNA and protein in a model of chronic liver inflammation. *Biochem. Biophys. Res. Commun.* **204,** 635–640.

Lüss, H., Li, R. K., Shapiro, R. A., Tzeng, E., McGowan, F. X., Yoneyama, T., Hatakeyama, K., Geller, D. A., Mickle, D. A., Simmons, R. L., and Billiar, T. R. (1997). Dedifferentiated human ventricular cardiac myocytes express inducible nitric oxide synthase mRNA but not protein in response to IL-1, TNF, IFN-γ, and LPS. *J. Mol. Cell. Cardiol.* **29,** 1153–1165.

Lyons, C. R., Orloff, G. J., and Cunningham, J. M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.* **267,** 6370–6374.

Ma, S. X., Holley, A. T., Sandra, A., Cassell, M. D., and Abboud, F. M. (1997). Increased expression of nitric oxide synthase in the gracile nucleus of aged rats. *Neuroscience* **76,** 659–663.

MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q. W., Sokol, K., Hutchinson, N., Chen, H., and Mudgett, J. S. (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81,** 641–650.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.

McQuillan, L. P., Leung, G. K., Marsden, P. A., Kostyk, S. K., and Kourembanas, S. (1994). Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. *Am. J. Physiol.* **267,** H1921–H1927.

Majano, P. L., Garcia-Monzon, C., Lopez-Cabrera, M., Lara-Pezzi, E., Fernandez-Ruiz, E., Garcia-Iglesias, C., Borque, M. J., and Moreno-Otero, R. (1998). Inducible nitric oxide synthase expression in chronic

viral hepatitis. Evidence for a virus-induced gene upregulation. *J. Clin. Invest.* **101,** 1343–1352.

Malek, A. M., Jiang, L. W., Lee, I., Sessa, W. C., Izumo, S., and Alpert, S. L. (1999). Induction of nitric oxide synthase mRNA by shear stress requires intracellular calcium and G-protein signals and is modulated by PI 3 kinase. *Biochem. Biophys. Res. Commun.* **254,** 231–242.

Marczin, N., Jilling, T., Papapetropoulos, A., Go, C., and Catravas, J. D. (1996). Cytoskeleton-dependent activation of the inducible nitric oxide synthase in cultured aortic smooth muscle cells. *Br. J. Pharmacol.* **118,** 1085–1094.

Marks-Konczalik, J., Chu, S. C., and Moss, J. (1998). Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor-κB-binding sites. *J. Biol. Chem.* **273,** 22201–22208.

Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J. Biol. Chem.* **268,** 17478–17488.

Martin, E., Nathan, C., and Xie, Q. W. (1994). Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med.* **180,** 977–984.

Martin, P. Y., Xu, D. L., Niederberger, M., Weigert, A., Tsai, P., John, J., Gines, P., and Schrier, R. W. (1996). Upregulation of endothelial constitutive NOS: A major role in the increased NO production in cirrhotic rats. *Am. J. Physiol.* **270,** F494–F499.

Mayer, B., John, M., and Böhme, E. (1990). Purification of a calcium/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor role of tetrahydrobiopterin. *FEBS Lett.* **277,** 215–219.

Melillo, G., Musso, T., Sica, A., Taylor, L. S., Cox, G. W., and Varesio, L. (1995). A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J. Exp. Med.* **182,** 1683–1693.

Melillo, G., Taylor, L. S., Brooks, A., Cox, G. W., and Varesio, L. (1996). Regulation of inducible nitric oxide synthase expression in IFN-γ-treated murine macrophages cultured under hypoxic conditions. *J. Immunol.* **157,** 2638–2644.

Melillo, G., Taylor, L. S., Brooks, A., Musso, T., Cox, G. W., and Varesio, L. (1997). Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. *J. Biol. Chem.* **272,** 12236–12243.

Meraz, M. A., White, J. M., Sheehan, K. C. F., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and Schreiber, R. D. (1996). Trageted disruption of the STAT1 gene in mice reveals unexpected physiologic specificity in the JAK-SAT signaling pathway. *Cell* **84,** 431–442.

Messmer, U. K., and Brüne, B. (1994). Modulation of inducible nitric oxide synthase in rinm5F cells. *Cell. Signal.* **6,** 17–24.

Miller, B. W., Baier, L. D., and Morrison, A. R. (1997). Overexpression of protein kinase Cζ isoform increases cyclooxygenase-2 and inducible nitric oxide synthase. *Am. J. Physiol.* **273,** C130–C136.

Miyahara, K., Kawamoto, T., Sase, K., Yui, Y., Toda, K., Yang, L. X., Hattori, R., Aoyama, T., Yamamoto, Y., Doi, Y., Ogoshi, S., Hashimoto, K., Kawai, C., Sasayama, S., and Shizuta, Y. (1994). Cloning and structural characterization of the human endothelial nitric-oxide-synthase gene. *Eur. J. Biochem.* **223,** 719–726.

Mollace, V., and Nistico, G. (1995). Release of nitric oxide from astroglial cells: A key mechanism in neuroimmune disorders. *Adv. Neuroimmunol.* **5,** 421–430.

Mühl, H., and Pfeilschifter, J. (1994). Possible role of protein kinase Cϵ isoenzyme in inhibition of interleukin-1β induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. J.* **303,** 607–612.

Mukaida, N., Morita, M., Ishikawa, Y., Rice, N., Okamoto, S., Kasahara, T., and Matsushima, K. (1994). Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-κB is target for glucocorticoid-mediated interleukin 8 gene repression. *J. Biol. Chem.* **269,** 13289–13295.

Muniyappa, R., Srinivas, P. R., Ram, J. L., Walsh, M. F., and Sowers, J. R. (1998). Calcium and protein kinase C mediate high-glucose-induced inhibition of inducible nitric oxide synthase in vascular smooth muscle cells. *Hypertension* **31,** 289–295.

Murphy, W. J., Muroi, M., Zhang, C. X., Suzuki, T., and Russell, S. W. (1996). Both basal and enhancer κB elements are required for full induction of the mouse inducible nitric oxide synthase gene. *J. Endotoxin Res.* **3,** 381–393.

Naassila, M., Roux, F., Beauge, F., and Daoust, M. (1996). Ethanol potentiates lipopolysaccharide- or interleukin-1β-induced nitric oxide generation in RBE4 cells. *Eur. J. Pharmacol.* **313,** 273–277.

Nakayama, I., Kawahara, Y., Tsuda, T., Okuda, M., and Yokoyama, M. (1994). Angiotensin-II inhibits cytokine-stimulated inducible nitric oxide synthase expression in vascular smooth muscle cells. *J. Biol. Chem.* **269,** 11628–11633.

Nathan, C. F., and Hibbs, J. B. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* **3,** 65–70.

Navarro-Antolin, J., Hernandez-Perera, O., Lopez Ongil, S., Rodriguez-Puyol, M., Rodriguez-Puyol, D., and Lamas, S. (1998). CsA and FK506 up-regulate eNOS expression: Role of reactive oxygen species and AP-1. *Kidney Int. Suppl.* **68,** S20–S24.

Neugarten, J., Ding, Q., Friedman, A., Lei, J., and Silbiger, S. (1997). Sex hormones and renal nitric oxide synthases. *J. Am. Soc. Nephrol.* **8,** 1240–1246.

Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W., and Murphy, T. J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.* **90,** 2092–2096.

Nishio, E., and Watanabe, Y. (1996). Glucose-induced down-regulation of NO production and inducible NOS expression in cultured rat aortic vascular smooth muscle cells: Role of protein kinase C. *Biochem. Biophys. Res. Commun.* **229,** 857–863.

North, A. J., Star, R. A., Brannon, T. S., Ujiie, K., Wells, L. B., Lowenstein, C. J., Snyder, S. H., and Shaul, P. W. (1994). Nitric oxide synthase type I and type III gene expression are developmentally regulated in rat lung. *Am. J. Physiol.* **266,** L635–L641.

Northington, F. J., Tobin, J. R., Harris, A. P., Traystman, R. J., and Koehler, R. C. (1997). Developmental and regional differences in nitric oxide synthase activity and blood flow in the sheep brain. *J. Cereb. Blood Flow Metab.* **17,** 109–115.

Nunokawa, Y., Ishida, N., and Tanaka, S. (1994). Promoter analysis of human inducible nitric oxide synthase gene associated with cardiovascular homeostasis. *Biochem. Biophys. Res. Commun.* **200,** 802–807.

Nunokawa, Y., Oikawa, S., and Tanaka, S. (1996). Human inducible nitric oxide synthase gene is transcriptionally regulated by nuclear factor-κB dependent mechanism. *Biochem. Biophys. Res. Commun.* **223,** 347–352.

Nunokawa, Y., Oikawa, S., and Tanaka, S. (1997). Expression of human inducible nitric oxide synthase is regulated by both promoter and 3'-regions. *Biochem. Biophys. Res. Commun.* **233,** 523–526.

O'Shea, R. D., and Gundlach, A. L. (1996). Food or water deprivation modulate nitric oxide synthase (NOS) activity and gene expression in rat hypothalamic neurones: Correlation with neurosecretory activity? *J. Neuroendocrinol.* **8,** 417–425.

Oda, H., Murayama, T., Sasaki, Y., Okada, T., and Nomura, Y. (1997). Endothelin enhances lipopolysaccharide-induced expression of inducible nitric oxide synthase in rat glial cells. *Eur. J. Pharmacol.* **339,** 253–260.

Oguchi, S., Weisz, A., and Esumi, H. (1994). Enhancement of inducible-type NO synthase gene transcription by protein synthesis inhibitors—activation of an intracellular signal transduction pathway by low concentrations of cycloheximide. *FEBS Lett.* **338,** 326–330.

Ogura, T., Nakayama, K., Fujisawa, H., and Esumi, H. (1996). Neuronal nitric oxide synthase expression in neuronal cell differentiation. *Neurosci. Lett.* **204,** 89–92.

Ohara, Y., Sayegh, H. S., Yamin, J. J., and Harrison, D. G. (1995). Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. *Hypertension* **25,** 415–420.

Olson, S. C., Dowds, T. A., Pino, P. A., Barry, M. T., and Burke-Wolin, T. (1997). ANG II stimulates endothelial nitric oxide synthase expression in bovine pulmonary artery endothelium. *Am. J. Physiol.* **273,** L315–L321.

Pahan, K., Sheikh, F. G., Namboodiri, A. M., and Singh, I. (1997). Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J. Clin. Invest.* **100,** 2671–2679.

Pahan, K., Sheikh, F. G., Khan, M., Namboodiri, A. M., and Singh, I. (1998a). Sphingomyelinase and ceramide stimulate the expression of inducible nitric-oxide synthase in rat primary astrocytes. *J. Biol. Chem.* **273,** 2591–2600.

Pahan, K., Sheikh, F. G., Namboodiri, A. M., and Singh, I. (1998b). Inhibitors of protein phosphatase 1 and 2A differentially regulate the expression of inducible nitric-oxide synthase in rat astrocytes and macrophages. *J. Biol. Chem.* **273,** 12219–12226.

Pahan, K., Raymond, J. R., and Singh, I. (1999). Inhibition of phosphatidylinositol 3-kinase induces nitric-oxide synthase in lipopolysaccharide- or cytokine-stimulated C6 glial cells. *J. Biol. Chem.* **274,** 7528–7536.

Palmer, L. A., Semenza, G. L., Stoler, M. H., and Johns, R. A. (1998). Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. *Am. J. Physiol.* **274,** L212–L219.

Paludan, S. R., Ellermann-Eriksen, S., and Mogensen, S. C. (1998). NF-κB activation is responsible for the synergistic effect of herpes simplex virus type 2 infection on interferon-gamma-induced nitric oxide production in macrophages. *J. Gen. Virol.* **79,** 2785–2793.

Paludan, S. R., Ellermann-Eriksen, S., Lovmand, J., and Mogensen, S. C. (1999). Interleukin-4-mediated inhibition of nitric oxide production in interferon-γ-treated and virus-infected macrophages. *Scand. J. Immunol.* **49,** 169–176.

Pang, L., and Hoult, J. R. (1997). Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E2 and other cyclic AMP stimulants in J774 macrophages. *Biochem. Pharmacol.* **53,** 493–500.

Park, C. S., Park, R., and Krishna, G. (1996). Constitutive expression and structural diversity of inducible isoform of nitric oxide synthase in human tissues. *Life Sci.* **59,** 219–225.

Pellacani, A., Chin, M. T., Wiesel, P., Ibanez, M., Patel, A., Yet, S. F., Hsieh, C. M., Paulauskis, J. D., Reeves, R., Lee, M. E., and Perrella, M. A. (1999). Induction of high mobility group-I(Y) protein by endotoxin and interleukin-1β in vascular smooth muscle cells. Role in activation of inducible nitric oxide synthase. *J. Biol. Chem.* **274,** 1525–1532.

Perrella, M. A., Pellacani, A., Wiesel, P., Chin, M. T., Foster, L. C., Ibanez, M., Hsieh, C. M., Reeves, R., Yet, S. F., and Lee, M. E. (1999). High mobility group-I(Y) protein facilitates nuclear factor-κB binding and transactivation of the inducible nitric-oxide synthase Promoter/Enhancer. *J. Biol. Chem.* **274,** 9045–9052.

Perrella, M. A., Yoshizumi, M., Fen, Z., Tsai, J. C., Hsieh, C. M., Kourembanas, S., and Lee, M. E. (1994). Transforming growth factor-β1, but not dexamethasone, down-regulates nitric-oxide synthase mRNA after its induction by interleukin-1β in rat smooth muscle cells. *J. Biol. Chem.* **269,** 14595–14600.

Phelan, M. W., and Faller, D. V. (1996). Hypoxia decreases constitutive nitric oxide synthase transcript and protein in cultured endothelial cells. *J. Cell. Physiol.* **167,** 469–476.

Pollock, J. S., Nakane, M., Buttery, L. K., Martinez, A., Springall, D., Polak, J. M., Förstermann, U., and Murad, F. (1993). Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. *Am. J. Physiol.* **265,** C1379–C1387.

Prabhakar, N. R., Rao, S., Premkumar, D., Pieramici, S. F., Kumar, G. K., and Kalaria, R. K. (1996). Regulation of neuronal nitric oxide synthase gene expression by hypoxia. Role of nitric oxide in respiratory adaptation to low pO_2. *Adv. Exp. Med. Biol.* **410,** 345–348.

Radomski, M. W., Palmer, R. M., and Moncada, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 10043–10047.

Ravichandran, L. V., and Johns, R. A. (1995). Up-regulation of endothelial nitric oxide synthase expression by cyclic guanosine 3',5'-monophosphate. *FEBS Lett.* **374,** 295–298.

Reilly, C. M., Zamorano, P., Stopper, V. S., and Mills, T. M. (1997). Androgenic regulation of NO availability in rat penile erection. *J. Androl.* **18,** 110–115.

Reiser, P. J., Kline, W. O., and Vaghy, P. L. (1997). Induction of neuronal type nitric oxide synthase in skeletal muscle by chronic electrical stimulation in vivo. *J. Appl. Physiol.* **82,** 1250–1255.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998). The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. *Nature* **391,** 79–82.

Rodriguez-Pascual, F., Kleinert, H., Hausding, M., and Förstermann, U. (1999). Changes in mRNA stability contribute to the expression of the human NO synthase II gene. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **359,** R 38.

Rossi, F., and Bianchini, E. (1996). Synergistic induction of nitric oxide by β-amyloid and cytokines in astrocytes. *Biochem. Biophys. Res. Commun.* **225,** 474–478.

Sakitani, K., Nishizawa, M., Inoue, K., Masu, Y., Okumura, T., and Ito, S. (1998). Synergistic regulation of inducible nitric oxide synthase gene by CCAAT/enhancer-binding protein beta and nuclear factor-κB in hepatocytes. *Genes Cells* **3,** 321–330.

Salvucci, O., Kolb, J. P., Dugas, B., Dugas, N., and Chouaib, S. (1998). The induction of nitric oxide by interleukin-12 and tumor necrosis factor-α in human natural killer cells: Relationship with the regulation of lytic activity. *Blood* **92,** 2093–2102.

Samdani, A. F., Dawson, T. M., and Dawson, V. L. (1997). Nitric oxide synthase in models of focal ischemia. *Stroke* **28,** 1283–1288.

Saura, M., Lopez, S., Rodriguez Puyol, M., Rodriguez Puyol, D., and Lamas, S. (1995). Regulation of inducible nitric oxide synthase expression in rat mesangial cells and isolated glomeruli. *Kidney Int.* **47,** 500–509.

Sawada, T., Falk, L. A., Rao, P., Murphy, W. J., and Pluznik, D. H. (1997). IL-6 induction of protein–DNA complexes via a novel regulatory region of the inducible nitric oxide synthase gene promoter—Role of octamer binding proteins. *J. Immunol.* **158,** 5267–5276.

Schaffer, M. R., Fuchs, N., Proksch, B., Bongartz, M., Beiter, T., and Becker, H. D. (1998). Tacrolimus impairs wound healing: A possible role of decreased nitric oxide synthesis. *Transplantation* **65,** 813–818.

Schmidt, H. H. H. W., Pollock, J. S., Nakane, M., Gorsky, L. D., Förstermann, U., and Murad, F. (1991). Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase. *Proc. Natl. Acad. Sci. USA* **88,** 365–369.

Schwarz, P. M., Gierten, B., Boissel, J. P., and Förstermann, U. (1998). Expressional down-regulation of neuronal-type nitric oxide synthase I by glucocorticoids in N1E-115 neuroblastoma cells. *Mol. Pharmacol.* **54,** 258–263.

Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J., and Hintze, T. H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* **74,** 349–353.

Sharma, H. S., Westman, J., Alm, P., Sjoquist, P. O., Cervos Navarro, J., and Nyberg, F. (1997). Involvement of nitric oxide in the pathophysiology of acute heat stress in the rat. Influence of a new antioxidant compound H-290/51. *Ann. N.Y. Acad. Sci.* **813,** 581–590.

Sharma, K., Danoff, T. M., Depiero, A., and Ziyadeh, F. N. (1995). Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: Possible mediation via protein kinase c+. *Biochem. Biophys. Res. Commun.* **207,** 80–88.

Shaul, P. W., North, A. J., Brannon, T. S., Ujiie, K., Wells, L. B., Nisen, P. A., Lowenstein, C. J., Snyder, S. H., and Star, R. A. (1995). Prolonged in vivo hypoxia enhances nitric oxide synthase type I and type III gene expression in adult rat lung. *Am. J. Respir. Cell Mol. Biol.* **13,** 167–174.

Sherman, M. P., Aeberhard, E. E., Wong, V. Z., Griscavage, J. M., and Ignarro, L. J. (1993). Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **191,** 1301–1308.

Sherman, T. S., Chen, Z., Yuhanna, I. S., Lau, K. S., Margraf, L. R., and Shaul, P. W. (1999). Nitric oxide synthase isoform expression in the developing lung epithelium. *Am. J. Physiol.* **276,** L383–L390.

Shimpo, M., Ikeda, U., Maeda, Y., Kurosaki, K., Okada, K., Saito, T., and Shimada, K. (1997). Serotonin inhibits nitric oxide synthesis in rat vascular smooth muscle cells stimulated with interleukin-1. *Eur. J. Pharmacol.* **338,** 97–104.

Shiraishi, A., Dudler, J., and Lotz, M. (1997). Role of IFN regulatory factor-1 in synovitis and nitric oxide production. *J. Immunol.* **159,** 3549–3554.

Silvagno, F., Xia, H., and Bredt, D. S. (1996). Neuronal nitric-oxide synthase-μ, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J. Biol. Chem.* **271,** 11204–11208.

Singer, I., Kawka, D. W., Scott, S., Weidner, J. R., Mumford, R. A., Riehl, T. E., and Stenson, W. F. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology* **111,** 871–885.

Singh, I., Grams, M., Wang, W. H., Yang, T., Killen, P., Smart, A., Schnermann, J., and Briggs, J. P. (1996a). Coordinate regulation of renal expression of nitric oxide synthase, renin, and angiotensinogen mRNA by dietary salt. *Am. J. Physiol.* **270,** F1027–F1037.

Singh, K., Balligand, J. L., Fischer, T. A., Smith, T. W., and Kelly, R. A. (1996b). Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells. Role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and STAT1 alpha. *J. Biol. Chem.* **271,** 1111–1117.

Smith, F. S., Ceppi, E. D., and Titheradge, M. A. (1997). Inhibition of cytokine-induced inducible nitric oxide synthase expression by glucagon and cAMP in cultured hepatocytes. *Biochem. J.* **326,** 187–192.

Spessert, R., Layes, E., Schollmayer, A., Reuss, S., and Vollrath, L. (1995). In the rat pineal gland, but not in the suprachiasmatic nucleus, the amount of constitutive neuronal nitric oxide synthase is regulated by environmental lighting conditions. *Biochem. Biophys. Res. Commun.* **212,** 70–76.

Spink, J., and Evans, T. (1997). Binding of the transcription factor interferon regulatory factor-1 to the inducible nitric-oxide synthase promoter. *J. Biol. Chem.* **272,** 24417–24425.

Spink, J. M., Cohen, J., and Evans, T. (1995). Delineation of the vsmc iNOS enhancer and its interaction with the transcription factor, relA. *Endothelium* **3,** S–50.

Spitsin, S. V., Farber, J. L., Bertovich, M., Moehren, G., Koprowski, H., and Michaels, F. H. (1997). Human- and mouse-inducible nitric oxide synthase promoters require activation of phosphatidylcholine-specific phospholipase C and NF-κB. *Mol. Med.* **3,** 315–326.

Spolarics, Z., Spitzer, J. J., Wang, J. F., Xie, J., Kolls, J., and Greenberg, S. (1993). Alcohol administration attenuates LPS-induced expression of inducible nitric oxide synthase in Kupffer and hepatic endothelial cells. *Biochem. Biophys. Res. Commun.* **197,** 606–611.

Stichtenoth, D. O., and Frölich, J. C. (1998). Nitric oxide and inflammatory joint diseases. *Br. J. Rheumatol.* **37,** 246–257.

Suschek, C., Rothe, H., Fehsel, K., Enczmann, J., and Kolb-Bachofen, V. (1993). Induction of a macrophage-like nitric oxide synthase in cultured rat aortic endothelial cells. IL-1β-mediated induction regulated by tumor necrosis factor-alpha and IFN-γ. *J. Immunol.* **151,** 3283–3291.

Suschek, C., Fehsel, K., Kröncke, K. D., Sommer, A., and Kolb-Bachofen, V. (1994). Primary cultures of rat islet capillary endothelial cells. Constitutive and cytokine-inducible macrophage-like nitric oxide synthases are expressed and activities regulated by glucose concentration. *Am. J. Pathol.* **145,** 685–695.

Szabo, C., Wong, H. R., Bauer, P. I., Kirsten, E., Oconnor, M., Zingarelli, B., Mendeleyev, J., Hasko, G., Vizi, E. S., Salzman, A. L., and Kun, E. (1997). Regulation of components of the inflammatory response by 5 iodo 6 amino 1,2 benzopyrone, an inhibitor of poly(adp ribose) synthetase and pleiotropic modifier of cellular signal pathways. *Int. J. Oncol.* **10,** 1093–1101.

Tascedda, F., Molteni, R., Racagni, G., and Riva, M. A. (1996). Acute and chronic changes in K(+)-induced depolarization alter NMDA and nNOS gene expression in cultured cerebellar granule cells. *Mol. Brain Res.* **40,** 171–174.

Taylor, B. S., Alarcon, L. H., and Billiar, T. R. (1998a). Inducible nitric oxide synthase in the liver: Regulation and function. *Biochemistry (Moscow)* **63,** 766–781.

Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1998b). Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* **273,** 15148–15156.

Teichert, A. M., Karantzoulis-Fegaras, F., Wang, Y., Mawji, I. A., Bei, X., Gnanapandithen, K., and Marsden, P. A. (1998). Characterization of the murine endothelial nitric oxide synthase promoter. *Biochim. Biophys. Acta* **1443,** 352–357.

Thompson, C. M., Bernhard, A. E., and Strobel, H. W. (1997). Barbiturate-induced expression of neuronal nitric oxide synthase in the rat cerebellum. *Brain Res.* **754,** 142–146.

Thomsen, L. L., Scott, J. M., Topley, P., Knowles, R. G., Keerie, A. J., and Frend, A. J. (1997). Selective inhibition of inducible nitric oxide synthase inhibits tumor growth in vivo: Studies with 1400W, a novel inhibitor. *Cancer Res.* **57,** 3300–3304.

Thornton, F. J., Schaffer, M. R., Witte, M. B., Moldawer, L. L., MacKay, S. L., Abouhamze, A., Tannahill, C. L., and Barbul, A. (1998). Enhanced collagen accumulation following direct transfection of the inducible nitric oxide synthase gene in cutaneous wounds. *Biochem. Biophys. Res. Commun.* **246,** 654–659.

Tojo, A., Madsen, K. M., and Wilcox, C. S. (1995). Expression of immunoreactive nitric oxide synthase isoforms in rat kidney. Effects of dietary salt and losartan. *Jpn. Heart J.* **36,** 389–398.

Torres, G., and Rivier, C. (1994). Induction of c-fos in rat brain by acute cocaine and fenfluramine exposure: A comparison study. *Brain Res.* **647,** 1–9.

Tsuchiya, T., Kishimoto, J., and Nakayama, Y. (1996). Marked increases in neuronal nitric oxide synthase (nNOS) mRNA and NADPH-diaphorase histostaining in adrenal cortex after immobilization stress in rats. *Psychoneuroendocrinology* **21,** 287–293.

Venema, R. C., Nishida, K., Alexander, R. W., Harrison, D. G., and Murphy, T. J. (1994). Organization of the bovine gene encoding the endothelial nitric oxide synthase. *Biochim. Biophys. Acta* **1218,** 413–420.

Villar, M. J., Ceccatelli, S., Bedecs, K., Bartfai, T., Bredt, D., Synder, S. H., and Hokfelt, T. (1994). Upregulation of nitric oxide synthase and galanin message-associated peptide in hypothalamic magnocellular neurons after hypophysectomy. Immunohistochemical and in situ hybridization studies. *Brain Res.* **650,** 219–228.

Vincent, S. R., and Hope, B. T. (1992). Neurons that say NO. *Trends Neurosci.* **15,** 108–113.

Vizzard, M. A. (1997). Increased expression of neuronal nitric oxide synthase in bladder afferent and spinal neurons following spinal cord injury. *Dev. Neurosci.* **19,** 232–246.

Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q. W., and Nathan, C. (1993). Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor-β. *J. Exp. Med.* **178,** 605–613.

Wadt, K. A., Larsen, C. M., Andersen, H. U., Nielsen, K., Karlsen, A. E., and Mandrup Poulsen, T. (1998). Ciliary neurotrophic factor potentiates the beta-cell inhibitory effect of IL-1β in rat pancreatic islets associated with increased nitric oxide synthesis and increased expression of inducible nitric oxide synthase. *Diabetes* **47,** 1602–1608.

Wang, Y., Goligorsky, M. S., Lin, M., Wilcox, J. N., and Marsden, P. A. (1997). A novel, testis-specific mRNA transcript encoding an NH_2-terminal truncated nitric-oxide synthase. *J. Biol. Chem.* **272,** 11392–11401.

Weber, C. M., Eke, B. C., and Maines, M. D. (1994). Corticosterone regulates heme oxygenase-2 and NO synthase transcription and protein expression in rat brain. *J. Neurochem.* **63,** 953–962.

Weiner, C. P., Knowles, R. G., and Moncada, S. (1994a). Induction of nitric oxide synthases early in pregnancy. *Am. J. Obstet. Gynecol.* **171,** 838–843.

Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994b). Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 5212–5216.

Weisz, A., Oguchi, S., Cicatiello, L., and Esumi, H. (1994). Dual mechanism for the control of inducible-type NO synthase gene expression in macrophages during activation by interferon-gamma and bacterial lipopolysaccharide—transcriptional and post-transcriptional regulation. *J. Biol. Chem.* **269,** 8324–8333.

Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S., and Keefer, J. K. (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254,** 1001–1003.

Wong, H. R., Finder, J. D., Wasserloos, K., and Pitt, B. R. (1995). Expression of iNOS in cultured rat pulmonary artery smooth muscle cells is inhibited by the heat shock response. *Am. J. Physiol.* **269,** L843–L848.

Woodman, C. R., Muller, J. M., Rush, J. W. E., Laughlin, M. H., and Price, E. M. (1999). Flow regulation of ecNOS and Cu/Zn SOD mRNA expression in porcine coronary arterioles. *Am. J. Physiol.* **276,** H1058–H1063.

Wright, K., Ward, S. G., Kolios, G., and Westwick, J. (1997). Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric-oxide synthase expression in epithelial cell line HT-29. *J. Biol. Chem.* **272,** 12626–12633.

Wu, K. K., Cieslik, K., Zembowicz, A., and Tang, J. L. (1997). Transcriptional activation of endothelial nitric oxide-synthase (eNOS) gene. *Jpn. J. Pharmacol.* **75,** A8.

Wu, W., Li, Y., and Schinco, F. P. (1994). Expression of c-jun and neuronal nitric oxide synthase in rat spinal motoneurons following axonal injury. *Neurosci. Lett.* **179,** 157–161.

Xiao, Z., Zhang, Z., and Diamond, S. L. (1997). Shear stress induction of the endothelial nitric oxide synthase gene is calcium-dependent but not calcium-activated. *J. Cell. Physiol.* **171,** 205–211.

Xie, K., Huang, S., Dong, Z., Gutman, M., and Fidler, I. J. (1995). Direct correlation between expression of endogenous inducible nitric oxide synthase and regression of M5076 reticulum cell sarcoma hepatic metastases in mice treated with liposomes containing lipopeptide CGP 31362. *Cancer Res.* **55,** 3123–3131.

Xie, Q. (1997). A novel lipopolysaccharide-response element contributes to induction of nitric oxide synthase. *J. Biol. Chem.* **272,** 14867–14872.

Xie, Q. W., Whisnant, R., and Nathan, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J. Exp. Med.* **177,** 1779–1784.

Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269,** 4705–4708.

Xu, D. L., Martin, P. Y., St. John, J., Tsai, P., Summer, S. N., Ohara, M., Kim, J. K., and Schrier, R. W. (1996). Upregulation of endothelial and neuronal constitutive nitric oxide synthase in pregnant rats. *Am. J. Physiol.* **271,** R1739–R1745.

Xue, C., Reynolds, P. R., and Johns, R. A. (1996). Developmental expression of NOS isoforms in fetal rat lung: Implications for transitional circulation and pulmonary angiogenesis. *Am. J. Physiol.* **270,** L88–L100.

Yamamoto, H., Lamphier, M. S., Fujita, T., Taniguchi, T., and Harada, H. (1994). The oncogenic transcription factor IRF-2 possesses a transcriptional repression and a latent activation domain. *Oncogene* **9,** 1423–1428.

Yamamoto K, Ikeda U, Okada K, Saito T, Kawahara Y, Okuda M, Yokoyama M., and Shimada K (1997). Arginine vasopressin increases nitric oxide synthesis in cytokine-stimulated rat cardiac myocytes. *Hypertension* **30,** 1112–1120.

Yamamoto, K., Dang, Q. N., Kelly, R. A., and Lee, R. T. (1998). Mechanical strain suppresses inducible nitric-oxide synthase in cardiac myocytes. *J. Biol. Chem.* **273,** 11862–11866.

Yamasaki, K., Edington, H. D., McClosky, C., Tzeng, E., Lizonova, A., Kovesdi, I., Steed, D. L., and Billiar, T. R. (1998). Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J. Clin. Invest.* **101,** 967–971.

Yoshizumi, M., Perrella, M. A., Burnett, J. C., and Lee, M. E. (1993). Tumor necrosis factor downregulates an endothelial nitric oxide synthase messenger RNA by shortening its half-life. *Circ. Res.* **73,** 205–209.

Yun, H. Y., Dawson, V. L., and Dawson, T. M. (1997). Nitric oxide in health and disease of the nervous system. *Mol. Psychiatry* **2,** 300–310.

Zembowicz, A., Tang, J. L., and Wu, K. K. (1995). Transcriptional induction of endothelial nitric oxide synthase type III by lysophosphatidylcholine. *J. Biol. Chem.* **270,** 17006–17010.

Zhang, H., Chen, X., Teng, X., Snead, C., and Catravas, J. D. (1998). Molecular cloning and analysis of the rat inducible nitric oxide synthase gene promoter in aortic smooth muscle cells. *Biochem. Pharmacol.* **55,** 1873–1880.

Zhang, R., Min, W., and Sessa, W. C. (1995). Functional analysis of the human endothelial nitric oxide synthase promoter. Sp1 and GATA factors are necessary for basal transcription in endothelial cells. *J. Biol. Chem.* **270,** 15320–15326.

Zhang, Z. G., Chopp, M., Zaloga, C., Pollock, J. S., and Förstermann, U. (1993). Cerebral endothelial nitric oxide synthase expression after focal cerebral ischemia in rats. *Stroke* **24,** 2016–2021.

Zhang, Z. G., Chopp, M., Gautam, S., Zaloga, C., Zhang, R. L., Schmidt, H. H., Pollock, J. S., and Förstermann, U. (1994). Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. *Brain Res.* **654,** 85–95.

Ziesche, R., Petkov, V., Williams, J., Zakeri, S. M., Mosgoller, W., Knofler, M., and Block, L. H. (1996). Lipopolysaccharide and interleukin 1 augment the effects of hypoxia and inflammation in human pulmonary arterial tissue. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 12478–12483.

Zimmermann, H., Kurzen, P., Klossner, W., Renner, E. L., and Marti, U. (1996). Decreased constitutive hepatic nitric oxide synthase expression in secondary biliary fibrosis and its changes after Roux-en-Y choledocho-jejunostomy in the rat. *J. Hepatol.* **25,** 567–573.

CHAPTER 8

Molecular Regulation of Inducible Nitric Oxide Synthase

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THE GENE FOR INDUCIBLE NITRIC OXIDE SYNTHASE GENE (iNOS) IS EXPRESSED BY MANY SPECIES AND IN A LARGE VARIETY OF CELL TYPES. TYPICALLY, THE EXPRESSION OF iNOS IS REPRESSED IN RESTING CELLS, REQUIRING ACTIVATION BY AN EXPANDING NUMBER OF BIOLOGICAL, CHEMICAL, OR PHYSICAL STIMULI. THE MOLECULAR REGULATION OF iNOS EXPRESSION IS COMPLEX AND OCCURS AT MULTIPLE SITES IN THE GENE EXPRESSION PATHWAY, WITH BOTH TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL CONTROL MECHANISMS. INDUCTION OF NITRIC OXIDE SYNTHESIS HAS BEEN SHOWN TO ELICIT EITHER BENEFICIAL OR DETRIMENTAL CONSEQUENCES DEPENDING ON THE PHYSIOLOGICAL OR PATHOPHYSIOLOGICAL CONDITION. THEREFORE, UNDERSTANDING THE SIGNALING PATHWAYS AND MOLECULAR MECHANISMS THAT REGULATE iNOS EXPRESSION IS CRITICAL FOR THE DEVELOPMENT OF EFFECTIVE THERAPIES TO MODULATE iNOS EXPRESSION IN DISEASE STATES. THE GOAL OF THIS CHAPTER IS TO SUMMARIZE THE MOLECULAR MECHANISMS THAT GOVERN iNOS GENE EXPRESSION, FOCUSING ON THE REGULATION OF iNOS TRANSCRIPTION. THE MAJOR DIFFERENCES BETWEEN HUMAN AND RODENT iNOS GENE REGULATION WILL BE HIGHLIGHTED AS WELL AS THE MECHANISMS THAT DOWNREGULATE iNOS EXPRESSION.

Introduction

Since the initial discovery that endothelium-derived relaxing factor (EDRF) is nitric oxide gas (NO) (Ignarro *et al.,* 1987), a great deal of scientific effort has been focused on elucidating the mechanisms that regulate nitric oxide synthesis and on understanding the physiological and pathophysiological consequences of biological NO production. Within cells, NO is produced from L-arginine by a family of NOS enzymes. The enzymes are the products of three distinct mammalian genes that are expressed in various cells and tissues. The three NOS genes and encoded proteins are subject to complex positive and negative regulatory inputs that determine the amount and duration of NO production. Table I summarizes the basic genetic organization of the human NOS genes with cell type-specific expression, chromosomal location, gene size, and exon–intron structure. The same organization is essentially apparent in the mouse, where chromosomal synteny and sequence homologies indicate that an evolutionarily conserved NOS gene family exists in vertebrates. This chapter will focus on the molecular regulation of the inducible NO synthase (iNOS, NOS2) gene.

The first NOS cDNA, cloned in 1991 by Bredt and Snyder from rat cerebellum, possessed sequence homology with the ''characterized'' cytochrome P-450 reductase. This knowledge provided important insight into the enzymology and cofactor requirements for NO synthesis (Bredt *et al.,* 1991). Subsequently, human brain nitric oxide synthase was cloned and observed to be expressed constitutively in both neuronal cells and skeletal muscle (Nakane *et al.,* 1993). The activity of the neuronal NOS (nNOS) enzyme is regulated

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Table I Gene Structure and Chromosomal Localization of the Human NO Synthase Genes

Human NOS gene	Cell types	Chromosome	Gene size	Gene structure
Constitutive NOS, neuronal type (ncNOS, nNOS, NOS-I, NOS1)	Neuronal cells, skeletal muscle cells, cardiac muscle	12	160kb	29 exons, 28 introns
Constitutive NOS, endothelial type (ecNOS, eNOS, NOS-III, NOS3)	Endothelial cells	7	21 kb	26 exons, 25 introns
Inducible NOS (iNOS, NOS-II, NOS2)	Many cell types	17	37 kb	26 exons, 25 introns

by a calcium-dependent interaction of calmodulin with nNOS, leading to synthesis of NO. The physiological importance of nNOS-derived NO as a signaling molecule with functions in the nervous system is well established (see Section III, NO and the Nervous System, in this book). Another distinct NOS isoform, endothelial NOS or (eNOS), was originally cloned from bovine and human endothelium (Janssens *et al.,* 1992; Lamas *et al.,* 1992; Marsden *et al.,* 1992; Nishida *et al.,* 1992; Sessa *et al.,* 1992). The expression of the eNOS gene is generally constitutive within endothelial cells and is regulated similarly to nNOS. As a result of the calcium dependency for nNOS and eNOS activity, the production of NO by these enzymes is generally transient, resulting in the production of small amounts of NO, which have been called NO puffs. The importance of the eNOS-derived NO in normal physiology is well established. NO produced by mammals was originally identified as the EDRF and regulates normal vascular tone (Ignarro *et al.,* 1987; Vallance *et al.,* 1989).

Cloning of the Murine and Human iNOS Genes

In contrast to the constitutive expression and predominant posttranslational regulation of nNOS and eNOS enzymatic activity, the existence of a bacterial lipopolysaccharide (LPS)- or cytokine-inducible NOS (iNOS) was initially described in mouse macrophage (Stuehr and Marletta, 1985, 1987). The murine iNOS cDNA was subsequently cloned from LPS and γ-interferon (IFNγ)-stimulated macrophages by three independent investigators (Lowenstein *et al.,* 1992; Lyons *et al.,* 1992; Xie *et al.,* 1992). The iNOS gene is typically not expressed in normal resting cells, but on exposure to infectious agents, microbial products, or cytokines, the expression of the iNOS gene is induced, resulting in a sustained, high-output production of NO (see Fig. 1). In contrast to the nNOS and eNOS enzymes, the production of NO by iNOS occurs in the absence of calcium signaling (Cho *et al.,* 1992). Although the dogma remains in place that NO production is regulated primarily by ''constitutive'' expression of neuronal or endothelial constitutive NOS (cNOS), or by ''inducible'' expression of iNOS, exceptions to the paradigm exist. For example, iNOS has been shown to be constitutively expressed in certain tissues (Guo *et al.,* 1995; Hoffman *et al.,* 1997; Mannick *et al.,* 1994). Additionally, although the cNOS protein is constitutively expressed in endothelial cells, full cNOS enzyme activity may depend on cytokine induction of GTP cyclohydrolase I to provide the essential NOS cofactor, tetrahydrobiopterin (Werner-Felmayer *et al.,* 1993). For a review of the expressional control of the cNOS isoforms, see Förstermann *et al.* (1998).

The existence of a human iNOS was initially implied by the increased levels of circulating nitrogen oxides in patients receiving cytokine-based tumor immunotherapy (Hibbs *et al.,* 1992; Ochoa, 1991a) and in trauma or bacterial sepsis (Ochoa *et al.,* 1991b). However, in contrast to murine macrophages, it was difficult to demonstrate that human macrophages expressed an LPS- or cytokine-inducible iNOS (Bertholet *et al.,* 1999). Human iNOS expression in a specific cell type was identified by Nussler in 1992 in primary human hepatocytes stimulated with a cytokine mixture (CM) of LPS, tumor necrosis factor (TNFα), interleukin 1β (IL-1β), and IFNγ (Nussler *et al.,* 1992). This then allowed our group to clone the first human iNOS cDNA from LPS- and

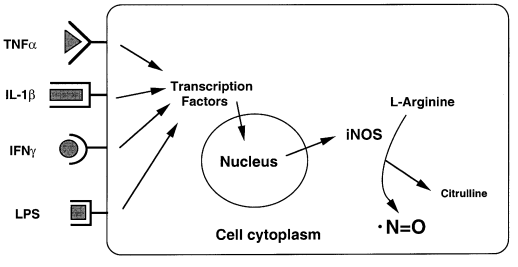


Figure 1 A simplified diagram representing the molecular pathways involved in the synthesis of nitric oxide by iNOS. The cytokines TNFα, IL-1β, IFNγ, and bacterial LPS are inducers of iNOS expression. On engagement of cell surface receptors, a signal transduction cascade is initiated, resulting in the activation of latent, cytoplasmic transcription factors. Activated transcription factors enter the nucleus, bind to promoter DNA sequences in the vicinity of target genes (i.e., iNOS), and activate the transcription of iNOS mRNA. The iNOS mRNA is then processed and translated into protein in the cytoplasm. Cytoplasmic iNOS protein requires a variety of cofactors for enzymatic activity. The guanidino nitrogen of the amino acid arginine is oxidized and cleaved, releasing NO and citrulline as end products of iNOS enzymatic activity. Although any point along this simplified biosynthetic pathway might constitute a limiting or regulatory step in the synthesis of NO by iNOS, it is clear that the predominant mode of regulating NO synthesis by iNOS occurs at the level of iNOS mRNA transcription.

cytokine-stimulated primary human hepatocytes in 1993 (Geller *et al.,* 1993a). The sequence of the human hepatocyte iNOS clone reveals a 4145-base pair (bp) cDNA, containing a 3459-bp open reading frame that encodes a polypeptide of 1153 amino acids with a calculated molecular mass of 131 kDa. Compared to human endothelial cNOS and human neuronal cNOS, human hepatocyte iNOS displays 51 and 53% amino acid sequence identity, respectively. Overall, human iNOS displays 80% sequence homology to murine macrophage iNOS. Similar to other NOS isoforms, hepatocyte iNOS contains consensus recognition sites for the cofactors flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH in the carboxyl half of the protein that have been shown to be important for iNOS enzyme activity. In addition, binding sites for heme, biopterin, and calmodulin are also present. The functional role of the human iNOS cDNA was confirmed by transfection of an iNOS cDNA expression vector into human kidney cells, which resulted in a substantial increase in NOS activity as detected by conversion of radiolabeled arginine to citrulline (Geller *et al.,* 1993a). Since the initial report, human iNOS cDNAs have been cloned from chondrocytes (Charles *et al.,* 1993; Maier *et al.,* 1994), the DLD-1 colon carcinoma cell line (P. A. Sherman *et al.,* 1993), the A-172 glioblastoma cell line (Hokari *et al.,* 1994), and human cardiac myocytes (Luss *et al.,* 1995). Each of these cDNAs are identical to the human hepatocyte iNOS cDNA with >99% sequence homology. Table II lists primary human cells, cell lines, or tissues that have been reliably documented to express human iNOS.

The human iNOS gene was cloned by Mudgett from a fibroblast genomic library (Chartrain *et al.,* 1994). All of the isolated clones were found to be part of a single genomic locus. The full-length human iNOS gene is 37 kb in length and is composed of 26 exons and 25 introns (Table I). This genomic structure is similar to that of human endothelial and neuronal NOS genes, and it suggests the divergence from a common ancestor. Primer extension analysis of LPS and cytokine-stimulated human hepatocyte RNA identified the transcriptional initiation site 30 bp downstream of the TATA box. Utilizing a somatic cell hybrid mapping panel and fluorescent *in situ* hybridization, the human iNOS gene was mapped to chromosome 17 at position 17 cen-q11.2 (Chartrain *et al.,* 1994). Human eNOS (Marsden *et al.,* 1993) and nNOS (Kishimoto *et al.,* 1992) reside on chromosome 7 and 12, respectively, confirming that the three NOS genes are distinct.

Protective and Pathophysiological Roles of iNOS Expression

The importance of iNOS expression in human physiological and pathophysiological conditions is increasingly clear and is the focus of a review by Kroncke *et al.,* (1998) and chapters in this book. We briefly mention that iNOS expression can result in both beneficial and detrimental actions to

Table II Human Cells and Tissues That Express iNOS

Primary cells	Cancers	Cell lines
Astrocytes	Breast	A-172 glioblastoma
Chondrocytes	Colon	AKN-1 liver epithelium
Cardiac myocytes	Melanoma	A549 lung epithelium
Eosinophils	Squamous cell	B cell lymphoma
Endometrium	Uterine	CaCO-2 adenocarcinoma
Fibroblasts	Lung	DLD-1 adenocarcinoma
Hepatocytes		MG 63 osteosarcoma
Islet cells		Neuroblastoma NB-39-nu
Lipocytes		SW480 colon carcinoma
Keratinocytes		SW620 colon carcinoma
Kupffer cells		
Lung epithelium		
Macrophages		
Monocytes		
Neurons		
Neutrophils		
Osteoblasts		
Osteoclasts		
Pancreatic ductal cells		
Placental trophoblasts		
Platelets		
Retinal epithelium		
Sertoli cells		
Skeletal muscle		
Vascular endothelial cells		
Vascular smooth muscle		

illustrate the importance of delineating the molecular mechanisms that govern iNOS expression. The large quantity of NO produced by iNOS has important antimicrobial functions (MacMicking *et al.,* 1997), and NO can inhibit the growth of viruses (Croen, 1993; Karupiah *et al.,* 1993; Saura *et al.,* 1999) and bacteria (Shiloh *et al.,* 1999). NO has been shown to have a protective effect in malaria (Anstey *et al.,* 1996; Nussler *et al.,* 1991), and polymorphisms have been identified in the human iNOS promoter that correlate with malaria ''disease susceptibility'' in certain populations (Burgner *et al.,* 1998; Kun *et al.,* 1998). In the immune system, NO reduces both neutrophil and platelet adhesion (Hickey and Kubes, 1997), and it has an immunoregulatory role in lymphocyte activation and allograft rejection (Bonham *et al.,* 1997; Hoffman *et al.,* 1990). In experimental endotoxemia, iNOS is widely expressed in many tissues (Salter *et al.,* 1991). The induced NO synthesis that occurs in vascular beds during sepsis is protective in solid organs such as the liver, kidney, and intestine (Harbrecht *et al.,* 1992; MacKendrick *et al.,* 1993; Markewitz *et al.,* 1993). NO is a potent vasodilator and anticoagulant that minimizes organ ischemia. Another major mechanism of iNOS protection is elicited by the antiapoptotic effects of NO (Li *et al.,* 1999). In an animal model of acute liver failure, a NO donor decreased liver damage and blocked apoptosis, whereas NOS inhibitors increased apoptosis in LPS-challenged animals (Ou *et al.,* 1997; Saavedra *et al.,* 1997). iNOS expression is also important in the normal process of wound healing (Heck *et al.,* 1992; Schaffer *et al.,* 1997; Yamasaki *et al.,* 1998).

Although NO appears to have many beneficial roles in the acute aspects of the septic response, overexpression of iNOS can be detrimental. Induced NO synthesis in blood vessel walls results in massive vasodilation and hypotension due to iNOS expression in smooth muscle cells (Busse and Mulsch, 1990; Nakayama *et al.,* 1992) and endothelial cells (Suschek *et al.,* 1993). Animal studies have shown that iNOS contributes to the vascular collapse in septic shock (Szabo and Thiemermann, 1994). Many animal models have shown that chronic, localized expression of iNOS has deleterious effects. In autoimmune diseases such as juvenile diabetes (Corbett and McDaniel, 1992; Eizirik *et al.,* 1994), arthritis (McCartney-Francis *et al.,* 1993), and immune-mediated nephritis (Weinberg *et al.,* 1994), NO is cytotoxic. Coincident expression of iNOS in neurodegenerative disease implicates NO in the pathogenesis of multiple sclerosis and other demyelinating central nervous system (CNS) lesions (Koprowski *et al.,* 1993). Furthermore, NO has been shown to cause DNA damage and is mutagenic (Wink *et al.,* 1991), and iNOS expression has been documented in several cancers (Ambs *et al.,* 1998a). Demonstrably, NO can have both beneficial and detrimental actions. Understanding the complex aspects of iNOS regulation has the potential of providing important insights and possible therapeutic strategies for a number of acute and chronic inflammatory diseases.

Characterization of iNOS mRNA Expression

There are both similarities and differences in the regulation of the human and murine iNOS genes. In general, rodent cells are more sensitive to various biological and pharmacological agents, and single agents will readily induce high-level iNOS expression. Figure 2 is a Northern blot for iNOS mRNA expression in cultured rat hepatocytes. IL-1β is the most potent single cytokine to stimulate rat hepatocyte iNOS

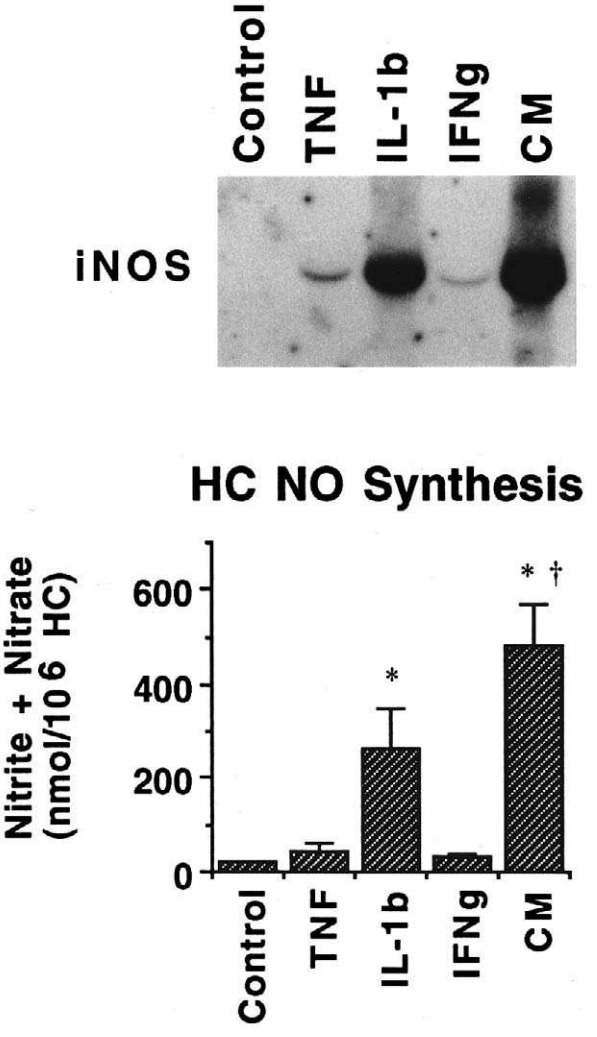


Figure 2 Effects of single cytokines and a cytokine mixture on the expression of rat hepatocyte iNOS mRNA and nitric oxide synthesis. The experiment pictured in this figure measured the expression of iNOS mRNA and the resulting nitrogen oxides that are generated in the culture medium as measured by Northern blotting and by the Greiss assay, respectively. The experiment measured the cellular iNOS mRNA and culture media nitrite + nitrate in response to media ± 500 U/ml of TNFα, IL-1β, or IFNγ as single agents, or together in a cytokine mixture (CM). Notice that IL-1β alone is sufficient for the induction of hepatocyte iNOS mRNA, whereas the CM is able to induce iNOS mRNA expression in a synergistic fashion. Also note that the level of NO produced, as measured by the Greiss assay, is a reflection of the expression of iNOS mRNA. * indicates $p < 0.05$ versus control; † indicates $p < 0.05$ versus IL-1β. Adapted with permission from Geller *et al.* (1995). *The Journal of Immunology* **155,** 4890–4898. Copyright 1995. The American Association of Immunologists.

mRNA expression and NO synthesis. TNFα or IFNγ alone weakly induce iNOS mRNA expression and have additive or synergistic effects when added together with IL-1β. LPS- or cytokine-induced NO synthesis was not easily identified in human macrophages, and a debate over whether humans even possessed an inducible NOS ensued. However, an LPS- and cytokine-inducible NOS activity was eventually identified in human hepatocytes (Geller *et al.,* 1993a; Nussler *et al.,* 1992), which led to the initial cloning of the human iNOS cDNA from human hepatocytes. Characterization of human iNOS expression showed fundamental differences between mouse and humans in the regulation of iNOS transcription by cytokines. For instance, isolation of the human iNOS cDNA in hepatocytes involved a cytokine mixture (CM) that included LPS, TNFα, IL-1β, and IFNγ to elicit maximal human iNOS mRNA expression. Subsequently, it was shown that various pairwise combinations of TNFα, IL-1β, and IFNγ functioned additively or synergistically for induction of human hepatocyte iNOS mRNA (de Vera *et al.,* 1996a; Geller *et al.,* 1993a). Pictured in Fig. 3A is a Northern blot analyzing human iNOS mRNA expression in the AKN-1 human liver cell line in response to various cytokine combinations. As depicted, iNOS mRNA expression is undetectable in resting cells. However, in response to a cytokine mixture of TNFα, IL-1β, and IFNγ, a robust iNOS mRNA expression is induced. A single mRNA band at ~4.5 kb first appeared 2 hours after stimulation, peaked at 4–6 hours, and was diminished by 24 hours. Similarly, NO_2^- and NO_3^- levels were measured from the culture supernatants and were found to increase 20- to 30-fold at 24 hours after stimulation. Whereas most human cell types require a combination of cytokines to activate iNOS expression, IL-1β alone at high doses can induce iNOS mRNA in cultures of primary human hepatocytes (Geller *et al.,* 1995) and chondrocytes (Charles *et al.,* 1993). As shown in Fig. 3B, IFNγ alone can induce only a low level of iNOS mRNA expression. However, either TNFα or IL-1β can synergize with IFNγ for increased iNOS expression, whereas CM is the strongest inducing combination. The molecular basis for the synergistic induction of iNOS gene expression by cytokines led to investigations into iNOS transcription and iNOS promoter activities.

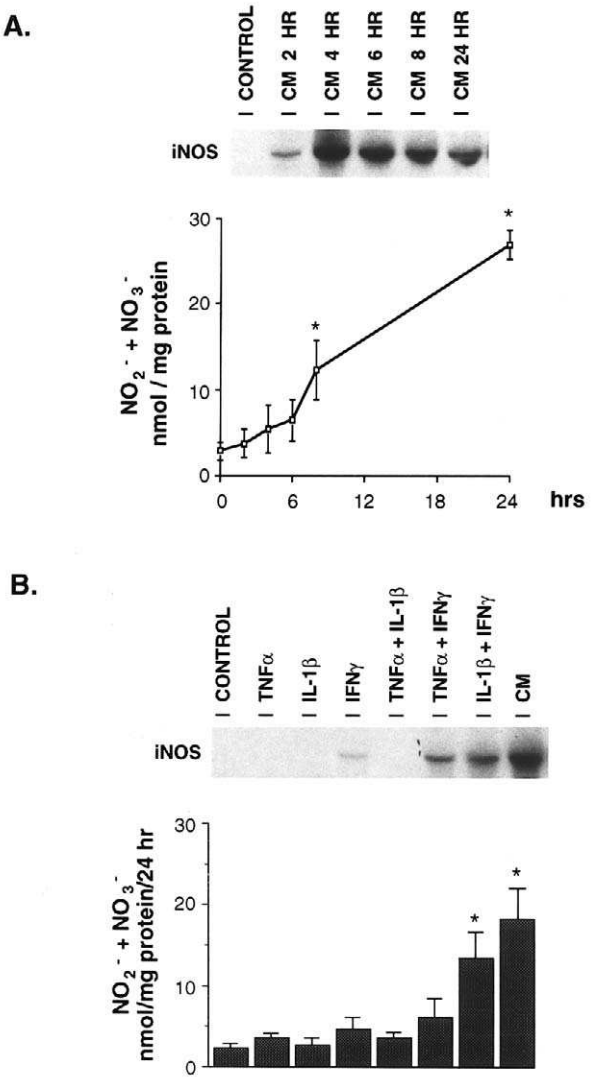


Figure 3 Multiple cytokines functioning in synergy are typically required for expression of human iNOS mRNA and subsequent NO synthesis. (A) A Northern blotting experiment for iNOS mRNA expression in AKN-1 cells was performed with RNA isolated at various time points in response to a cytokine mixture containing 1000 U/ml of TNFα, 100 U/ml of IL-1β, and 250 U/ml of IFNγ. Note that the peak of iNOS mRNA induction occurs around 4 hours after cytokine stimulation, and that the peak production of NO lags behind that of iNOS mRNA synthesis and continues to increase at 24 hours. (B) Multiple cytokines were tested singly, and in combination, for the ability to induce iNOS mRNA expression by Northern blotting, and for NO synthesis by the Greiss reaction. Single cytokines such as TNFα, IL-1β, or IFNγ are not effective for induction of iNOS mRNA by themselves. However, cytokine synergy for the induction of iNOS mRNA is observed when either TNFα or IL-1β is combined with IFNγ. Note that CM is the strongest inducing combination for both the synthesis of iNOS mRNA and for NO production. Adapted with permission from de Vera *et al.* (1996a).

Deletional Analysis of the Murine and Human iNOS Promoters

Since the cloning of the mouse and human iNOS cDNAs, it is evident that the predominant mode of regulation for iNOS expression is at the level of mRNA transcription initiation (de Vera *et al.,* 1996a; Lorsbach *et al.,* 1993; Xie *et al.,* 1993). Nuclear run-on assays have shown a three- to fivefold increase in iNOS transcription rate in murine or human cells after LPS and cytokine stimulation (de Vera *et al.,* 1996a; Lowenstein *et al.,* 1993; Xie *et al.,* 1993). Interestingly, a basal nuclear transcript was seen in the unstimulated human cells and indicates constitutive, low-level human iNOS transcription (de Vera *et al.,* 1996a). This was unexpected, given that a basal iNOS mRNA expression is not observed in Northern blot experiments using resting primary human cells or most cell lines (see Fig. 3). This suggests that the iNOS mRNA transcript is highly labile and is rapidly degraded in

the absence of cytokine stimulation, indicating the probability of posttranscriptional regulatory mechanisms in addition to transcriptional regulation.

To illustrate the differences between the molecular regulation of the rodent and human iNOS genes, a summary of the murine and human iNOS promoters will be reviewed. In work by Xie (Xie *et al.,* 1993) and Lowenstein (Lowenstein *et al.,* 1993), a 1.7-kb segment of the 5'-flanking region of the murine iNOS gene was found to contain LPS and cytokine-responsive promoter elements. Following transfection of deletional iNOS promoter–reporter gene constructs into the macrophage cell line RAW 264.7, two discrete upstream regions (region I and II) were found to mediate inducibility by LPS and IFNγ.

These results contrast markedly with the transcriptional regulation of the human iNOS promoter where our group reported that far upstream elements in the 5'-flanking region of the human iNOS gene were required to confer cytokine-inducibility (de Vera *et al.,* 1996a). To delineate the cytokine-responsive regions of the human iNOS promoter, we isolated ~16 kb of the human iNOS gene 5'-flanking region and generated deletional iNOS promoter constructs ranging in size from 1.3 to 16 kb. These deletional segments were then ligated in front of the luciferase reporter gene. After liposomal transfection of the iNOS promoter–luciferase reporter constructs into human liver (AKN-1) or lung (A549) epithelial cells, analysis of the first 4.7 kb upstream-demonstrated basal promoter expression, but failed to show any cytokine-inducible activity (see Fig. 4A). However, a three- to fourfold increase in promoter activity was observed in constructs extending up to −5.8 and −7.2 kb, and a ninefold increase in promoter activity was induced by cytokines after transfection of the −16-kb construct. Therefore, the functional cytokine-responsive promoter elements are located upstream from −4.7 kb in the human iNOS promoter. The three- to ninefold increase in cytokine-stimulated promoter activity with the larger constructs correlated reasonably well with the ~fivefold induction of transcription that we detected by nuclear run-on assay. To further establish that the proximal 5'-flanking region was not a critical component of the human iNOS promoter, we deleted 2.6 kb of DNA from the 5'-flanking region (−2.1 to −4.7 kb) in the context of

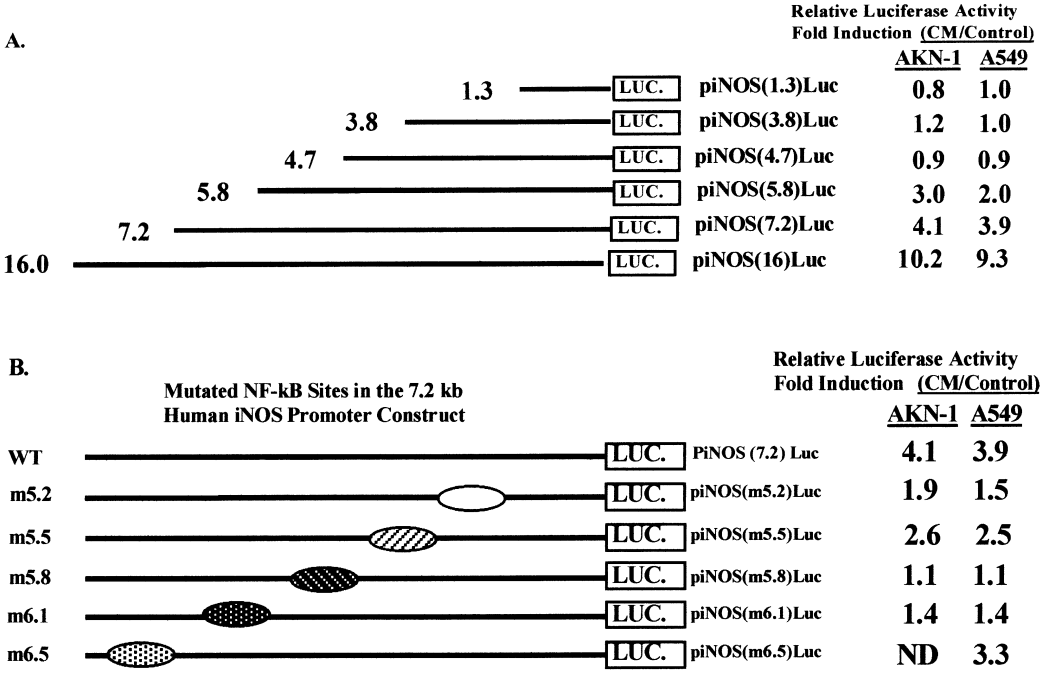


Figure 4 A 5' deletion and mutation analysis of iNOS promoter–luciferase reporter gene constructs localizes cytokine-responsive NF-κB binding sequences far upstream in the human iNOS promoter. (A) Various lengths of iNOS promoter DNA (−1.3, −3.8, −4.7, −5.8, −7.2, and −16 kb) were fused to a luciferase reporter vector, transfected into either AKN-1 or A549 cells, and either unstimulated or stimulated with a cytokine mixture (CM) containing 1000 U/ml TNFα, 100 U/ml IL-1β, and 250 U/ml IFNγ. Values are expressed as -fold induction of light units from CM-stimulated cells divided by the light units from unstimulated cells. The relative light units were normalized to the expression of β-galactosidase units expressed from a cotransfected, internal control β-gal expression plasmid. Notice that the cytokine-inducibility of the luciferase reporter plasmids requires iNOS promoter DNA sequence upstream of −4.7 kb. (B) DNA sequence analysis and site-directed mutagenesis of iNOS promoter–reporter plasmids identify multiple, cytokine-responsive NF-κB sequence elements. Site-directed mutagenesis of the −7.2 kb iNOS promoter–reporter plasmids was utilized to assess the function of five putative NF-κB promoter elements localized between −5.2 and −6.5 kb of 5'-flanking iNOS promoter DNA. Normalized values are expressed as relative light units from CM-stimulated cells divided by the relative light units from unstimulated cells. Notice that mutation of the different NF-κB elements eliminates the cytokine inducibility of the iNOS promoter–reporter plasmids to varying extents. Adapted with permission from Taylor *et al.* (1998).

the 7.2 kb iNOS–luciferase promoter construct. Transfection and cytokine stimulation in either human cell line maintained the same ~fourfold induction as the full-length 7.2-kb iNOS–luciferase promoter construct, confirming that this proximal region was dispensable (de Vera *et al.,* 1996a; Taylor *et al.,* 1998).

Studies by other groups have verified that the upstream regions are required for cytokine-inducible human iNOS promoter activity. Similar to our results in human liver and lung cells, Laubach and Marks were unable to demonstrate any inducible activity with a 3.7-kb iNOS promoter construct in human DLD-1 colon cells (Laubach *et al.,* 1997) and human A549 cells (Marks-Konczalik *et al.,* 1998). Kolyada has also demonstrated no LPS and IFNγ inducibility in human vascular smooth muscle cells transfected with a 1.1-kb human iNOS promoter segment (Kolyada *et al.,* 1996). Work by Linn identified an enhancer region from −8.7 to −10.7 kb in the human iNOS promoter that confers IL-1β and IFNγ responsiveness in DLD-1 cells (Linn *et al.,* 1997). Since the initial isolation of the murine and human iNOS promoters, much work has been done to define the *cis*- and *trans*-activating factors important for regulating iNOS transcription. A summary of these findings will be reviewed.

The Critical Importance of the NF-κB Signaling Pathway

The mechanisms by which the NF-κB family of DNA binding proteins function to regulate gene transcription has been the subject of intense scientific scrutiny for more than a decade. The NF-κB family of DNA binding proteins are typically resident in the cytoplasm in various homo- and heterodimeric associations and are retained in the cytoplasm by the inhibitory κB (IκB) family of proteins until an appropriate signal is transduced. A large set of environmental and biological signals can initiate the NF-κB signal transduction cascade. Signal transduction results in the phosphorylation and subsequent ubiquitination of the IκB family of proteins, which targets the IκB protein for proteolytic degradation by the ''proteosome.'' As a result of IκB degradation, the dimeric NF-κB proteins are now free to enter the nucleus, bind to specific DNA sequences, and regulate gene transcription by interactions with basal transcription factors.

Until more recently, the manner in which various agonists signal the phosphorylation and subsequent degradation of IκB has remained a mystery. However, progress has identified the signaling pathways through which TNFα or IL-1β-receptor interactions mediate the phosphorylation of serine-32 and serine-36 in the IκB protein. Phosphorylation of serine-32 and serine-36 in the IκB proteins targets the IκB for ubiquitination and subsequent degradation by the proteosome. Following receptor engagement, TNFα receptor–associated factors (TRAF 2 or 6) interact with and activate the NF-κB inducing kinase (NIK) complex. Then, the activated NIK complex phosphorylates and activates the inhibitory κB kinase (IKK) complex. Activated IKK can directly phosphorylate serine-32 and serine-36 of the IκB protein, which is then ubiquitinated and degraded by the proteosome, thereby releasing the NF-κB dimer to enter the nucleus. Other kinases have also been shown to phosphorylate serine-32 and serine-36 or other amino acid residues in the IκB proteins. However, the functional importance of serine-32 and serine-36 phosphorylation in IκB degradation and NF-κB activation is well established. For reviews of the NF-κB signal transduction cascade, see Baldwin (1996), Ghosh *et al.* (1998), and Mercurio and Manning (1999).

Early studies identified NF-κB as an important transcription factor in the LPS-induced expression of iNOS in the murine RAW 264.7 macrophage cell line. Deletional analyses of iNOS promoter–reporter plasmids were utilized in mapping the LPS- and IFNγ-inducible elements to within −1.7 kb upstream of the mouse iNOS mRNA start site (Lowenstein *et al.,* 1993; Xie *et al.,* 1993). A more detailed mutational analysis revealed the presence of a functional NF-κB DNA-binding sequence at −85 to −76 bp from the transcription start site that, when mutated, lost the ability to bind NF-κB proteins and to activate LPS-induced reporter expression (Xie *et al.,* 1994).

Other investigators looking at LPS- or cytokine-induced iNOS transcription also discovered an important role for NF-κB. However, important differences were being identified in the regulation of iNOS expression in different cell types. For instance, in mouse macrophages, the downstream NF-κB site between −85 to −76 is apparently more important than the upstream NF-κB site at −971 to −962 for LPS inducibility (Xie *et al.,* 1994). However, in IL-1β; TNFα; and IFNγ-stimulated vascular smooth muscle, the upstream NF-κB site at −971 to −962 is more functional (Spink *et al.,* 1995). Furthermore, both LPS induction and the synergistic response to LPS plus IFNγ were mapped to an upstream enhancer DNA sequence that included the upstream NF-κB site at −971 to −962 (Alley *et al.,* 1995; Lowenstein *et al.,* 1993). Both the upstream and the downstream NF-κB sequence elements have been shown by numerous investigators to bind various NF-κB family members *in vitro* in different cell types and in response to differing stimuli. The compiled results of the *in vitro* NF-κB protein–iNOS promoter DNA interactions are summarized by Murphy (1999). In general, these studies reveal that particular NF-κB proteins bind to different NF-κB sequences with distinct affinities in a cell type- and stimulus-dependent fashion, revealing a fine complexity in the NF-κB regulation that is not fully understood at this time.

The apparent differences between regulation of iNOS gene transcription in different cell types and in different species has been a source of controversy, while providing an impetus to understand these differences on a mechanistic basis. Nonetheless, an important role for the NF-κB proteins in the regulation of human iNOS transcription has been demonstrated. When we sequenced the human iNOS promoter region upstream to −7.2 kb, numerous putative NF-κB response elements were identified. Pharmacological NF-κB inhibitors significantly suppressed cytokine-stimulated iNOS

mRNA expression and NO synthesis, suggesting that NF-κB is involved in the induction of the human iNOS gene. To determine whether any of the putative NF-κB elements in the region upstream from −4.7 kb were functional, site-directed mutagenesis was used to generate five additional 7.2-kb constructs, each with a 2-bp mutation in the core sequence of the NF-κB element (see Fig. 4B). These mutant NF-κB promoter constructs were transfected into human cells and then stimulated with cytokines to detect inducible promoter activity. The mutation at −5.8 kb resulted in loss of both basal and inducible promoter activity, whereas mutations of the sites at −5.2, −5.5, and −6.1 kb decreased inducible promoter activity by 60, 45, and 65%, respectively (Taylor *et al.,* 1998). The NF-κB mutant at site −6.5 kb retained the full cytokine inducibility typically seen with the 7.2-kb construct. The results showed that the NF-κB motif at −5.8 kb is required for cytokine-induced promoter activity, whereas the sites at −5.2, −5.5, and −6.1 kb have a cooperative effect. Another NF-κB site at −8.2 kb in the human iNOS promoter has also been shown to be functional (Marks-Konczalik *et al.,* 1998).

To demonstrate NF-κB DNA-binding activity in human liver (AKN-1) and lung (A549) cells, gel shift assays were done using the site-specific NF-κB oligonucleotide at −5.8 kb. Basal levels of NF-κB DNA binding were seen in control cells (see Fig. 5). The addition of the cytokine TNFα or IL-1β induced a strong gel shift complex for NF-κB, whereas IFNγ had no effect. All three agents together (CM) also induced DNA-binding activity for NF-κB, and the appearance of this complex was markedly suppressed by the addition of the antioxidant pyrrolidine dithiocarbamate (PDTC), further implicating NF-κB in iNOS expression. Specificity for NF-κB was demonstrated by competition assay with a 100-fold excess of unlabeled NF-κB oligonucleotide. Competition assays with excess unlabeled mutant oligonucleotide failed to compete for the NF-κB–DNA complex, demonstrating specificity for NF-κB. Antibody supershift studies in the A549 lung cells showed the presence of both p50 and p65 subunits of NF-κB in the complex. Additional studies indicate that the proximal promoter NF-κB sequence located at −106 to −115 bp in the human iNOS promoter DNA is not sufficient to confer cytokine inducibility to iNOS transcription (de Vera *et al.,* 1996a; X. Zhang *et al.,* 1996). However, this NF-κB sequence appears to be important for basal expression of iNOS and can functionally cooperate with other NF-κB sequences for cytokine inducibility (Marks-Konczalik *et al.,* 1998; Nunokawa *et al.,* 1994).

It should be noted that a variety of agents besides LPS, TNFα, or IL-1β can induce the nuclear translocation and activation of NF-κB. Importantly, many of these inducers of NF-κB are also inducers of iNOS expression in some cell types (see Table III). For instance, the β-amyloid protein observed in Alzheimer's disease is an efficient inducer of both NF-κB activation and iNOS expression in mouse astroglial cells (Akama *et al.,* 1998; Bonaiuto *et al.,* 1997). Furthermore, gp120 of human immunodeficiency virus (HIV) induces NF-κB in immune cells (Briant *et al.,* 1998a,b; Shatrov *et al.,* 1996), and both gp41 and gp120 activate iNOS expression (Adamson *et al.,* 1996; Kong *et al.,* 1996). Other agents that induce NF-κB activation and subsequent iNOS expression include FcE receptor signaling by antigen–IgE immune complexes (Bayon *et al.,* 1997; Bidri *et al.,* 1997), insulin-like growth factor-II (IGF-II) (Kaliman *et al.,* 1999), double-stranded RNA (dsRNA) (Heitmeier *et al.,* 1998), reactive oxygen species (ROS) (Adcock *et al.,* 1994; Hoffmann *et al.,* 1996), lipid secondary messengers (Katsuyama *et al.,* 1998; Pahan *et al.,* 1998; Welsh, 1996), cAMP analogs (Kleinert *et al.,* 1996a,b; Pahan *et al.,* 1997), IL-12 (Grohmann *et al.,* 1998; Salvucci *et al.,* 1998), phorbol esters (Diaz-Guerra *et al.,* 1996a), low environmental pH (Bellocq *et al.,* 1998), hyaluronic acid derivatives (McKee *et al.,* 1997), and parasite-derived peptides (Velasco *et al.,* 1997).

In vivo footprinting technology has also suggested an important role for the NF-κB proteins in iNOS expression in living mouse cells. In particular, the downstream NF-κB sequence is occupied by proteins under basal conditions and in LPS-stimulated cells, whereas the upstream sequence is only occupied in response to LPS (Goldring *et al.,* 1996, 1998). Importantly, p65–NF-κB deficient mice die *in utero* of massive hepatic apoptosis, whereas the p50 and c-Rel null mice survive to term but are immunologically compromised. It is significant that activated macrophages from c-Rel knockout mice are partially defective for iNOS expression (Grigoriadis *et al.,* 1996). In addition, data utilizing adenovirus gene transfer expressing either a dominant negative IκBα protein (Jobin *et al.,* 1998) or a dominant negative RelA (p65) NF-κB protein (Soares *et al.,* 1998) support a critical role for NF-κB in cytokine-induced iNOS expression in endothelial cells. Our data indicate the same is true in primary rat hepatocytes. In particular, expression of the dominant negative IκBα protein in rat hepatocytes prevented cytokine-induced NF-κB activation and subsequent iNOS expression (Taylor *et al.,* 1999). Furthermore, TNFα is a well-studied cytokine that signals through the NF-κB pathway, and TNFα receptor knockout mice are defective in the induction of iNOS by cytokines (Da Silva *et al.,* 1997; Salkowski *et al.,* 1996; Vieira *et al.,* 1996). In summary, a tremendous variety of data indicate a critical role for NF-κB proteins in the regulation of rodent and human iNOS transcription in a variety of cell types in response to various signals. Although NF-κB is a critical transcription factor mediating iNOS expression, it is clear that other signaling pathways are also involved.

The Importance of the Interferon–Jak–Stat Pathway for iNOS Induction

γ-Interferon is a well-characterized inducer of iNOS transcription in many different cell systems, particularly murine macrophage cell lines. Signal transduction by the type I interferons (IFNα/β) and type II interferon (IFNγ) has been

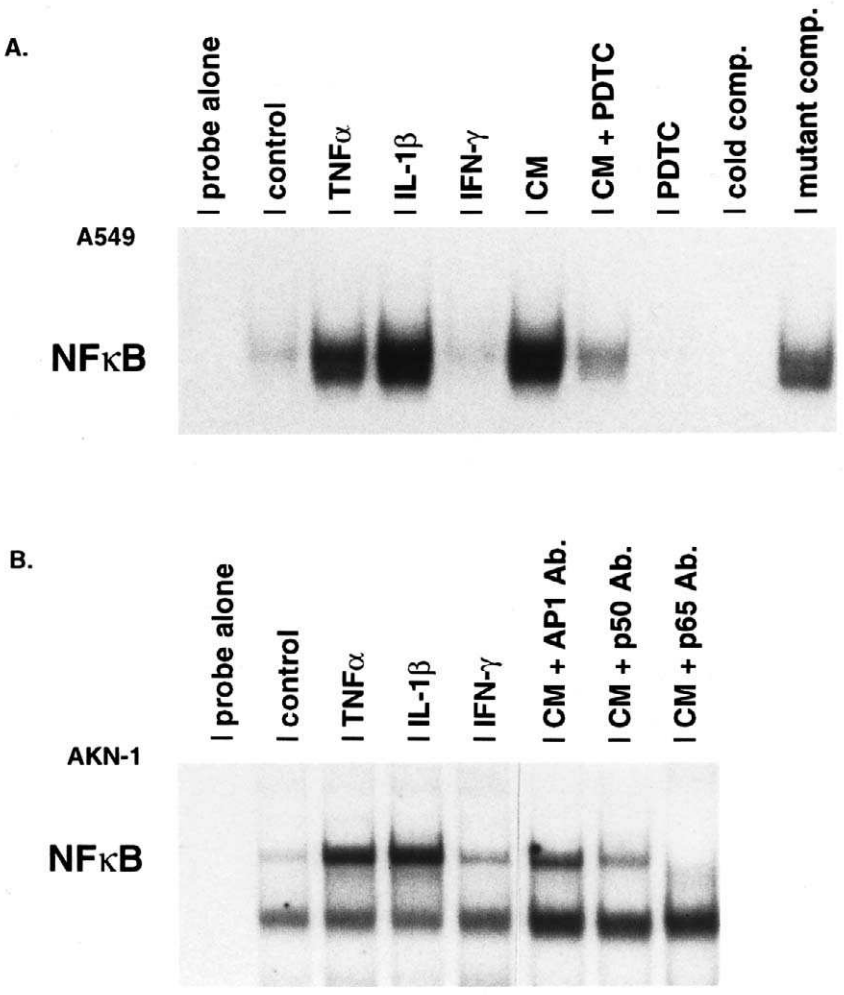


Figure 5 The functional NF-κB-like element at −5.8 kb of the human iNOS promoter is a DNA binding sequence for cytokine-induced nuclear NF-κB proteins. The experiments pictured are gel mobility shift assays which measure nuclear protein binding to radiolabeled DNA sequence, resulting in the formation of a protein–DNA complex. Protein–DNA complexes migrate more slowly than free DNA during native gel electrophoresis. Only the protein–DNA complexes are depicted. Notice that the lane labeled, ''probe alone'' does not result in a protein–DNA complex. Additionally, the nuclear DNA binding proteins that are ''induced'' on the addition of either TNFα, IL-1β, or CM to A549 human lung epithelium (A) or AKN-1 human liver (B) cell cultures are inhibited by addition of the NF-κB inhibitor PDTC. The radioactive protein–DNA complex is a specific molecular interaction, since an excess of nonradioactive DNA can compete with the radioactive probe for protein binding (cold comp). The addition of a DNA sequence mutated in the NF-κB recognition sequence (mutant comp) fails to compete with the radiolabeled probe, indicating that the protein binding to the DNA has sequence specificity for the NF-κB element. Additionally, notice that the addition of antibody to p50 and p65 NF-κB will specifically eliminate the formation of the NF-κB protein–DNA complex, whereas the addition of control AP-1 antibodies does not affect formation of the NF-κB–DNA complex. Adapted with permission from Taylor *et al.* (1998).

shown to involve the Jak–Stat signaling pathway. Typically, the binding of interferons to specific cell surface receptors results in the activation of an intrinsic or receptor-associated tyrosine kinase (i.e., a Jak or a Tyk kinase). Subsequently, the active tyrosine kinase(s) will phosphorylate important tyrosine residues in the Stat proteins. Tyrosine-phosphorylated Stat proteins can then form homo- or heterodimers with other Stat proteins. Tyrosine phosphorylation of Stat proteins is required for dimerization, nuclear entry, and DNA binding activities. Once in the nucleus, the active Stat dimers bind to particular sequence elements in the vicinity of target genes, interact with general transcription factors, and thereby regulate differential gene transcription. In the case of IFNγ, receptor engagement typically involves activation of Jak1 and Jak2 kinases, which then phosphorylate and activate Stat1 homodimers that enter the nucleus and bind to the IFNγ activating sequence (GAS) elements in regulated target genes. As for IFNα or IFNβ, receptor engagement activates

Table III Various Agents That Induce the Nuclear Translocation and Activation of NF-κB[a]

Activating stimuli	Signaling pathways	Downstream effectors
1. LPS and CD14	NIK/IKK???	NF-κB,
LPS and Toll receptors	NIK/IKK???	NF-κB
	Jak	Stat
	???	CREB, C/EBP, Oct/Brn3
2. TNFα	Traf 2-NIK/IKK	NF-κB
	Src–IκB	NF-κB
	Jak	Stat 1
3. IL-1B	Traf 6–NIK/IKK	NF-κB,
4. INFγ	Jak–Stat	Stat1
5. IFNα/B	Jak–Stat	Stat1, Stat2, IRFs
6. IL-6	???	CREB, C/EBP, Oct/Brn3
7. IGF-II	PI-3-kinase	NF-κB
8. IL-12	???	NF-κB
9. IgE–antigen–FCR complex	???	NF-κB
	Jak???	Stats
10. dsRNA	NIK/IKK and/or PKR???	NF-κB, Stats
11. Viral proteins (HIV gp120)	NIK/IKK	NF-κB
12. Parasite-derived peptide	???	NF-κB
13. Hyaluronic acid fragment	???	NF-κB
14. β Amyloid protein	???	NF-κB
15. cAMP agonists	PKA???	NF-κB
	PKA	CREB
16. Lipid secondary messengers	PLC–PKC–MAP kinase	AP-1 family???
	???	NF-κB
17. Hypoxia and picolinic acid	???	HIF-1
	???	NF-κB
18. ROS	Glutathione status???	NF-κB
	jak kinase???	Stat

[a] ??? indicates that the precise signaling pathways or downstream effectors by which the activating stimuli are transduced are either speculative or unknown.

the Jak2 and/or Tyk2 kinases, which typically tyrosine phosphorylate both Stat1 and Stat2. In this case, Stat1–Stat2 heterodimers are formed that subsequently interact with the interferon regulatory factor (IRF) family of DNA binding proteins, enter the nucleus, and bind to a distinct DNA sequence element called IRSE (interferon response sequence element). Additionally, IFNα and IFNβ signaling can also result in the activation of Stat1 homodimers that interact with GAS elements. For excellent reviews of the interferon-responsive Jak–Stat–IRF pathway, see Darnell (1998) and Stark *et al.* (1998).

Sequencing and mutagenesis of murine iNOS promoter–reporter constructs have identified functionally important interferon responsive elements that are required for the synergistic activation of iNOS transcription by LPS and IFNγ (Lowenstein *et al.,* 1993; Xie *et al.,* 1993). A functional ISRE element at −913 to −923 was shown to bind LPS and IFNγ-activated ISGF3, which contains Stat1, Stat2, and IRF-1 proteins in a ternary complex with DNA (Martin *et al.,* 1994). Furthermore, IRF-1 knockout mice were shown to be defective in the induction of iNOS transcription in macrophages in response to LPS and IFNγ (Fujimura *et al.,* 1997; Kamijo *et al.,* 1994; Martin *et al.,* 1994; Salkowski *et al.,* 1996). In addition, both IFNγ knockout mice (Vieira *et al.,* 1997) and IFNγ receptor knockout mice (Kamijo *et al.,* 1993) are also defective in iNOS induction. Finally, a functionally important GAS element that binds IFNγ-induced Stat1 homodimers has been identified in the murine iNOS promoter and is important for LPS plus IFNγ-induced iNOS expression in mouse macrophages (Gao *et al.,* 1997). While a requirement for Stat1 or Stat2 in IFNγ-induced iNOS expression has not been reported utilizing gene knockout technology, the importance of the IFN–Jak–Stat pathway is well established in the experiments described above.

For human iNOS expression, identification of the pathways involved in the response to IFNγ for iNOS induction is an active area of investigation. One report has suggested the involvement of the IFNγ–Jak2–Stat1 pathway (Kleinert

et al., 1998), but the iNOS promoter elements that bind either Stat1 dimers or the ISGF3 complex have not been described. However, many reports have investigated IFNγ-induced Jak kinase and Stat activation by relying on the use of tyrosine kinase inhibitors to suggest the involvement of the IFNγ–Jak2–Stat1 pathway for iNOS expression. It should be noted that the tyrosine kinase inhibitors are rather nonspecific. Indeed, herbimicin A is an effective inhibitor of Jak2 kinase, but it is also an effective inhibitor of NF-κB DNA binding activity (Iwasaki *et al.,* 1992; Mahon and O'Neill, 1995; Nishiya *et al.,* 1995). Additionally, whereas tyrophostin B42 is reportedly a specific inhibitor of Jak2 kinase, it is also an effective blocker of NF-κB activation (B. S. Lee *et al.,* 1997). Therefore, reliance on these inhibitors to assign strict causality to the IFNγ–Jak2–Stat1 pathway for iNOS induction should be cautioned.

Figure 6 is a gel shift assay for Stat1α DNA-binding activity in A549 human lung cells using a consensus oligonucleotide (hSIE) for Stat1α. IFNγ alone or as a component of cm induces Stat1 activity. We have identified a Stat1 binding sequence at approximately −5.2 kb in the human iNOS promoter that is required for induction of a luciferase reporter construct in response to CM (Taylor *et al.,* 1998). The element is predicted to be a NF-κB binding sequence overlapping with a GAS element. Gel shift data indicate that the −5.2 element is a very weak NF-κB binding element, but it will bind to nuclear Stat1 protein. Similar to the mouse promoter, but located far upstream in the human promoter, functionally important NF-κB (−5.8 kb) and Stat1 (−5.2 kb) binding elements are found in relative close proximity. Current experiments utilizing wild-type and dominant negative Stat1 expression plasmids, and also human 2fTGH fibroblasts and their Stat1 mutant derivative (U3A), indicate an important role for Stat1 in regulation of human iNOS mRNA expression.

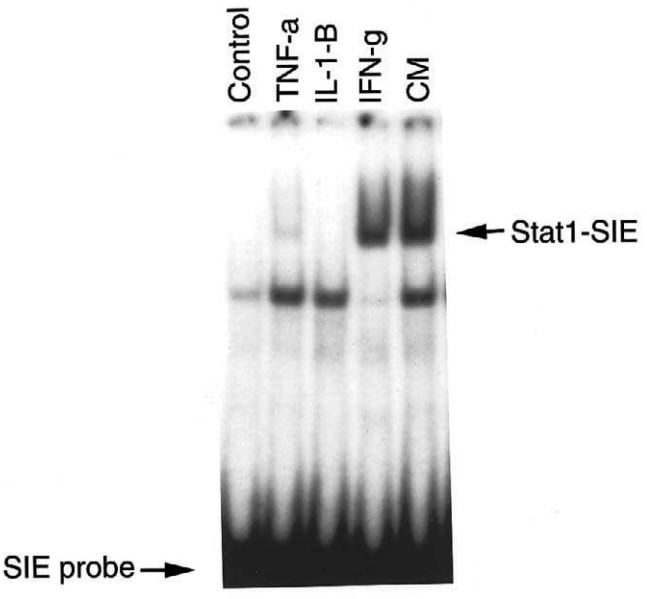


Figure 6 γ-Interferon induces nuclear, Stat1 DNA-binding activity. The gel shift assay depicted utilizes nuclear proteins isolated from human A549 lung epithelium stimulated for 2 hours with TNFα, IL-1β, IFNγ, or CM as indicated. The experiment utilized a radiolabeled consensus SIE DNA sequence, which is a high affinity binding site for Stat1α homodimers. In A549 cells, only IFNγ or CM induces nuclear, Stat1 DNA-binding activity. The identification of these protein–DNA interactions as Stat1 protein–DNA interactions has been verified by use of competition assays and by antibody supershift assays.

LPS is an important inducer of iNOS expression, and the ability of LPS to activate signaling pathways and iNOS expression remains an intense area of investigation. In this regard, LPS has been identified as an inducer of NF-κB in murine and human macrophages (Denlinger *et al.,* 1998; Goldring *et al.,* 1998). LPS is also an efficient inducer of *de novo* IFNα and IFNβ synthesis in macrophages. The ability of LPS to induce iNOS in mouse macrophages requires the synthesis of and subsequent autocrine signaling by IFNβ (Gao *et al.,* 1998). Therefore, LPS not only induces NF-κB, but can also induce the IFNβ–Jak–Stat pathway, resulting in the activation of the ISGF3 transcription factor complex.

The host response to viral infection involves the immunoregulatory function of IFNγ as a T helper cell type I (Th1) cytokine. Furthermore, dsRNAs produced by viruses are potent inducers of the type II interferons, IFNα/β, which are important in the antiviral response. This response involves the action of the dsRNA-activated protein kinase (PKR). It has been reported that IFNγ can activate the expression of PKR, whereas IFNα/B or dsRNA are potent inducers of PKR enzyme activity. An important function for dsRNA-activated PKR is to shut down the host protein translation machinery in virus-infected cells. This involves the dsRNA-activated auto-phosphorylation of PKR and subsequent PKR-dependent phosphorylation of the ribosomal eukaryotic translation initiation factor IIA (see Darnell, 1998 and Stark *et al.,* 1998). Interestingly, dsRNA is an important inducer of NF-κB in astroglial cells through effecting IκB degradation, and this results in iNOS expression in cells also exposed to IFNγ (Heitmeier *et al.,* 1998). Intriguingly, macrophages from PKR knockout mice are defective for NF-κB activation and IRF-1 induction by dsRNA and IFNγ (Kumar *et al.,* 1997). Additionally, PKR has been shown to activate NF-κB by inducing IκB degradation (Kumar *et al.,* 1994). Therefore, it appears that the interferons could function through multiple pathways in regulating iNOS expression. Viral RNA can activate NF-κB, PKR, and induce interferon expression. IFNγ induces Jak–Stat1 pathway activation and PKR expression. The type II interferons IFNα/β are effective in ISGF3 activation and could signal a PKR-dependent NF-κB activation. These findings signify a very complex immune response that would involve the interferons in Th1 type immunoregulation, interferon-induced iNOS, and NO-induced antimicrobial activities and that would involve the interelated actions of the PKR, the Jak–Stat, and the NF-κB signal transduction pathways.

Cytokine Synergy for Induction of iNOS mRNA Involves NF-κB and Interferon–Jak–Stat–IRF Pathways

Although IFNγ alone can efficiently induce iNOS expression in some murine cell types, it is generally not effective alone for iNOS mRNA induction in human cell types. However, IFNγ has been shown to cooperate for iNOS expression with LPS (Deng *et al.,* 1993; Geller *et al.,* 1993b; Lorsbach *et al.,* 1993; Lowenstein *et al.,* 1993; Stuehr and Marletta, 1987; Xie *et al.,* 1993), TNFα (Cox *et al.,* 1992), IL-1β (Geller *et al.,* 1993b, 1995), IL-2 (Cox *et al.,* 1992; Deng *et al.,* 1993), dsRNA (Heitmeier *et al.,* 1998), β-amyloid protein (Bonaiuto *et al.,* 1997), phorbol esters (Jun *et al.,* 1994; Yoon *et al.,* 1994), and agents that elevate or mimic intracellular calcium (Raddassi *et al.,* 1994) or cAMP (Kleinert *et al.,* 1996a,b; Koide *et al.,* 1993; Kunz *et al.,* 1994).

Intriguingly, many different rodent cell types have been shown to express iNOS in response to single biological agents, including LPS, TNFα, IL-1β, or IFNγ. However, in mouse macrophages, LPS-induced iNOS expression requires the *de novo* synthesis of and autocrine actions of IFNβ, since antibodies to IFNβ can specifically attenuate the LPS-induced iNOS expression (Fujihara *et al.,* 1994a; Gao *et al.,* 1998). Additionally, IL-1β alone is sufficient to induce iNOS expression in rat hepatocytes (Geller *et al.,* 1995). However, the IL-1β-induced iNOS expression in rat hepatocytes requires the autocrine synthesis and function of IFNγ, since antibodies or antisense RNA strategies directed against IFNγ will block the IL-1β-induced iNOS expression (Schroeder *et al.,* 1998). IFNγ alone has been shown to induce at least some iNOS expression in many different rodent cell types including hepatocytes and vascular smooth muscle (VSM), and in a few human cell types such as hepatocytes and the AKN-1 and DLD-1 cell lines. However, it is interesting that there is at least some level of ''constitutive'' nuclear NF-κB protein in VSM (Lawrence *et al.,* 1994; Shin *et al.,* 1996), hepatocytes (R. W. Ganster and D. A. Geller, personal observation), AKN-1 cells (Taylor *et al.,* 1998), and DLD-1 cells (Kleinert *et al.,* 1998). Furthermore, IFNγ is a weak inducer of NF-κB in rat hepatocytes (Duval *et al.,* 1996) and a strong inducer of NF-κB in the mouse renal epithelium cell line MCT (Amoah-Apraku *et al.,* 1995). Finally, TNFα alone is an effective inducer of NF-κB activation and iNOS expression in mouse macrophages, and it is interesting that TNFα can efficiently activate NF-κB and is also an activator of the Jak2–Stat1 pathway (Guo *et al.,* 1998). In summary, iNOS expression is typically regulated in a synergistic manner by a combination of inducers of the NF-κB pathway and by the interferon–Jak–Stat pathway. In the instances where single agents are effective for iNOS induction, both the NF-κB and Jak–Stat pathways are activated by the stimuli, or one pathway is constitutively activated. Several mechanisms describing the synergistic effects of NF-κB inducers with Jak–Stat pathway inducers in regulating gene expression have been suggested (Drew *et al.,* 1995; Lowenstein *et al.,* 1993; Neish *et al.,* 1995; Ohmori *et al.,* 1997; Pine, 1997; Shen and Stavnezer, 1998; Taylor *et al.,* 1998). However, the molecular basis of this cytokine-induced transcriptional synergy is an important and unresolved question and likely involves substantial integrations of the NF-κB and Jak–Stat pathways.

The Complex Effects of the cAMP-Dependent Protein Kinase a Pathway on iNOS Transcription

Although the cAMP response pathway has been investigated for decades, many questions remain about the manner by which cAMP regulates mRNA transcription (for review, see Montminy, 1997). Likewise, the effects of cAMP and agonists that increase the intracellular concentration of cAMP on iNOS transcription are complex and the molecular mechanisms remain obscure. For instance, in rat mesangial (Eberhardt *et al.,* 1998; Kunz *et al.,* 1994) or VSM cells (Imai *et al.,* 1994; Koide *et al.,* 1993), agents that mimic or elevate intracellular cAMP concentrations such as cholera toxin, forskolin, isoproterenol, or cAMP analogs all activate the transcription of iNOS. Furthermore, agents that activate the cAMP pathway can cooperate with TNFα, IL-1β, IFNγ, or activators of protein kinase C in an additive or synergistic fashion for the induction of iNOS transcription in a variety of cell types (Imai *et al.,* 1994; Koide *et al.,* 1993; Kunz *et al.,* 1994; Mullet *et al.,* 1997; Scott-Burden *et al.,* 1994). In contrast, agonists that activate the cAMP–protein kinase A (PKA) pathway in brain astroglial cells do not induce iNOS expression and are inhibitory to the LPS- or cytokine-induced iNOS transcription (Pahan *et al.,* 1997). In liver Kupffer cells and hepatocytes, prostaglandins or cAMP–PKA activators inhibit the LPS- or cytokine-induced iNOS expression (Harbrecht *et al.,* 1997; Mustafa and Olson, 1998). In macrophage cell lines and primary astroglia, the effects of cAMP–PKA agonists and mimics are predominantly inhibitory for iNOS expression (Harbrecht *et al.,* 1997; Morris *et al.,* 1998; Mullet *et al.,* 1997; Mustafa and Olson, 1998; Pahan *et al.,* 1997). However, in peritoneal macrophages, iNOS transcription is reported to be activated by cAMP or PKA activators (Pahan *et al.,* 1997). In primary murine splenic macrophages, cAMP or prostaglandin E_2 can synergize with TNFα plus IFNγ to activate iNOS expression, whereas these same agents are inhibitory for LPS-induced iNOS (Schwacha *et al.,* 1998). The conflicting actions of cAMP on iNOS expression probably reflect differential effects of cAMP on cell metabolism as well as differences in cell type or culture conditions. Furthermore, the complex effects of cAMP on iNOS transcription in different cells types may relate both to direct effects of the cAMP–PKA pathway on iNOS transcription and to indirect effects of the cAMP–PKA pathway on iNOS expression via cross talk with both the NF-κB and the Jak–Stat pathway.

Direct regulation of iNOS expression by the cAMP–PKA pathway has been described in mesangial cells. Either IL-1β

or cAMP can activate and cooperate for iNOS transcription in these cells, which is associated with the induction of DNA binding by the NF-κB, CREB, and CCAAT/enhancer binding protein (C/EBP) family of DNA-binding proteins. Furthermore, a functionally responsive C/EBP element between −277 and −111 bp upstream of the rat transcription start site has been described that is capable of interacting with CREB and C/EBP family members (Eberhardt *et al.,* 1998). Importantly, cAMP is a strong inducer of CREB and C/EBP, whereas IL-1β is a strong activator of NF-κB. Interestingly, IL-1β is a weak activator of induced CREB and C/EBP DNA-binding activities and is also a positive effector in upregulation of C/EBP expression (Eberhardt *et al.,* 1998). Another possible mechanism for the positive effect of cAMP on iNOS expression includes the following: protein kinase A phosphorylates *in vitro* a carboxyl-terminal serine residue in the transcription activation domain of the p65 subunit of NF-κB, which is required for interaction with the general coactivator CBP/p300 and for transcription activation (Gerritsen *et al.,* 1997; Zhong *et al.,* 1998). Additionally, the cytoplasmic PKAc subunit is identified in a ternary association with the cytoplasmic NF-κB–IκB complex, and the phosphorylation of p65 by PKAc is independent of cAMP (Zhong *et al.,* 1998).

Activators of cAMP signaling or cAMP mimics can inhibit LPS- or cytokine-induced iNOS transcription. The basis for these effects are not well described. However, some studies have suggested that cAMP–PKA-dependent signaling inhibits the degradation of the IκB induced by LPS or cytokines and thereby inhibits the ability of NF-κB to enter the nucleus and activate iNOS transcription (Mustafa and Olson, 1998; Pahan *et al.,* 1997). Furthermore, cAMP has been shown to decrease the expression and activity of Stat1 in PHA-stimulated T cells (Ivashkiv *et al.,* 1996). Additionally, cAMP has been shown to inhibit IFNγ-induced Jak1 kinase activation and subsequent Stat1 DNA binding activity, an effect which may be associated with the recruitment of SHP-2 phosphatase to the Jak1–IFNγ-receptor complexes (Sengupta *et al.,* 1996; You *et al.,* 1999).

Another possible level of complexity is the differential cell type expression of various positive and negative CREB/CREM/ICER gene family members. Additionally, CREB proteins can directly interact with various members of the AP-1 transcription factor family, interactions that result in protein complexes which bind to distinct DNA sequences (Morooka *et al.,* 1995; Zhou and Engel, 1995). An interesting speculation as to the apparent opposite effects of cAMP on iNOS expression in different cell types would propose that NF-κB, Stat1, CREB, AP-1/ATF, and the HMG families of transcription factors are all potentially competing for limiting amounts of CBP/p300 (Bisotto *et al.,* 1996; Gerritsen *et al.,* 1997; Horvai *et al.,* 1997; Kamei *et al.,* 1996; Lee *et al.,* 1998; Parry and Mackman, 1997; Swope *et al.,* 1996; J. J. Zhang *et al.,* 1996; Zhong *et al.,* 1998). CBP/p300 encodes a general transcription coactivator complex which intrinsically possesses and also recruits histone acetylase and deacetylase enzyme complexes to reorganize chromatin structures. If, for instance, the binding of activated CREB protein to the iNOS promoter DNA were blocked (via any sort of repressive chromatin), then the induced CREB protein would be forced to bind to other ''target genes'' and could potentially sequester the essential CBP/p300 proteins to other loci. Clearly, the complex cell-specific effects of cAMP on iNOS expression need to be further examined and should provide interesting information regarding signaling pathway cooperation and interference.

Additional Signaling Pathways That Regulate iNOS Expression

Molecular dissection of the requirements for regulated iNOS expression has indicated a very important and direct role for the NF-κB signaling pathway, the interferon–Jak–Stat pathway, and also the cAMP–PKA–CREB–C/EBP pathway. However, a variety of data suggests the involvement of other important signal transduction molecules and pathways that include cell surface receptors, IL-6 and the Oct/POU/Brn transcription factor family, the hypoxia–hypoxia inducible factor (HIF)-1 pathway, phospholipase C (PLC), protein kinase C (PKC), and the Ras–mitogen activated protein (MAP) kinase cascade. Therefore, a brief summary of these data will ensue with particular emphasis on how these ''auxiliary'' pathways might regulate iNOS expression either directly, or through cross talk with the NF-κB or Jak–Stat pathways.

Treatment of immature macrophages with IL-6 can induce maturation that is coincident with iNOS expression. Furthermore, LPS or IL-6 can induce nuclear C/EBP and ''octamer'' DNA-binding activities on the iNOS promoter as assessed *in vitro* (Sawada *et al.,* 1997) and by *in vivo* footprinting technology (Goldring *et al.,* 1996). The signaling pathways regulated by IL-6 that result in altered DNA binding activities have not been adequately described. However, the protein binding to the octamer-like iNOS promoter sequence has been identified as Brn3a (Gay *et al.,* 1998). Importantly, promoter–reporter experiments indicate that mutation of the octamer element eliminates IL-6-induced iNOS promoter–reporter activity, and furthermore, overexpression of Brn3A can potentiate expression of iNOS mRNA. These data indicate the involvement of an IL-6 responsive signaling pathway that can induce the nuclear DNA binding activities of both the NF-IL-6 (i.e., C/EBP) and Brn3a and Brn3b proteins. Importantly, NF-κB and C/EBP have been shown to cooperate for IL-8 (Stein and Baldwin, 1993), HIV (Ruocco *et al.,* 1996), and iNOS transcription (Eberhardt *et al.,* 1998; Sakitani *et al.,* 1998). Additionally, it appears that only Brn3, and not Oct-1 or Oct-2, are capable of *trans*-activation from the iNOS promoter (Gay *et al.,* 1998), revealing a degree of specificity in the responsiveness of the iNOS promoter to particular members of the Oct/Brn/POU family of DNA-binding transcription factors.

Recent data have highlighted the role of hypoxia in the induction of iNOS expression. Hypoxia is a stimulus for the

induction of iNOS expression in rat lung *in vivo* (Xue *et al.,* 1994). Although the intracellular signaling mechanisms that regulate gene expression in response to hypoxia are only recently being dissected, a role for HIF-1 is established (Wenger and Gassmann, 1997). A hypoxia response element in the mouse iNOS promoter at position −227 to −209 is required for the response to hypoxia and to picolinic acid in the presence of IFNγ. Additionally, this elememt is also required for the synergistic iNOS induction by iron chelators and IFNγ (Melillo *et al.,* 1997). Similar data have not yet been demonstrated for the human system in regard to hypoxic induction of iNOS mRNA. However, the mouse hypoxic response element is not identified in the corresponding location in the human proximal promoter sequence.

Influence of the Cell Cycle Regulatory Proteins on iNOS Expression

Recent data have shown the involvement of cell cycle regulatory components on iNOS expression including the functions of certain proto-oncogenes such as PKC, Ras–Raf–MAP kinase cascade, Fos/Jun/ATF (AP-1) family, as well as the tumor suppressor p53. Frequently observed in studies analyzing the cell cycle machinery, the particular proteins involved apparently function to varying extents in different cell types. Clearly, further investigations are essential to clarify the role of these proteins on iNOS expression.

Several studies have investigated the involvement of the MAP kinases as positive effectors of LPS- or cytokine-induced iNOS expression (Chan *et al.,* 1999; Da Silva *et al.,* 1997; Nishiya *et al.,* 1997; Pahan *et al.,* 1998; Singh *et al.,* 1996). Importantly, both pharmacological and genetic evidence has implicated various MAP kinases in the induction of mouse iNOS expression. For instance, in mouse astrocytes, a drug (FHPI) that specifically inhibits the p38 MAP kinase can block iNOS induction in response to TNFα and IL-1β (Da Silva *et al.,* 1997). In mouse macrophages, expression of dominant negative mutants of MEKK1 and MKK4, which act upststream of p46/54 JNK/SAPK, abrogated the TNFα plus IFNγ-induced iNOS expression (Chan *et al.,* 1999). In cardiac myocytes and vascular endothelium, the p42/p44 extracellular signal-related kinase (ERK)1/ERK2 kinases have been implicated in the activation of iNOS expression by either IL-1β or IFNγ (Singh *et al.,* 1996). Separate studies in rat C6 gliomas exposed to LPS and IFNγ suggest that expression of a dominant negative Ras protein or pharmacological inhibition of the ERK kinases do not effect iNOS expression (Nishiya *et al.,* 1997). However, others suggest that cytokine- or LPS-stimulated C6 gliomas or primary astrocytes will synergistically express iNOS in the presence of ceramides. Furthermore, this expression is inhibited by PD98059 (a MEK kinase inhibitor) or by FPT II, which is a farnesyl protein transferase inhibitor, suggesting involvement of a Ras–Raf–MAP kinase pathway for iNOS expression (Pahan *et al.,* 1998). A separate study utilizing specific inhibitors of either protein farnesylation of geranylgeranylation in IL-1β-induced rat primary aortic smooth muscle or hepatocytes suggested that a farnesylated protein other than Ras is required for IL-1β-induced iNOS induction, and that a geranylgeranylated protein inhibited IL-1β-induced iNOS expression (Finder *et al.,* 1997). Likewise, PKC inhibitors and farnesyltransferase inhibitors have also implicated a PKC–Ras–Raf–ERK kinase pathway in venticular myocytes (Singh *et al.,* 1996). The discrepancies could possibly relate to cell type and culture differences, as well as to differences in the stimuli used to elicit iNOS induction. Additionally, it is dangerous to rely soley on pharmacological inhibition experiments to assign direct causality in connecting particular MAP kinases to iNOS expression. Importantly, the MAP kinase cascades have been implicated in the activation of NF-κB (Briant *et al.,* 1998a,b; Ho *et al.,* 1997; F. S. Lee *et al.,* 1997; Meyer *et al.,* 1996; Nakano *et al.,* 1998; Schulze-Osthoff *et al.,* 1997; Yin *et al.,* 1998). Furthermore, MEKK1 protein is directly involved in the phosphorylation-induced activation of the IKK complex and subsequent degradation of IκB (F. S. Lee *et al.,* 1997; Nakano *et al.,* 1998; Yin *et al.,* 1998). Hence, modulation of NF-κB activation is likely to be an important mechanism in the regulation of iNOS induction by the MAP kinase cascades.

The Fos/Jun/ATF family of nuclear transcription factors play very important roles in the regulation of gene expression, and several of these proteins are involved in cell growth and cell cycle control (Foletta *et al.,* 1998). Indeed, the Fos and Jun proteins, which make up the heterodimeric transcription factor called AP-1, were originally identified as viral oncoproteins and are intimately involved in cell cycle regulation. Furthermore, the AP-1 proteins are very important targets of the MAP kinase cascades (Whitmarsh and Davis, 1996); hence the name JNK for the p38/Jun N-terminal kinase, which is also named the stress-activated protein kinase. Recent data suggest an important role for AP-1 in regulating iNOS expression. For instance, in response to the cytokine mixture, AP-1 DNA-binding activity was decreased while iNOS mRNA expression was induced in human DLD-1 cells. Additionally, pharmacological activators of AP-1 repressed iNOS expression in response to cytokines, implicating AP-1 as a repressor of cytokine-induced iNOS expression in DLD-1 cells. Furthermore, cotransfection of both Fos and Jun expression vectors repressed the cytokine-induced expression of a 7.2-kb human iNOS promoter–reporter construct (Kleinert *et al.,* 1998). Additional support for a negative effect of AP-1 on iNOS expression in the mouse macrophage is revealed by an increase in iNOS promoter-driven reporter expression in constructs that deleted a putative AP-1 sequence (Lowenstein *et al.,* 1993). In contrast, another study implicated AP-1-like proteins as positive effectors of iNOS transcription in human A549 lung epithelium cells. CM treatment of A549 cells increased the DNA-binding activity of Jun D and Fra-2. Additionally, AP-1 binding sequences were identified at −5301 and at −5115 bp in promoter–reporter mutagenesis experiments, and the sequences were important for cytokine-induced iNOS ex-

pression (Marks-Konczalik *et al.,* 1998). The basis for the opposite effects of AP-1 for iNOS expression is not clear but might relate to the differential expression of Fos/Jun/ATF family members in DLD-1 versus A549 cells.

The phospholipase C and protein kinase C pathway has been shown to have a dual role on iNOS expression. Studies have described either a positive (Bosca and Lazo, 1994; Diaz-Guerra *et al.,* 1996a,b; Hortelano *et al.,* 1992, 1993; Jun *et al.,* 1994; Welsh, 1996) or a negative (Fujihara *et al.,* 1994b; Hortelano *et al.,* 1992; Muhl and Pfeilschifter, 1994; Nakayama *et al.,* 1994) regulatory effect on iNOS expression. The basis for the opposite effects has not been described. However, different members of the PKC family are differentially expressed in cells and have been shown to function differentially (Fujihara *et al.,* 1994b; Muhl and Pfeilschifter, 1994). Importantly, agents that function through PKC include various lipids such as sphingomyelin, ceramide, diacylglycerol, or phorbol esters which activate PLC and hence PKC, but can also influence NF-κB activation to varying extents (Diaz-Guerra *et al.,* 1996a; Katsuyama *et al.,* 1998; Pahan *et al.,* 1998). Additionally, these same lipids and second messengers also effect the MAP kinase cascades in an ill-defined mechanism (Pahan *et al.,* 1998; Welsh, 1996; Whitmarsh and Davis, 1996). Complicating matters further is the differential expression and functions of the Fos/Jun/ATF (AP-1) family of transcription factors, which are regulated in part by PKA, PKC, and by MAP kinases. For reviews of the cAMP–PKA, PLC–PKC, MAP kinase cascades, and AP-1 family of transcription factors see Foletta *et al.* (1998), Mons *et al.* (1998), Montminy (1997), Toker (1998), Whitmarsh and Davis (1996), and Wilkinson and Nixon (1998).

Transcription Factor Interactions That Mediate iNOS Transcription

Many of the models for regulated gene transcription involve the ability of regulated transcription factors to bind to particular DNA sequences in the vicinity of the regulated target gene. Subsequently, the transcription factors recruit various general transcription factors to the promoter via protein–protein interactions. Important proteins that are recruited to promoters by regulatory transcription factors include the TATA box binding protein (TBP) and the TBP-associated factors (TAFs) in the general transcription factor complex named TFIID. TFIID is a limiting factor that facilitates the assembly of a functional transcription initiation complex containing the RNA polymerase II (for review, see Hampsey, 1998, and Hoffmann *et al.,* 1997). Another important and limiting target of regulatory DNA binding proteins is the coactivator CBP/p300, which is recruited to promoters and facilitates the organization of a permissive chromatin environment via recruitment of histone acetylase/deacetylase complexes (for review, see Hampsey, 1998, and Imhof *et al.,* 1997). Recruitment of general transcription factors and reorganization of nucleoprotein environments are important activities that transcription factors have evolved via protein–protein interactions with enzymatic machineries that are limiting and essential.

Importantly, NF-κB has been shown to interact directly and functionally with the TBP (Kerr *et al.,* 1993; Schmitz *et al.,* 1995; Xu *et al.,* 1993). Other transcription factors that are important in the regulation of iNOS expression that interact directly with TBP include AP-1 family members (Metz *et al.,* 1994; Ransone *et al.,* 1993) and HMG proteins (Ge and Roeder, 1994; Sutrias-Grau *et al.,* 1999). Additionally, NF-κB p65 protein can interact with TAFs, including TAFII105 (Yamit-Hezi and Dikstein, 1998). CREB proteins also functionally interact *in vitro* with general transcription factors TFIIB and TFIID, but not TBP, indicating that CREB interactions with TFIID require the TAFs (Xing *et al.,* 1995). Importantly, NF-κB p65, Stat1, IRF proteins, CREB, AP-1 proteins, and HMG-I can directly interact with p300/CBP, which functions in the reorganization of nucleosomal DNA as a histone acetyltransferase (Bisotto *et al.,* 1996; Gerritsen *et al.,* 1997; Horvai *et al.,* 1997; Kamei *et al.,* 1996; Lee *et al.,* 1998; Parry and Mackman, 1997; Swope *et al.,* 1996; J. J. Zhang *et al.,* 1996; Zhong *et al.,* 1998). These data suggest that activation of iNOS mRNA transcription is likely to involve multiple interactions with and hence recruitment of the general transcription machinery via TBP–TAF–TATA box associations, but also involves recruitment of chromatin remodeling machines that antagonize repressive chromatin structures.

The transcription of several other genes besides iNOS are regulated synergistically by the combination of a factor (TNFα, IL-1β, or dsRNA) and IFNγ, including ICA*M*−1, IP-10, and IRF-1 (Majumder *et al.,* 1998; Ohmori and Hamilton, 1995; Ohmori *et al.,* 1997), or involve the actions of several shared transcription factors, as in the regulation of IFNβ expression (Wathelet *et al.,* 1998). The transcription of iNOS, ICA*M*−1, IP-10, and IFNβ involves the coordinated activities of the NF-κB, Stat, and IRF families of transcription factors. Therefore, it is probable that the expression of these genes is subject to similar regulatory mechanisms. Direct protein–protein interactions between transcription factors have been shown to contribute to cooperativity in the combinatorial regulation of gene transcription by multiple signaling pathways. Protein–protein transcription factor interactions that are demonstrated to be functionally important include IRF-1–Stat (Darnell, 1998; Kamijo *et al.,* 1994; Martin *et al.,* 1994; Qureshi *et al.,* 1995; Stark *et al.,* 1998), IRF-1–NF-κB (Drew *et al.,* 1995; Neish *et al.,* 1995), IRF-1–HMG (Neish *et al.,* 1995), NF-κB–HMG (Lehming *et al.,* 1994; Lewis *et al.,* 1994; Perrella *et al.,* 1999; Thanos and Maniatis, 1992), and AP-1–HMG (Du and Maniatis, 1994). Many of these interactions have been described as components of a multiprotein complex named the ''enhanceosome.'' Enhanceosome functions are very important in the synergistic regulation of IFNβ transcription in response to dsRNA or cytokines. It seems surprising that the Stat proteins have not been described as an integral component of

the enhanceosome, since Stat proteins have been shown to functionally and physically interact with CBP/p300, IRF-1, AP-1, and HMG-I proteins. Furthermore, Stat6 has been shown to functionally interact with NF-κB proteins (Bennett *et al.,* 1997; Messner *et al.,* 1997), and a direct NF-κB–Stat6 protein–protein interaction has been identified (Shen and Stavnezer, 1998). Current biochemical and genetic experiments are suggesting direct functional interactions between the TNFα–NF-κB pathway and the IFNγ–Stat1 pathway in human A549 epithelial and in the 2fTGH fibroblast cell lines. The regulation of iNOS, ICAM-1, IP-10, and IFNβ transcription has been very valuable in the generation of information regarding mechanisms of cytokine-induced, transcriptional synergism.

In summary, significant progress has been achieved in understanding the signaling pathways, transcription factors, and promoter elements that regulate iNOS transcription. However, it is clear that the regulation of iNOS expression is very complex, and further research is required to decipher the precise signal transduction pathways and molecular mechanisms that are critical for regulated iNOS expression. Fundamental differences have been identified in the regulation of iNOS expression in different cell types and in the regulation of human versus murine iNOS gene expression. Figure 7 is a summary of the major signaling pathways and nuclear transcription factors that mediate induction of murine and human iNOS gene transcription. It should be noted that there are no reports of direct investigation into the mouse iNOS promoter with regulatory DNA that extends as far upstream as −16 kb, as is demonstrated for the human iNOS promoter. Nonetheless, one report describes an iNOS knockout mouse that deletes the ''proximal promoter'' region and eliminates exons 1 through 4; intriguingly, this mouse expresses a nonfunctional, truncated iNOS protein in proper developmental fashion in response to LPS administration *in vivo* (Loihl *et al.,* 1999). These data suggest that the mouse iNOS promoter may contain cytokine responsive elements that extend beyond what has currently been investigated, and it would be interesting to know whether the NF-κB elements that have been mapped in the human promoter from −5.2 to −6.1 kb exist in the mouse. It is also clear that the murine and human systems do share common signal transduction pathways in regulating iNOS expression, and although the differences observed are significant, these differences may reflect a subtle, quantitative difference and not necessarily a fundamental regulatory and evolutionary divergence.

Downregulators of iNOS Expression

In addition to defining the factors that upregulate iNOS gene expression, there has been great interest to define those factors that downregulate iNOS expression. Glucocorticoids are known to inhibit induced NO synthesis in several cell types (Geller *et al.,* 1993b; Knowles *et al.,* 1990), and this effect appears to be tissue and species specific. For example, Kleinert and co-workers suggested that dexametha-

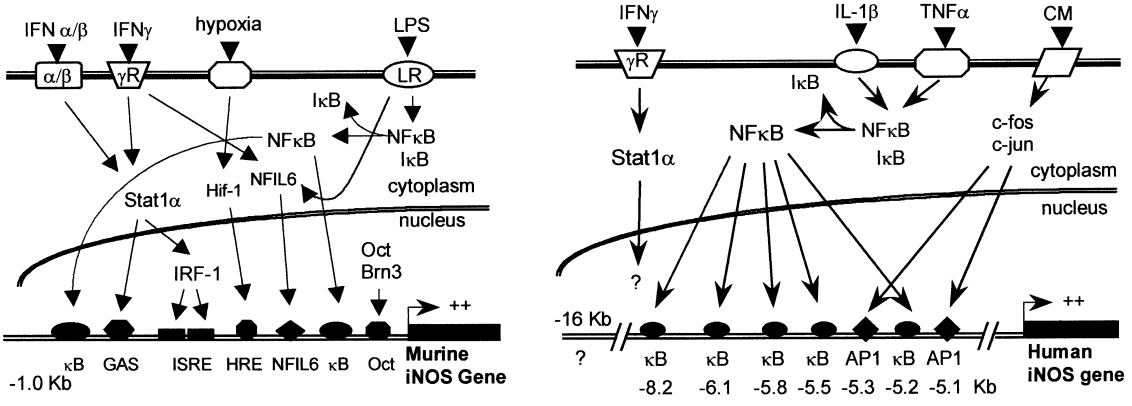


Figure 7 A generic summary and comparison of the major inducers, transcription factors, and promoter elements that mediate iNOS gene expression in human and murine cells. Notice that the cytokine responsive promoter elements that have been identified in the murine iNOS promoter are all located within −1 kb of 5' iNOS promoter DNA, whereas functional promoter elements in the human system have been localized between −5.1 and −8.2 kb of 5'-promoter DNA. In both systems, NF-κB elements are critical to the regulation of iNOS transcription. Functionally important elements in the response to interferons that bind to Stat1 homodimers (GAS) or Stat1–Stat2 heterodimers in association with IRF-1 proteins (ISRE) have been identified in the mouse promoter. A role for the interferon–Jak–Stat–IRF-1 pathway in humans cells is clear, but the promoter elements that mediate this regulation have yet to be described. The hypoxia response element (HRE) identified in the murine promoter is not conserved in the human iNOS promoter, and a role for hypoxia and Hif-1 protein in human iNOS transcription has not been described. Likewise, NF-IL6 (i.e., CAAT/EBP) and Brn3 proteins and promoter elements have been localized in the murine promoter, and it is not yet known whether these promoter elements participate in the regulation of human iNOS transcription. Conversely, a complex role for AP-1 proteins has been described for the human system, and functional AP-1 binding sites have been identified only in the human promoter. It should be recognized that the precise signal transduction pathways and molecular interactions by which the extracellular signals are conveyed to the nuclear transcription machinery are very active topics of many investigations.

sone inhibits cytokine-induced iNOS mRNA in human A549 epithelial cells by downregulating nuclear NF-κB DNA-binding activity without an increase in IκBα mRNA levels, and they suggested a glucocorticoid receptor–NFκB protein–protein interaction (Kleinert *et al.,* 1996a). Our group reported that dexamethasone decreased iNOS mRNA levels in rat hepatocytes and that this downregulation occurred at the level of transcription (de Vera *et al.,* 1997). This effect is a result of increased cytosolic IκBα levels and a concomitant decrease in nuclear p65 translocation in the presence of dexamethasone. In human colon carcinoma DLD-1 cells, Salzman reported that dexamethasone exhibited only a slight decrease in iNOS mRNA expression (Salzman *et al.,* 1996), and Kleinert found no inhibition of iNOS mRNA levels by dexamethasone in the same DLD-1 cells (Kleinert *et al.,* 1998). Perrella found that dexamethasone decreased iNOS mRNA levels in rat aortic vascular smooth muscle cells only when given before IL-1β and not after (Perrella *et al.,* 1994). In contrast, Kunz reported that dexamethasone did not alter IL-1β-induced iNOS mRNA in rat mesangial cells. Surprisingly, nuclear run-on experiments showed that dexamethasone markedly attenuated IL-1β-induced iNOS gene transcription, and this was counteracted by a prolongation in iNOS mRNA half-life from 1 to 2.5 hours (Kunz *et al.,* 1996). In RAW 264.7 cells, dexamethasone inhibited the IFNγ-induced expression of both iNOS mRNA and protein. The inhibition resulted from a reduced level of mRNA transcription, lessened mRNA stability, decreased mRNA translation, and an increase in calpain-mediated proteolysis of iNOS protein (Walker *et al.,* 1997). A possible explanation for the cell type-specific differences in dexamethasone-mediated repression of cytokine-induced iNOS transcription would invoke that the glucocorticoid receptor and NF-κB are in competition for limiting amounts of the general transcription coactivator CBP/p300 (Sheppard *et al.,* 1998). The observed differences could relate to various outcomes of the competition for CBP/p300, as well as to cell type differences in the regulation of iNOS mRNA half-life, translation, and protein stability. Despite extensive clinical use of glucocorticoids as immunosuppressive agents in patients with autoimmune diseases or after organ transplants, we have only begun to understand the mechanisms by which these drugs elicit their anti-inflammatory actions. Downregulation of iNOS expression during inflammation is one way by which steroids may exert their therapeutic effects.

A large variety of agents have been shown both to reduce cytokine-induced NF-κB activation and to inhibit cytokine-induced iNOS transcription. Important biological inhibitors of NF-κB activation and iNOS expression include cAMP-elevating agents (Mustafa and Olson, 1998; Pahan *et al.,* 1997; Parry and Mackman, 1997), steroids (Miller *et al.,* 1996), atrial natriuretic peptide (Kiemer and Vollmar, 1998), melatonin (Gilad *et al.,* 1998), the PPAR receptor (Ricote *et al.,* 1998), and IL-11 (Trepicchio *et al.,* 1997). Important environmental or pharmacological inhibitors of both NF-κB activation and iNOS expression include heat shock (de Vera *et al.,* 1996b; Feinstein *et al.,* 1996), reactive oxygen species scavengers (de Vera *et al.,* 1996b; Feinstein *et al.,* 1996; Liu *et al.,* 1997; Mulsch *et al.,* 1993; M. P. Sherman *et al.,* 1993), calcineurin inhihitors (Kunz *et al.,* 1995; Muhl *et al.,* 1993), aspirin (Farivar and Brecher, 1996), and cannabinoids (Jeon *et al.,* 1996). However, the manner in which the various agents inhibit the activation of NF-κB is unknown.

Transforming growth factor β (TGF-β) also partially prevents induced NO synthesis. Suppression of iNOS expression by TGF-β *in vitro* has been shown in macrophages (Ding *et al.,* 1990), mesangial cells (Pfeilschifter and Vosbeck, 1991), and cardiac myocytes (Roberts *et al.,* 1992). In rat aortic smooth muscle cells, TGF-β decreased IL-1β-stimulated iNOS mRNA by decreasing iNOS transcription (Perrella *et al.,* 1994) and promoter activity (Perrella *et al.,* 1996). On the basis of studies in primary murine macrophages, Vodovotz reported that this inhibition occurred at the posttranscriptional level and not by a direct effect on transcription: TGF-β suppressed macrophage iNOS expression by decreasing iNOS mRNA stability and translational efficiency, and by decreasing stability of iNOS protein (Vodovotz *et al.,* 1993). TGF-β inhibited induced NO synthesis in murine HC (77%), rat HC (17%), and human HC (10%), highlighting the significant variation between species (Nussler *et al.,* 1995). Interestingly, TGF-β actually enhances induction of iNOS mRNA in Swiss 3T3 fibroblasts (Gilbert and Herschman, 1993; Kleinert *et al.,* 1996b) and human keratinocytes (Arany *et al.,* 1996). Importantly, the expression of iNOS mRNA and protein are increased in the heart and kidney of the TGF-β null mouse, and retinal pigmented epithelium expresses higher levels of iNOS mRNA and protein in response to LPS plus IFNγ (Vodovotz *et al.,* 1996a,b). Furthermore, transgenic mice engineered to overexpress TGF-β from an albumin promoter display decreased serum nitrite/nitrate and decreased cytokine-induced iNOS mRNA and protein in peritoneal macrophages. Paradoxically, these same mice show increased iNOS mRNA and protein expression in the liver and kidney (Vodovotz *et al.,* 1998). These results underscore the complexity of iNOS regulation and lend support to the notion that species- and cell-specific mechanisms are likely to be important in controlling iNOS expression.

Similar to the feedback regulation of many cellular mediators, NO has been shown to exert a negative regulatory effect on iNOS gene expression (Colasanti *et al.,* 1995; Griscavage *et al.,* 1993; Park *et al.,* 1997; Peng *et al.,* 1995; Taylor *et al.,* 1997). Taylor demonstrated that addition of the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) markedly decreased steady-state iNOS mRNA and protein levels in rat and human hepatocytes. Nuclear run-on analysis revealed that SNAP inhibited CM-stimulated iNOS gene transcription by 80%, whereas gel shift assays of the nuclear extracts revealed that SNAP decreased CM-induced DNA-binding activity for NF-κB in a concentration-dependent manner. These studies identify a negative feedback loop whereby NO downregulates iNOS gene expression, possibly

to limit overproduction during pathophysiological conditions. Ignarro's group has shown that NO can also inhibit iNOS enzyme activity (Griscavage *et al.,* 1993), indicating another level of negative feedback regulation for posttranslational control of induced NO synthesis.

The tumor suppressor protein p53 encodes an important nuclear transcription factor that is induced in response to a variety of environmental and biological stressors, including exogenous mutagenic agents. Importantly, the p53 gene is mutated in up to 50% of all human cancers. Intriguingly, a variety of human cancer cell lines are shown to constitutively express high levels of iNOS, suggesting that p53 is a repressor of iNOS expression (Ambs *et al.,* 1997, 1998a). As NO has been shown to be mutagenic (Wink *et al.,* 1991), it was hypothesized that NO donors would induce p53 expression. Exposure of human cells to a NO donor resulted in nuclear p53 accumulation. Furthermore, overexpression of wild-type but not mutant p53 resulted in downregulation of iNOS transcription in a variety of human and rodent cells (Forrester *et al.,* 1996). These data imply a novel negative feedback loop where NO-induced DNA damage activates p53 expression which then *trans*-represses iNOS transcription, functioning as a safeguard against NO-induced DNA damage. As a follow-up to this *in vitro* study, the relationship between iNOS and p53 was examined *in vivo* in cancer-prone p53 knockout mice. Untreated p53 knockout mice excreted 70% more urinary nitrate than wild-type controls, and iNOS protein expression was constitutively detected only in the spleen of knockout mice and not in the spleens of wild-type mice. Following *C. parvum* injection, urinary nitrate excretion in the knockout mice exceeded that of the wild-type controls by 200% (Ambs *et al.,* 1998b). These data corroborate the *in vitro* findings and indicate that p53 is an important *trans*-repressor of basal and stimulated iNOS expression *in vivo.* The ability of p53 to repress iNOS transcription has been mapped in promoter–reporter experiments to the proximal iNOS promoter in the vicinity of the TATA box (Forrester *et al.,* 1996). Importantly, it has been reported that p53 can repress basal and activated transcription through direct physical interactions with the TATA box binding protein (TBP) (Farmer *et al.,* 1996; Liu *et al.,* 1993; Seto *et al.,* 1992; Thut *et al.,* 1997). Intriguingly, p53 can also compete against p65 NF-κB for limiting amounts of the general transcription coactivator, CBP/p300 (Ravi *et al.,* 1998).

Mechanisms involved in the ''activation'' of iNOS transcription may function in part by altering repressive chromatin structures as well as in the direct ''activation'' of transcription mediated by interactions with the general transcription machinery. Reorganization of chromatin during the expression of HIV has been shown to involve NF-κB sequences in the HIV long terminal repeat, and NF-κB-dependent chromatin reorganization has been described using *in vitro* (Pazin *et al.,* 1996; Steger and Workman, 1997; Widlak *et al.,* 1997) and *in vivo* footprinting technology (Demarchi *et al.,* 1996). The factors that function in the establishment of repressive chromatin structures have not been described, although it should be noted that HMG-I (Y) protein is an architectural component of chromatin and that many HMG proteins are repressive to basal transcription (Stelzer *et al.,* 1994). HMG proteins can either block or facilitate the DNA binding and/or the *trans*-activating potentials of other transcription factors including both NF-κB and ATF transcription factors (Du and Maniatis, 1994; Lehming *et al.,* 1994; Lewis *et al.,* 1994; Munshi *et al.,* 1998; Thanos and Maniatis, 1992). Recently, an AT-rich HMG-I (Y) DNA binding element has been localized at −61 to −54 in the mouse promoter just downstream of the −85 to −76 NF-κB sequence and overlapping with the Oct/Brn3A element that is important for iNOS promoter reporter activation by LPS and IL-1β. Furthermore, HMG-I (Y) and p50 NF-κB were observed in a ternary complex with DNA in gel shift assays. Overexpression of HMG-I has been shown to potentiate activation of the iNOS promoter by transfected p50 or p65 NF-κB expression plasmids (Perrella *et al.,* 1999). These data demonstrate functional cooperation between NF-κB and HMG-I (Y) for iNOS induction and highlight that transcription factors function in the context of an ill-defined nucleoprotein environment.

Many of the biological and pharmacological agents that exert a negative effect on iNOS expression have been shown to reduce the activation of either NF-κB, Stat1, or IRF-1 as described previously. However, a variety of other agents, including hormones (Miller *et al.,* 1996), growth factors (Goureau *et al.,* 1993; Heck *et al.,* 1992; Kunz *et al.,* 1997; Liu *et al.,* 1996; Schini *et al.,* 1994), and cytokines (Bogdan *et al.,* 1994; Cunha *et al.,* 1992; Sands *et al.,* 1994), can inhibit the expression of iNOS mRNA through mechanisms that have not been described. Recent data indicate the involvement of the phosphatidylinositol-3-kinase (PI-3-kinase) in the inhibitory regulation of iNOS by either IL-13 (Doyle *et al.,* 1994; Wright *et al.,* 1997) or macrophage stimulating protein (MSP) activation of the RON receptor tyrosine kinase (Chen *et al.,* 1998). However, the downstream target(s) of PI-3-kinase that mediates the negative regulation of iNOS expression is not defined. Moreover, other data indicate functional interactions between a variety of cell surface receptors including the IL-4 and IL-13 receptors and the tyrosine phosphatases SHP-1 and SHP-2, which also interact with and dephosphorylate the PI-3-kinase (Gesbert *et al.,* 1998; Imani *et al.,* 1997; Yu *et al.,* 1998). Other data indicate that IL-1, IGF-II, or hypoxia can activate NF-κB downstream of the PI-3-kinase (Kaliman *et al.,* 1999; Sands *et al.,* 1994; Sizemore *et al.,* 1999), possibly by phosphorylating and activating the *trans*-activation domain of NF-κB p65 (Sizemore *et al.,* 1999). Importantly, SHP-1 is also a negative regulator of NF-κB activation (Massa and Wu, 1998). These data suggest the possibility that IL-4, IL-10, and IL-13 may negatively regulate NF-κB activity by dephosphorylation of PI-3-kinase mediated by SHP-1 or SHP-2, serving to limit the *trans*-activation potential of NF-κB. Intriguingly, SHP-1 (Imani *et al.,* 1997; Ram and Waxman, 1997; You and Zhao, 1997; Yu *et al.,* 1998) or SHP-2 (Gesbert *et al.,* 1998; Servidei *et al.,* 1998; Yin *et al.,* 1997; You *et al.,* 1999)

are also negative regulators of the IFN–Jak–Stat pathway, suggesting that SHP proteins may negatively regulate the NF-κB pathway though the dephosphorylation of PI-3-kinase, and the IFN–Jak–Stat pathway by dephosphorylation of Jak and/or Stat proteins.

Posttranscriptional Regulation of iNOS Expression

In addition to transcriptional control, posttranscriptional regulation of the iNOS gene has been identified. As already mentioned, TGF-β suppresses macrophage iNOS expression by posttranscriptional mechanisms (Vodovotz *et al.,* 1993). Whereas dexamethasone decreases iNOS transcription, it has also been shown to exert posttranscriptional effects by increasing iNOS mRNA stability in IL-1β-stimulated rat mesangial cells and vascular smooth muscle cells (Kunz *et al.,* 1996; Perrella *et al.,* 1994) and to increase calpain-like proteolysis of iNOS protein in RAW 246.7 macrophages (Walker *et al.,* 1997). The precise mechanisms for these posttranscriptional effects have not been elucidated. The 3'-untranslated region (UTR) of the human and rodent iNOS cDNAs contains several AT-rich sequences that correspond to the AU sequences in the mRNA (Geller *et al.,* 1993a). These ATTTA sequence motifs have been shown in many labile cytokine and protooncogene transcripts and have been shown to destabilize mRNA (Caput *et al.,* 1986; Shaw and Kamen, 1986) and inhibit translational effiency (Han *et al.,* 1990). Nunokawa demonstrated a cooperative interaction between the 5'-promoter and the 3'-region of the human iNOS gene using luciferase promoter constructs containing both the iNOS 5'-flanking region and the 3'-UTR (Nunokawa *et al.,* 1997). Transfection of the constructs containing both the 5'- and 3'-regions resulted in a decrease in constitutive reporter gene activity and an increase in cytokine responsiveness.

Recently, Luss and co-workers cloned and characterized the expression of iNOS in human cardiac myocytes (Luss *et al.,* 1995). The human cardiac myocyte iNOS cDNA is >99% identical to the human hepatocyte iNOS cDNA that was previously cloned. Interestingly, although Luss *et al.* (1995) readily detected iNOS mRNA in cytokine-stimulated myocytes, they were unable to detect iNOS protein or NO synthesis. These data suggest that a translational defect exists for expression of iNOS in human cardiac myocytes, implying that cell-specific posttranscriptional mechanisms may be operating here. Whether this observation reflects rapid degradation of iNOS mRNA or a block in translational machinery has not been established.

Chu and colleagues have identified structural diversity in the 5'-UTR of the human iNOS gene. Despite the presence of a TATA box in the promoter, multiple transcription initiation sites were observed in ~6% of iNOS mRNAs expressed in cytokine-stimulated human A549 lung epithelial cells (Chu *et al.,* 1995). The TATA-independent iNOS mRNAs were also upregulated by cytokines. Alternative splicing in the 5'-UTR also accounted for further structural diversity owing to the presence of eight partially overlapping open reading frames (ORFs) upstream from the major iNOS ATG. These ORFs may or may not have an important role in the translational regulation of the human iNOS mRNA.

In addition to alternative splicing in the 5'-UTR, Eissa *et al.* (1996) identified alternative mRNA splicing within the human iNOS mRNA. Four alternative mRNA transcripts were seen with internal deletions of exon 5, exons 8 and 9, exons 9–11, and exons 15 and 16. The deletion of exon 5 resulted in a truncated protein, whereas the other three variants produced in-frame deletions. Interestingly, expression of the exon $8^{-}9^{-}$ iNOS cDNA in a human cell line produced iNOS protein that lacked functional NOS activity and did not result in production of NO (Eissa *et al.,* 1998). The molecular basis for this observation was the lack of iNOS protein dimerization as a result of the deleted exons, indicating a critical role of the exon 8–9 region for iNOS protein dimerization.

References

Adamson, D. C., Wildemann, B., Sasaki, M., Glass, J. D., McArthur, J. C., Christov, V. I., Dawson, T. M., and Dawson, V. L. (1996). Immunologic NO synthase: Elevation in severe AIDS dementia and induction by HIV-1 gp41. *Science* **274,** 1917–1921.

Adcock, I. M., Brown, C. R., Kwon, O., and Barnes, P. J. (1994). Oxidative stress induces NF κ B DNA binding and inducible NOS mRNA in human epithelial cells. *Biochem. Biophys. Res. Commun.* **199,** 1518–1524.

Akama, K. T., Albanese, C., Pestell, R. G., and Van Eldik, L. J. (1998). Amyloid β-peptide stimulates nitric oxide production in astrocytes through an NFκB-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5795–5800.

Alley, E. W., Murphy, W. J., and Russell, S. W. (1995). A classical enhancer element responsive to both lipopolysaccharide and interferon-γ augments induction of the iNOS gene in mouse macrophages. *Gene* **158,** 247–251.

Ambs, S., Hussain, S. P., and Harris, C. C. (1997). Interactive effects of nitric oxide and the p53 tumor suppressor gene in carcinogenesis and tumor progression. *FASEB J.* **11,** 443–448.

Ambs, S., Merriam, W. G., Bennett, W. P., Felley-Bosco, E., Ogunfusika, M. O., Oser, S. M., Klein, S., Shields, P. G., Billiar, T. R., and Harris, C. C. (1998a). Frequent nitric oxide synthase-2 expression in human colon adenomas: Implication for tumor angiogenesis and colon cancer progression. *Cancer Res.* **58,** 334–341.

Ambs, S., Ogunfusika, M. O., Merriam, W. G., Bennett, W. P., Billiar, T. R., and Harris, C. C. (1998b). Up-regulation of inducible nitric oxide synthase expression in cancer-prone p53 knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8823–8828.

Amoah-Apraku, B., Chandler, L. J., Harrison, J. K., Tang, S. S., Ingelfinger, J. R., and Guzman, N. J. (1995). NF-κ B and transcriptional control of renal epithelial-inducible nitric oxide synthase. *Kidney Int.* **48,** 674–682.

Anstey, N. M., Weinberg, J. B., Hassanali, M. Y., Mwaikambo, E. D., Manyenga, D., Misukonis, M. A., Arnelle, D. R., Hollis, D., McDonald, M. I., and Granger, D. L. (1996). Nitric oxide in Tanzanian children with malaria: Inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J. Exp. Med.* **184,** 557–567.

Arany, I., Brysk, M. M., Brysk, H., and Tyring, S. K. (1996). Regulation of inducible nitric oxide synthase mRNA levels by differentiation and cytokines in human keratinocytes. *Biochem. Biophys. Res. Commun.* **220,** 618–622.

Baldwin, A. S. (1996). NFkB review. *Annu. Rev. Immunol.* **14,** 649–681.

Bayon, Y., Alonso, A., and Sanchez Crespo, M. (1997). Stimulation of Fc γ receptors in rat peritoneal macrophages induces the expression of nitric oxide synthase and chemokines by mechanisms showing different sensitivities to antioxidants and nitric oxide donors. *J. Immunol.* **159,** 887–894.

Bellocq, A., Suberville, S., Philippe, C., Bertrand, F., Perez, J., Fouqueray, B., Cherqui, G., and Baud, L. (1998). Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages. Evidence for involvement of nuclear factor-κB activation. *J. Biol. Chem.* **273,** 5086–5092.

Bennett, B. L., Cruz, R., Lacson, R. G., and Manning, A. M. (1997). Interleukin-4 suppression of tumor necrosis factor α-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-κB. *J. Biol. Chem.* **272,** 10212–10219.

Bertholet, S., Tzeng, E., Felley-Bosco, E., and Mauel, J. (1999). Expression of the inducible NO synthase in human monocytic U937 cells allows high output nitric oxide production. *J. Leukocyte Biol.* **65,** 50–58.

Bidri, M., Ktorza, S., Vouldoukis, I., Le Goff, L., Debre, P., Guillosson, J. J., and Arock, M. (1997). Nitric oxide pathway is induced by Fc ϵ RI and up-regulated by stem cell factor in mouse mast cells. *Eur. J. Immunol.* **27,** 2907–2913.

Bisotto, S., Minorgan, S., and Rehfuss, R. P. (1996). Identification and characterization of a novel transcriptional activation domain in the CREB-binding protein. *J. Biol. Chem.* **271,** 17746–17750.

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q. W., and Nathan, C. (1994). Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukocyte Biol.* **55,** 227–233.

Bonaiuto, C., McDonald, P. P., Rossi, F., and Cassatella, M. A. (1997). Activation of nuclear factor-κ B by β-amyloid peptides and interferon-γ in murine microglia. *J. Neuroimmunol.* **77,** 51–56.

Bonham, C. A., Lu, L., Hoffman, R. A., Simmons, R. L., and Thomson, A. W. (1997). Generation of nitric oxide by mouse dendritic cells and its implications for immune response regulation. *Adv. Exp. Med. Biol.* **417,** 283–290.

Bosca, L., and Lazo, P. A. (1994). Induction of nitric oxide release by MRC OX-44 (anti-CD53) through a protein kinase C-dependent pathway in rat macrophages. *J. Exp. Med.* **179,** 1119–1126.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351,** 714–718.

Briant, L., Roberthebmann, V., Acquaviva, C., Pelchenmatthews, A., Marsh, M., and Devaux, C. (1998a). The protein tyrosine kinase p56(Lck) is required for triggering Nf-κ-B activation upon interaction of human immunodeficiency virus type 1 envelope glycoprotein gp120 with cell surface Cd4. *J. Virol.* **72,** 6207–6214.

Briant, L., Roberthebmann, V., Sivan, V., Brunet, A., Pouyssegur, J., and Devaux, C. (1998b). Involvement of extracellular signal-regulated kinase module in hiv-mediated Cd4 signals controlling activation of nuclear factor-κ-B and ap-1 transcription factors. *J. Immunol.* **160,** 1875–1885.

Burgner, D., Xu, W., Rockett, K., Gravenor, M., Charles, I. G., Hill, A. V., and Kwiatkowski, D. (1998). Inducible nitric oxide synthase polymorphism and fatal cerebral malaria. *Lancet* **352,** 1193–1194.

Busse, R., and Mulsch, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.* **275,** 87–90.

Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1986). Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 1670–1674.

Chan, E. D., Winston, B. W., Uh, S. T., Wynes, M. W., Rose, D. M., and Riches, D. W. H. (1999). Evaluation of the role of mitogen-activated protein kinases in the expression of inducible nitric oxide synthase by IFN-γ and TNF-α in mouse macrophages. *J. Immunol.* **162,** 415–422.

Charles, I. G., Palmer, R. M., Hickery, M. S., Bayliss, M. T., Chubb, A. P., Hall, V. S., Moss, D. W., and Moncada, S. (1993). Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11419–11423.

Chartrain, N. A., Geller, D. A., Koty, P. P., Sitrin, N. F., Nussler, A. K., Hoffman, E. P., Billiar, T. R., Hutchinson, N. I., and Mudgett, J. S. (1994). Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* **269,** 6765–6772.

Chen, Y. Q., Fisher, J. H., and Wang, M. H. (1998). Activation of the RON receptor tyrosine kinase inhibits inducible nitric oxide synthase (iNOS) expression by murine peritoneal exudate macrophages: Phosphatidylinositol-3 kinase is required for RON-mediated inhibition of iNOS expression. *J. Immunol.* **161,** 4950–4959.

Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* **176,** 599–604.

Chu, S. C., Wu, H. P., Banks, T. C., Eissa, N. T., and Moss, J. (1995). Structural diversity in the 5'-untranslated region of cytokine-stimulated human inducible nitric oxide synthase mRNA. *J. Biol. Chem.* **270,** 10625–10630.

Colasanti, M., Persichini, T., Menegazzi, M., Mariotto, S., Giordano, E., Caldarera, C. M., Sogos, V., Lauro, G. M., and Suzuki, H. (1995). Induction of nitric oxide synthase mRNA expression. Suppression by exogenous nitric oxide. *J. Biol. Chem.* **270,** 26731–26733.

Corbett, J. A., and McDaniel, M. L. (1992). Does nitric oxide mediate autoimmune destruction of β-cells? Possible therapeutic interventions in IDDM. *Diabetes* **41,** 897–903.

Cox, G. W., Melillo, G., Chattopadhyay, U., Mullet, D., Fertel, R. H., and Varesio, L. (1992). Tumor necrosis factor-α-dependent production of reactive nitrogen intermediates mediates IFN-γ plus IL-2-induced murine macrophage tumoricidal activity. *J. Immunol.* **149,** 3290–3296.

Croen, K. D. (1993). Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J. Clin. Invest.* **91,** 2446–2452.

Cunha, F. Q., Moncada, S., and Liew, F. Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. *Biochem. Biophys. Res. Commun.* **182,** 1155–1159.

Da Silva, J., Pierrat, B., Mary, J. L., and Lesslauer, W. (1997). Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes. *J. Biol. Chem.* **272,** 28373–28380.

Darnell, J. E., Jr. (1998). Studies of IFN-induced transcriptional activation uncover the Jak–Stat pathway. *J. Interferon Cytokine Res.* **18,** 549–554.

de Vera, M. E., Shapiro, R. A., Nussler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1996a). Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1054–1059.

de Vera, M. E., Wong, J. M., Zhou, J. Y., Tzeng, E., Wong, H. R., Billiar, T. R., and Geller, D. A. (1996b). Cytokine-induced nitric oxide synthase gene transcription is blocked by the heat shock response in human liver cells. *Surgery* **120,** 144–149.

de Vera, M. E., Taylor, B. S., Wang, Q., Shapiro, R. A., Billiar, T. R., and Geller, D. A. (1997). Dexamethasone suppresses iNOS gene expression by upregulating I-κ B α and inhibiting NF-κ B. *Am. J. Physiol.* **273,** G1290–G1296.

Demarchi, F., Bovenzi, P., Di Luca, D., and Giacca, M. (1996). Transcriptional activation of human immunodeficiency virus type 1 by herpesvirus infection: An in vivo footprinting study. *Intervirology* **39,** 236–241.

Deng, W., Thiel, B., Tannenbaum, C. S., Hamilton, T. A., and Stuehr, D. J. (1993). Synergistic cooperation between T cell lymphokines for induction of the nitric oxide synthase gene in murine peritoneal macrophages. *J. Immunol.* **151,** 322–329.

Denlinger, L. C., Garis, K. A., Sommer, J. A., Guadarrama, A. G., Proctor, R. A., and Bertics, P. J. (1998). Nuclear translocation of NF-κB in lipopolysaccharide-treated macrophages fails to correspond to endotoxicity: Evidence suggesting a requirement for a γ interferon-like signal. *Infect. Immun.* **66,** 1638–1647.

Diaz-Guerra, M. J. M., Velasco, M., Martin-Sanz, P., and Bosca, L. (1996a). Evidence for common mechanisms in the transcriptional control of type II nitric oxide synthase in isolated hepatocytes. Requirement of NF-κB activation after stimulation with bacterial cell wall products and phorbol esters. *J. Biol. Chem.* **271,** 30114–30120.

Diaz-Guerra, M. J. M., Bodelon, O. G., Velasco, M., Whelan, R., Parker, P. J., and Bosca, L. (1996b). Up-regulation of protein kinase C-ϵ promotes the expression of cytokine-inducible nitric oxide synthase in RAW 264.7 cells. *J. Biol. Chem.* **271,** 32028–32033.

Ding, A., Nathan, C. F., Graycar, J., Derynck, R., Stuehr, D. J., and Srimal, S. (1990). Macrophage deactivating factor and transforming growth factors-β 1 -β 2 and -β 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. *J. Immunol.* **145,** 940–944.

Doyle, A. G., Herbein, G., Montaner, L. J., Minty, A. J., Caput, D., Ferrara, P., and Gordon, S. (1994). Interleukin-13 alters the activation state of murine macrophages in vitro: Comparison with interleukin-4 and interferon-γ. *Eur. J. Immunol.* **24,** 1441–1445.

Drew, P. D., Franzoso, G., Becker, K. G., Bours, V., Carlson, L. M., Siebenlist, U., and Ozato, K. (1995). NF κ B and interferon regulatory factor 1 physically interact and synergistically induce major histocompatibility class I gene expression. *J. Interferon Cytokine Res.* **15,** 1037–1045.

Du, W., and Maniatis, T. (1994). The high mobility group protein HMG I(Y) can stimulate or inhibit DNA binding of distinct transcription factor ATF-2 isoforms. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11318–11322.

Duval, D. L., Miller, D. R., Collier, J., and Billings, R. E. (1996). Characterization of hepatic nitric oxide synthase: Identification as the cytokine-inducible form primarily regulated by oxidants. *Mol. Pharmacol.* **50,** 277–284.

Eberhardt, W., Pluss, C., Hummel, R., and Pfeilschifter, J. (1998). Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J. Immunol.* **160,** 4961–4969.

Eissa, N. T., Strauss, A. J., Haggerty, C. M., Choo, E. K., Chu, S. C., and Moss, J. (1996). Alternative splicing of human inducible nitric-oxide synthase mRNA. Tissue-specific regulation and induction by cytokines. *J. Biol. Chem.* **271,** 27184–27187.

Eissa, N. T., Yuan, J. W., Haggerty, C. M., Choo, E. K., Palmer, C. D., and Moss, J. (1998). Cloning and characterization of human inducible nitric oxide synthase splice variants: A domain, encoded by exons 8 and 9, is critical for dimerization. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7625–7630.

Eizirik, D. L., Sandler, S., Welsh, N., Cetkovic-Cvrlje, M., Nieman, A., Geller, D. A., Pipeleers, D. G., Bendtzen, K., and Hellerstrom, C. (1994). Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *J. Clin. Invest.* **93,** 1968–1974.

Farivar, R. S., and Brecher, P. (1996). Salicylate is a transcriptional inhibitor of the inducible nitric oxide synthase in cultured cardiac fibroblasts. *J. Biol. Chem.* **271,** 31585–31592.

Farmer, G., Friedlander, P., Colgan, J., Manley, J. L., and Prives, C. (1996). Transcriptional repression by p53 involves molecular interactions distinct from those with the TATA box binding protein. *Nucleic Acids Res.* **24,** 4281–4288.

Feinstein, D. L., Galea, E., Aquino, D. A., Li, G. C., Xu, H., and Reis, D. J. (1996). Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFκB activation. *J. Biol. Chem.* **271,** 17724–17732.

Finder, J. D., Litz, J. L., Blaskovich, M. A., McGuire, T. F., Qian, Y., Hamilton, A. D., Davies, P., and Sebti, S. M. (1997). Inhibition of protein geranylgeranylation causes a superinduction of nitric-oxide synthase-2 by interleukin-1β in vascular smooth muscle cells. *J. Biol. Chem.* **272,** 13484–13488.

Foletta, V. C., Segal, D. H., and Cohen, D. R. (1998). Transcriptional regulation in the immune system: All roads lead to AP-1. *J. Leukocyte Biol.* **63,** 139–152.

Forrester, K., Ambs, S., Lupold, S. E., Kapust, R. B., Spillare, E. A., Weinberg, W. C., Felley-Bosco, E., Wang, X. W., Geller, D. A., Tzeng, E., Billiar, T. R., and Harris, C. C. (1996). Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2442–2447.

Förstermann, U., Boissel, J. P., and Kleinert, H. (1998). Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J.* **12,** 773–790.

Fujihara, M., Ito, N., Pace, J. L., Watanabe, Y., Russell, S. W., and Suzuki, T. (1994a). Role of endogenous interferon-β in lipopolysaccharide-triggered activation of the inducible nitric-oxide synthase gene in a mouse macrophage cell line, J774. *J. Biol. Chem.* **269,** 12773–12778.

Fujihara, M., Connolly, N., Ito, N., and Suzuki, T. (1994b). Properties of protein kinase C isoforms (β II, ϵ, and ζ) in a macrophage cell line (J774) and their roles in LPS-induced nitric oxide production. *J. Immunol.* **152,** 1898–1906.

Fujimura, M., Tominaga, T., Kato, I., Takasawa, S., Kawase, M., Taniguchi, T., Okamoto, H., and Yoshimoto, T. (1997). Attenuation of nitric oxide synthase induction in IRF-1-deficient glial cells. *Brain Res.* **759,** 247–250.

Ganster, R. W., and Geller, D. A. (2000). In press.

Gao, J., Morrison, D. C., Parmely, T. J., Russell, S. W., and Murphy, W. J. (1997). An interferon-γ-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-γ and lipopolysaccharide. *J. Biol. Chem.* **272,** 1226–1230.

Gao, J. J., Filla, M. B., Fultz, M. J., Vogel, S. N., Russell, S. W., and Murphy, W. J. (1998). Autocrine/paracrine IFN-αβ mediates the lipopolysaccharide-induced activation of transcription factor Stat1α in mouse macrophages: Pivotal role of Stat1α in induction of the inducible nitric oxide synthase gene. *J. Immunol.* **161,** 4803–4810.

Gay, R. D., Dawson, S. J., Murphy, W. J., Russell, S. W., and Latchman, D. S. (1998). Activation of the iNOS gene promoter by Brn-3 POU family transcription factors is dependent upon the octamer motif in the promoter. *Biochim. Biophys. Acta* **1443,** 315–322.

Ge, H., and Roeder, R. G. (1994). The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein. *J. Biol. Chem.* **269,** 17136–17140.

Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Billiar, T. R. (1993a). Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3491–3495.

Geller, D. A., Nussler, A. K., Di Silvio, M., Lowenstein, C. J., Shapiro, R. A., Wang, S. C., Simmons, R. L., and Billiar, T. R. (1993b). Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 522–526.

Geller, D. A., DeVera, M. E., Russell, D., Shapiro, R. A., Nussler, A. K., Simmons, R. L., and Billiar, T. R. (1995). A central role for interleukin-1-β in the *in vitro* and *in vivo* regulation of hepatic inducible nitric oxide synthase. *J. Immunol.* **155,** 4890–4898.

Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2927–2932.

Gesbert, F., Guenzi, C., and Bertoglio, J. (1998). A new tyrosine-phosphorylated 97-kDa adaptor protein mediates interleukin-2-induced association of SHP-2 with p85-phosphatidylinositol 3-kinase in human T lymphocytes. *J. Biol. Chem.* **273,** 18273–18281.

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-κ B and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–260.

Gilad, E., Wong, H. R., Zingarelli, B., Virag, L., O'Connor, M., Salzman, A. L., and Szabo, C. (1998). Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: Role of inhibition of NFκB activation. *FASEB J.* **12,** 685–693.

Gilbert, R. S., and Herschman, H. R. (1993). Transforming growth factor β differentially modulates the inducible nitric oxide synthase gene in distinct cell types. *Biochem. Biophys. Res. Commun.* **195,** 380–384.

Goldring, C. E., Reveneau, S., Algarte, M., and Jeannin, J. F. (1996). In vivo footprinting of the mouse inducible nitric oxide synthase gene: Inducible protein occupation of numerous sites including Oct and NF-IL6. *Nucleic Acids Res.* **24,** 1682–1687.

Goldring, C. E., Reveneau, S., Pinard, D., and Jeannin, J. F. (1998). Hyporesponsiveness to lipopolysaccharide alters the composition of NF-κB binding to the regulatory regions of inducible nitric oxide synthase gene. *Eur. J. Immunol.* **28,** 2960–2970.

Goureau, O., Lepoivre, M., Becquet, F., and Courtois, Y. (1993). Differential regulation of inducible nitric oxide synthase by fibroblast growth factors and transforming growth factor β in bovine retinal pigmented epithelial cells: Inverse correlation with cellular proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 4276–4280.

Grigoriadis, G., Zhan, Y., Grumont, R. J., Metcalf, D., Handman, E., Cheers, C., and Gerondakis, S. (1996). The Rel subunit of NF-κB-like transcription factors is a positive and negative regulator of macrophage gene expression: Distinct roles for Rel in different macrophage populations. *EMBO J.* **15,** 7099–7107.

Griscavage, J. M., Rogers, N. E., Sherman, M. P., and Ignarro, L. J. (1993). Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. *J. Immunol.* **151,** 6329–6337.

Grohmann, U., Belladonna, M. L., Bianchi, R., Orabona, C., Ayroldi, E., Fioretti, M. C., and Puccetti, P. (1998). Il-12 acts directly on Dc to promote nuclear localization of NF-κ-B and primes Dc for Il-12 production. *Immunity* **9,** 315–323.

Guo, D., Dunbar, J. D., Yang, C. H., Pfeffer, L. M., and Donner, D. B. (1998). Induction of Jak/STAT signaling by activation of the type 1 TNF receptor. *J. Immunol.* **160,** 2742–2750.

Guo, F. H., De Raeve, H. R., Rice, T. W., Stuehr, D. J., Thunnissen, F. B., and Erzurum, S. C. (1995). Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7809–7813.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. *Microbiol. Mol. Biol. Rev.* **62,** 465–503.

Han, J., Brown, T., and Beutler, B. (1990). Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level [published erratum appears in *J. Exp. Med.* (1990) Mar. 1; **171**(3): 971–972]. *J. Exp. Med.* **171,** 465–475.

Harbrecht, B. G., Billiar, T. R., Stadler, J., Demetris, A. J., Ochoa, J., Curran, R. D., and Simmons, R. L. (1992). Inhibition of nitric oxide synthesis during endotoxemia promotes intrahepatic thrombosis and an oxygen radical-mediated hepatic injury. *J. Leukocyte Biol.* **52,** 390–394.

Harbrecht, B. G., Kim, Y. M., Wirant, E. A., Simmons, R. L., and Billiar, T. R. (1997). Timing of prostaglandin exposure is critical for the inhibition of LPS- or IFN-γ-induced macrophage NO synthesis by PGE2. *J. Leukocyte Biol.* **61,** 712–720.

Heck, D. E., Laskin, D. L., Gardner, C. R., and Laskin, J. D. (1992). Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes. Potential role for nitric oxide in the regulation of wound healing. *J. Biol. Chem.* **267,** 21277–21280.

Heitmeier, M. R., Scarim, A. L., and Corbett, J. A. (1998). Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF-κB activation. *J. Biol. Chem.* **273,** 15301–15307.

Hibbs, J. B., Jr., Westenfelder, C., Taintor, R., Vavrin, Z., Kablitz, C., Baranowski, R. L., Ward, J. H., Menlove, R. L., McMurry, M. P., Kushner, J. P., and *et al.* (1992). Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy [published erratum appears in *J. Clin. Invest.* (1992) Jul.; **90**(1): 295]. *J. Clin. Invest.* **89,** 867–877.

Hickey, M. J., and Kubes, P. (1997). Role of nitric oxide in regulation of leucocyte–endothelial cell interactions. *Exp. Physiol.* **82,** 339–348.

Ho, H. Y., Lee, H. H., and Lai, M. Z. (1997). Overexpression of mitogen-activated protein kinase kinase kinase reversed cAMP inhibition of NF-κB in T cells. *Eur. J. Immunol.* **27,** 222–226.

Hoffman, R. A., Langrehr, J. M., Billiar, T. R., Curran, R. D., and Simmons, R. L. (1990). Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J. Immunol.* **145,** 2220–2226.

Hoffman, R. A., Zhang, G., Nussler, N. C., Gleixner, S. L., Ford, H. R., Simmons, R. L., and Watkins, S. C. (1997). Constitutive expression of inducible nitric oxide synthase in the mouse ileal mucosa. *Am. J. Physiol.* **272,** G383–G392.

Hoffmann, A., Oelgeschlager, T., and Roeder, R. G. (1997). Considerations of transcriptional control mechanisms: Do TFIID–core promoter complexes recapitulate nucleosome-like functions? *Proc. Natl. Acad. Sci. U.S.A.* **94,** 8928–8935.

Hoffmann, G., Schobersberger, W., Frede, S., Pelzer, L., Fandrey, J., Wachter, H., Fuchs, D., and Grote, J. (1996). Neopterin activates transcription factor nuclear factor-κ B in vascular smooth muscle cells. *FEBS Lett.* **391,** 181–184.

Hokari, A., Zeniya, M., and Esumi, H. (1994). Cloning and functional expression of human inducible nitric oxide synthase (NOS) cDNA from a glioblastoma cell line A-172. *J. Biochem. (Tokyo)* **116,** 575–581.

Hortelano, S., Genaro, A. M., and Bosca, L. (1992). Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. Antagonism with the induction elicited by lipopolysaccharide. *J. Biol. Chem.* **267,** 24937–24940.

Hortelano, S., Genaro, A. M., and Bosca, L. (1993). Phorbol esters induce nitric oxide synthase and increase arginine influx in cultured peritoneal macrophages. *FEBS Lett.* **320,** 135–139.

Horvai, A. E., Xu, L., Korzus, E., Brard, G., Kalafus, D., Mullen, T. M., Rose, D. W., Rosenfeld, M. G., and Glass, C. K. (1997). Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 1074–1079.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Imai, T., Hirata, Y., Kanno, K., and Marumo, F. (1994). Induction of nitric oxide synthase by cyclic AMP in rat vascular smooth muscle cells. *J. Clin. Invest.* **93,** 543–549.

Imani, F., Rager, K. J., Catipovic, B., and Marsh, D. G. (1997). Interleukin-4 (IL-4) induces phosphatidylinositol 3-kinase (p85) dephosphorylation. Implications for the role of SHP-1 in the IL-4-induced signals in human B cells. *J. Biol. Chem.* **272,** 7927–7931.

Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., and Ge, H. (1997). Acetylation of general transcription factors by histone acetyltransferases. *Curr. Biol.* **7,** 689–692.

Ivashkiv, L. B., Schmitt, E. M., and Castro, A. (1996). Inhibition of transcription factor Stat1 activity in mononuclear cell cultures and T cells by the cyclic AMP signaling pathway. *J. Immunol.* **157,** 1415–1421.

Iwasaki, T., Uehara, Y., Graves, L., Rachie, N., and Bomsztyk, K. (1992). Herbimycin A blocks IL-1-induced NF-κ B DNA-binding activity in lymphoid cell lines. *FEBS Lett.* **298,** 240–244.

Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B., and Bloch, K. D. (1992). Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase [published erratum appears in *J. Biol. Chem.* (1992) Nov. 5; **267**(31): 22694]. *J. Biol. Chem.* **267,** 14519–14522.

Jeon, Y. J., Yang, K. H., Pulaski, J. T., and Kaminski, N. E. (1996). Attenuation of inducible nitric oxide synthase gene expression by Δ^9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor- κ B/Rel activation. *Mol. Pharmacol.* **50,** 334–341.

Jobin, C., Panja, A., Hellerbrand, C., Iimuro, Y., Didonato, J., Brenner, D. A., and Sartor, R. B. (1998). Inhibition of proinflammatory molecule production by adenovirus-mediated expression of a nuclear factor κB super-repressor in human intestinal epithelial cells. *J. Immunol.* **160,** 410–418.

Jun, C. D., Choi, B. M., Hoon, R., Um, J. Y., Kwak, H. J., Lee, B. S., Paik, S. G., Kim, H. M., and Chung, H. T. (1994). Synergistic cooperation between phorbol ester and IFN-γ for induction of nitric oxide synthesis in murine peritoneal macrophages. *J. Immunol.* **153,** 3684–3690.

Kaliman, P., Canicio, J., Testar, X., Palacin, M., and Zorzano, A. (1999). Insulin-like growth factor-II, phosphatidylinositol 3-kinase, nuclear factor-κB and inducible nitric-oxide synthase define a common myogenic signaling pathway [In Process Citation]. *J. Biol. Chem.* **274,** 17437–17444.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85,** 403–414.

Kamijo, R., Shapiro, D., Le, J., Huang, S., Aguet, M., and Vilcek, J. (1993). Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon γ receptor. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6626–6630.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S. I., Kimura, T., Green, S. J., and *et al.* (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* **263,** 1612–1615.

Karupiah, G., Xie, Q. W., Buller, R. M., Nathan, C., Duarte, C., and MacMicking, J. D. (1993). Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science* **261,** 1445–1448.

Katsuyama, K., Shichiri, M., Marumo, F., and Hirata, Y. (1998). Role of nuclear factor-κB activation in cytokine- and sphingomyelinase-stimulated inducible nitric oxide synthase gene expression in vascular smooth muscle cells. *Endocrinology* **139,** 4506–4512.

Kerr, L. D., Ransone, L. J., Wamsley, P., Schmitt, M. J., Boyer, T. G., Zhou, Q., Berk, A. J., and Verma, I. M. (1993). Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-κ B. *Nature* **365,** 412–419.

Kiemer, A. K., and Vollmar, A. M. (1998). Autocrine regulation of inducible nitric-oxide synthase in macrophages by atrial natriuretic peptide. *J. Biol. Chem.* **273,** 13444–13451.

Kishimoto, J., Spurr, N., Liao, M., Lizhi, L., Emson, P., and Xu, W. (1992). Localization of brain nitric oxide synthase (NOS) to human chromosome 12 [published erratum appears in *Genomics* (1993) Feb.; **15**(2): 465]. *Genomics* **14,** 802–804.

Kleinert, H., Euchenhofer, C., Ihrig-Biedert, I., and Förstermann, U. (1996a). Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κ B. *Mol. Pharmacol.* **49,** 15–21.

Kleinert, H., Euchenhofer, C., Ihrig-Biedert, I., and Förstermann, U. (1996b). In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF-κB. *J. Biol. Chem.* **271,** 6039–6044.

Kleinert, H., Wallerath, T., Fritz, G., Ihrig-Biedert, I., Rodriguez-Pascual, F., Geller, D. A., and Förstermann, U. (1998). Cytokine induction of NO synthase II in human DLD-1 cells: Roles of the JAK-STAT, AP-1 and NF-κB-signaling pathways. *Br. J. Pharmacol.* **125,** 193–201.

Knowles, R. G., Salter, M., Brooks, S. L., and Moncada, S. (1990). Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem. Biophys. Res. Commun.* **172,** 1042–1048.

Koide, M., Kawahara, Y., Nakayama, I., Tsuda, T., and Yokoyama, M. (1993). Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells. Synergism with the induction elicited by inflammatory cytokines. *J. Biol. Chem.* **268,** 24959–24966.

Kolyada, A. Y., Savikovsky, N., and Madias, N. E. (1996). Transcriptional regulation of the human iNOS gene in vascular-smooth-muscle cells and macrophages: Evidence for tissue specificity. *Biochem. Biophys. Res. Commun.* **220,** 600–605.

Kong, L. Y., Wilson, B. C., McMillian, M. K., Bing, G., Hudson, P. M., and Hong, J. S. (1996). The effects of the HIV-1 envelope protein gp120 on the production of nitric oxide and proinflammatory cytokines in mixed glial cell cultures. *Cell. Immunol.* **172,** 77–83.

Koprowski, H., Zheng, Y. M., Heber-Katz, E., Fraser, N., Rorke, L., Fu, Z. F., Hanlon, C., and Dietzschold, B. (1993). In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases [published erratum appears in *Proc. Natl. Acad. Sci. U.S.A.* (1993) June 1; **90**(11): 5378]. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3024–3027.

Kroncke, K. D., Fehsel, K., and Kolb-Bachofen, V. (1998). Inducible nitric oxide synthase in human diseases. *Clin. Exp. Immunol.* **113,** 147–156.

Kumar, A., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. (1994). Double-stranded RNA-dependent protein kinase activates transcription factor NF-κ B by phosphorylating I κ B. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6288–6292.

Kumar, A., Yang, Y. L., Flati, V., Der, S., Kadereit, S., Deb, A., Haque, J., Reis, L., Weissmann, C., and Williams, B. R. (1997). Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: Role of IRF-1 and NF-κB. *EMBO J.* **16,** 406–416.

Kun, J. F., Mordmuller, B., Lell, B., Lehman, L. G., Luckner, D., and Kremsner, P. G. (1998). Polymorphism in promoter region of inducible nitric oxide synthase gene and protection against malaria [letter] [see comments]. *Lancet* **351,** 265–266.

Kunz, D., Muhl, H., Walker, G., and Pfeilschifter, J. (1994). Two distinct signaling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 5387–5391.

Kunz, D., Walker, G., Eberhardt, W., Nitsch, D., and Pfeilschifter, J. (1995). Interleukin 1 β-induced expression of nitric oxide synthase in rat renal mesangial cells is suppressed by cyclosporin A. *Biochem. Biophys. Res. Commun.* **216,** 438–446.

Kunz, D., Walker, G., Eberhardt, W., and Pfeilschifter, J. (1996). Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1 β-stimulated mesangial cells: Evidence for the involvement of transcriptional and posttranscriptional regulation. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 255–259.

Kunz, D., Walker, G., Eberhardt, W., Messmer, U. K., Huwiler, A., and Pfeilschifter, J. (1997). Platelet-derived growth factor and fibroblast growth factor differentially regulate interleukin 1β- and cAMP-induced nitric oxide synthase expression in rat renal mesangial cells. *J. Clin. Invest.* **100,** 2800–2809.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6348–6352.

Laubach, V. E., Zhang, C. X., Russell, S. W., Murphy, W. J., and Sherman, P. A. (1997). Analysis of expression and promoter function of the human inducible nitric oxide synthase gene in DLD-1 cells and monkey hepatocytes. *Biochim. Biophys. Acta* **1351,** 287–295.

Lawrence, R., Chang, L. J., Siebenlist, U., Bressler, P., and Sonenshein, G. E. (1994). Vascular smooth muscle cells express a constitutive NF-κ B-like activity. *J. Biol. Chem.* **269,** 28913–28918.

Lee, B. S., Kang, H. S., Pyun, K. H., and Choi, I. (1997). Roles of tyrosine kinases in the regulation of nitric oxide synthesis in murine liver cells: Modulation of NF-κ B activity by tyrosine kinases. *Hepatology* **25,** 913–919.

Lee, F. S., Hagler, J., Chen, Z. J., and Maniatis, T. (1997). Activation of the IκB α kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* **88,** 213–222.

Lee, S. K., Kim, H. J., Na, S. Y., Kim, T. S., Choi, H. S., Im, S. Y., and Lee, J. W. (1998). Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. *J. Biol. Chem.* **273,** 16651–16654.

Lehming, N., Thanos, D., Brickman, J. M., Ma, J., Maniatis, T., and Ptashne, M. (1994). An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature* **371,** 175–179.

Lewis, H., Kaszubska, W., DeLamarter, J. F., and Whelan, J. (1994). Cooperativity between two NF-κ B complexes, mediated by high-mobility-group protein I(Y), is essential for cytokine-induced expression of the E-selectin promoter. *Mol. Cell. Biol.* **14,** 5701–5709.

Li, J., Bombeck, C. A., Yang, S., Kim, Y. M., and Billiar, T. R. (1999). Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes [In Process Citation]. *J. Biol. Chem.* **274,** 17325–17333.

Linn, S. C., Morelli, P. J., Edry, I., Cottongim, S. E., Szabo, C., and Salzman, A. L. (1997). Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am. J. Physiol.* **272,** G1499–G1508.

Liu, S. F., Ye, X., and Malik, A. B. (1997). In vivo inhibition of nuclear factor-κ B activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. *J. Immunol.* **159,** 3976–3983.

Liu, X., Miller, C. W., Koeffler, P. H., and Berk, A. J. (1993). The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription. *Mol. Cell. Biol.* **13,** 3291–3300.

Liu, Z. Z., Cui, S., Billiar, T. R., Dorko, K., Halfter, W., Geller, D. A., Michalopoulos, G., Beger, H. G., Albina, J., and Nussler, A. K. (1996). Effects of hepatocellular mitogens on cytokine-induced nitric oxide synthesis in human hepatocytes. *J. Leukocyte Biol.* **60,** 382–388.

Loihl, A. K., Whalen, S., Campbell, I. L., Mudgett, J. S., and Murphy, S. (1999). Transcriptional activation following cerebral ischemia in mice of a promoter-deleted nitric oxide synthase-2 gene. *J. Biol. Chem.* **274,** 8844–8849.

Lorsbach, R. B., Murphy, W. J., Lowenstein, C. J., Snyder, S. H., and Russell, S. W. (1993). Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-γ and lipopolysaccharide. *J. Biol. Chem.* **268,** 1908–1913.

Lowenstein, C. J., Glatt, C. S., Bredt, D. S., and Snyder, S. H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6711–6715.

Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., and Murphy, W. J. (1993). Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon γ and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9730–9734.

Luss, H., Watkins, S. C., Freeswick, P. D., Imro, A. K., Nussler, A. K., Billiar, T. R., Simmons, R. L., del Nido, P. J., and McGowan, F. X., Jr. (1995). Characterization of inducible nitric oxide synthase expression in endotoxemic rat cardiac myocytes in vivo and following cytokine exposure in vitro. *J. Mol. Cell. Cardiol.* **27,** 2015–2029.

Lyons, C. R., Orloff, G. J., and Cunningham, J. M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.* **267,** 6370–6374.

McCartney-Francis, N., Allen, J. B., Mizel, D. E., Albina, J. E., Xie, Q. W., Nathan, C. F., and Wahl, S. M. (1993). Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* **178,** 749–754.

McKee, C. M., Lowenstein, C. J., Horton, M. R., Wu, J., Bao, C., Chin, B. Y., Choi, A. M., and Noble, P. W. (1997). Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor κB-dependent mechanism. *J. Biol. Chem.* **272,** 8013–8018.

MacKendrick, W., Caplan, M., and Hsueh, W. (1993). Endogenous nitric oxide protects against platelet-activating factor-induced bowel injury in the rat. *Pediatr. Res.* **34,** 222–228.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.

Mahon, T. M., and O'Neill, L. A. (1995). Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NF-κ B activation in T lymphocytes. Evidence for covalent modification of the p50 subunit. *J. Biol. Chem.* **270,** 28557–28564.

Maier, R., Bilbe, G., Rediske, J., and Lotz, M. (1994). Inducible nitric oxide synthase from human articular chondrocytes: cDNA cloning and analysis of mRNA expression. *Biochim. Biophys. Acta* **1208,** 145–150.

Majumder, S., Zhou, L. Z., Chaturvedi, P., Babcock, G., Aras, S., and Ransohoff, R. M. (1998). p48/STAT-1α-containing complexes play a predominant role in induction of IFN-γ-inducible protein, 10 kDa (IP-10) by IFN-γ alone or in synergy with TNF-α. *J. Immunol.* **161,** 4736–4744.

Mannick, J. B., Asano, K., Izumi, K., Kieff, E., and Stamler, J. S. (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* **79,** 1137–1146.

Markewitz, B. A., Michael, J. R., and Kohan, D. E. (1993). Cytokine-induced expression of a nitric oxide synthase in rat renal tubule cells. *J. Clin. Invest.* **91,** 2138–2143.

Marks-Konczalik, J., Chu, S. C., and Moss, J. (1998). Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor κB-binding sites. *J. Biol. Chem.* **273,** 22201–22208.

Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S., and Michel, T. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett.* **307,** 287–293.

Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J. Biol. Chem.* **268,** 17478–17488.

Martin, E., Nathan, C., and Xie, Q. W. (1994). Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med.* **180,** 977–984.

Massa, P. T., and Wu, C. (1998). Increased inducible activation of NF-κB and responsive genes in astrocytes deficient in the protein tyrosine phosphatase SHP-1. *J. Interferon Cytokine Res.* **18,** 499–507.

Melillo, G., Taylor, L. S., Brooks, A., Musso, T., Cox, G. W., and Varesio, L. (1997). Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. *J. Biol. Chem.* **272,** 12236–12243.

Mercurio, F., and Manning, A. M. (1999). Multiple signals converging on NF-κB. *Curr. Opin. Cell Biol.* **11,** 226–232.

Messner, B., Stutz, A. M., Albrecht, B., Peiritsch, S., and Woisetschlager, M. (1997). Cooperation of binding sites for STAT6 and NF κ B/rel in the IL-4-induced up-regulation of the human IgE germline promoter. *J. Immunol.* **159,** 3330–3337.

Metz, R., Kouzarides, T., and Bravo, R. (1994). A C-terminal domain in FosB, absent in FosB/SF and Fra-1, which is able to interact with the TATA binding protein, is required for altered cell growth. *EMBO J.* **13,** 3832–3842.

Meyer, C. F., Wang, X., Chang, C., Templeton, D., and Tan, T. H. (1996). Interaction between c-Rel and the mitogen-activated protein kinase kinase kinase 1 signaling cascade in mediating κB enhancer activation. *J. Biol. Chem.* **271,** 8971–8976.

Miller, L., Alley, E. W., Murphy, W. J., Russell, S. W., and Hunt, J. S. (1996). Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. *J. Leukocyte Biol.* **59,** 442–450.

Mons, N., Decorte, L., Jaffard, R., and Cooper, D. M. (1998). Ca^{2+}-sensitive adenylyl cyclases, key integrators of cellular signalling. *Life Sci.* **62,** 1647–1652.

Montminy, M. (1997). Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem.* **66,** 807–822.

Morooka, H., Bonventre, J. V., Pombo, C. M., Kyriakis, J. M., and Force, T. (1995). Ischemia and reperfusion enhance ATF-2 and c-Jun binding to cAMP response elements and to an AP-1 binding site from the c-jun promoter. *J. Biol. Chem.* **270,** 30084–30092.

Morris, S. M., Jr., Kepka-Lenhart, D., and Chen, L. C. (1998). Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. *Am. J. Physiol.* **275,** E740–E747.

Muhl, H., and Pfeilschifter, J. (1994). Possible role of protein kinase C-ϵ isoenzyme in inhibition of interleukin 1 β induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. J.* **303,** 607–612.

Muhl, H., Kunz, D., Rob, P., and Pfeilschifter, J. (1993). Cyclosporin derivatives inhibit interleukin 1 β induction of nitric oxide synthase in renal mesangial cells. *Eur. J. Pharmacol.* **249,** 95–100.

Mullet, D., Fertel, R. H., Kniss, D., and Cox, G. W. (1997). An increase in intracellular cyclic AMP modulates nitric oxide production in IFN-γ-treated macrophages. *J. Immunol.* **158,** 897–904.

Mulsch, A., Schray-Utz, B., Mordvintcev, P. I., Hauschildt, S., and Busse, R. (1993). Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett.* **321,** 215–218.

Munshi, N., Merika, M., Yie, J., Senger, K., Chen, G., and Thanos, D. (1998). Acetylation of HMG I(Y) by CBP turns off IFN β expression by disrupting the enhanceosome. *Mol. Cell* **2,** 457–467.

Murphy, W. J. (1999). ''Transcriptional Regulation of the Genes Encoding Nitric Oxide Synthase'' (W. J. Murphy, ed.), pp. 1–57.

Mustafa, S. B., and Olson, M. S. (1998). Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. *J. Biol. Chem.* **273,** 5073–5080.

Nakane, M., Schmidt, H. H., Pollock, J. S., Förstermann, U., and Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett.* **316,** 175–180.

Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998). Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 3537–3542.

Nakayama, D. K., Geller, D. A., Lowenstein, C. J., Chern, H. D., Davies, P., Pitt, B. R., Simmons, R. L., and Billiar, T. R. (1992). Cytokines and lipopolysaccharide induce nitric oxide synthase in cultured rat pulmonary artery smooth muscle [published erratum appears in *Am. J. Respir. Cell Mol. Biol.* (1993) Aug.; **9**(2): following 229]. *Am. J. Respir. Cell Mol. Biol.* **7,** 471–476.

Nakayama, I., Kawahara, Y., Tsuda, T., Okuda, M., and Yokoyama, M. (1994). Angiotensin II inhibits cytokine-stimulated inducible nitric oxide synthase expression in vascular smooth muscle cells. *J. Biol. Chem.* **269,** 11628–11633.

Neish, A. S., Read, M. A., Thanos, D., Pine, R., Maniatis, T., and Collins, T. (1995). Endothelial interferon regulatory factor 1 cooperates with NF-κ B as a transcriptional activator of vascular cell adhesion molecule 1. *Mol. Cell. Biol.* **15,** 2558–2569.

Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W., and Murphy, T. J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.* **90,** 2092–2096.

Nishiya, T., Uehara, T., and Nomura, Y. (1995). Herbimycin A suppresses NF-κ B activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in C6 glioma cells. *FEBS Lett.* **371,** 333–336.

Nishiya, T., Uehara, T., Edamatsu, H., Kaziro, Y., Itoh, H., and Nomura, Y. (1997). Activation of Stat1 and subsequent transcription of inducible nitric oxide synthase gene in C6 glioma cells is independent of interferon-γ-induced MAPK activation that is mediated by p21ras. *FEBS Lett.* **408,** 33–38.

Nunokawa, Y., Ishida, N., and Tanaka, S. (1994). Promoter analysis of human inducible nitric oxide synthase gene associated with cardiovascular homeostasis. *Biochem. Biophys. Res. Commun.* **200,** 802–807.

Nunokawa, Y., Oikawa, S., and Tanaka, S. (1997). Expression of human inducible nitric oxide synthase is regulated by both promoter and 3'-regions. *Biochem. Biophys. Res. Commun.* **233,** 523–526.

Nussler, A., Drapier, J. C., Renia, L., Pied, S., Miltgen, F., Gentilini, M., and Mazier, D. (1991). L-arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin 6 stimulation. *Eur. J. Immunol.* **21,** 227–230.

Nussler, A. K., DiSilvio, M., Billiar, T. R., Hoffman, R. A., Geller, D. A., Selby, R., Madariaga, J., and Simmons, R. L. (1992). Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. *J. Exp. Med.* **176,** 261–264.

Nussler, A. K., Di Silvio, M., Liu, Z. Z., Geller, D. A., Freeswick, P., Dorko, K., Bartoli, F., and Billiar, T. R. (1995). Further characterization and comparison of inducible nitric oxide synthase in mouse, rat, and human hepatocytes. *Hepatology* **21,** 1552–1560.

Ochoa, J. B., Curtis, B., Peitzman, A. B., Simmons, R. L., Billiar, T. R., Hoffman, R., Rault, R., Longo, D. L., Urba, W. J., and Ochoa, A. C. (1991a). Increased circulating nitrogen oxide levels after human tumor immunotherapy: Correlation with toxic hemodynamic changes. *J. Natl. Cancer Inst.* **84,** 864–867.

Ochoa, J. B., Udekwu, A. O., Billiar, T. R., Curran, R. D., Cerra, F. B., Simmons, R. L., and Peitzman, A. B. (1991b). Nitrogen oxide levels in patients after trauma and during sepsis. *Ann. Surgery* **214,** 621–626.

Ohmori, Y., and Hamilton, T. A. (1995). The interferon-stimulated response element and a κ B site mediate synergistic induction of murine IP-10 gene transcription by IFN-γ and TNF-α. *J. Immunol.* **154,** 5235–5244.

Ohmori, Y., Schreiber, R. D., and Hamilton, T. A. (1997). Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κB. *J. Biol. Chem.* **272,** 14899–14907.

Ou, J., Carlos, T. M., Watkins, S. C., Saavedra, J. E., Keefer, L. K., Kim, Y. M., Harbrecht, B. G., and Billiar, T. R. (1997). Differential effects of nonselective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. *Nitric Oxide* **1,** 404–416.

Pahan, K., Namboodiri, A. M., Sheikh, F. G., Smith, B. T., and Singh, I. (1997). Increasing cAMP attenuates induction of inducible nitric-oxide synthase in rat primary astrocytes. *J. Biol. Chem.* **272,** 7786–7791.

Pahan, K., Sheikh, F. G., Khan, M., Namboodiri, A. M., and Singh, I. (1998). Sphingomyelinase and ceramide stimulate the expression of inducible nitric-oxide synthase in rat primary astrocytes. *J. Biol. Chem.* **273,** 2591–2600.

Park, S. K., Lin, H. L., and Murphy, S. (1997). Nitric oxide regulates nitric oxide synthase-2 gene expression by inhibiting NF-κB binding to DNA. *Biochem. J.* **322,** 609–613.

Parry, G. C., and Mackman, N. (1997). Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-κB-mediated transcription. *J. Immunol.* **159,** 5450–5456.

Pazin, M. J., Sheridan, P. L., Cannon, K., Cao, Z., Keck, J. G., Kadonaga, J. T., and Jones, K. A. (1996). NF-κ B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. *Genes Dev.* **10,** 37–49.

Peng, H. B., Libby, P., and Liao, J. K. (1995). Induction and stabilization of I κ B α by nitric oxide mediates inhibition of NF-κ B. *J. Biol. Chem.* **270,** 14214–14219.

Perrella, M. A., Yoshizumi, M., Fen, Z., Tsai, J. C., Hsieh, C. M., Kourembanas, S., and Lee, M. E. (1994). Transforming growth factor-β 1, but not dexamethasone, down-regulates nitric-oxide synthase mRNA after its induction by interleukin-1 β in rat smooth muscle cells. *J. Biol. Chem.* **269,** 14595–14600.

Perrella, M. A., Patterson, C., Tan, L., Yet, S. F., Hsieh, C. M., Yoshizumi, M., and Lee, M. E. (1996). Suppression of interleukin-1β-induced nitric-oxide synthase promoter/enhancer activity by transforming growth factor-β1 in vascular smooth muscle cells. Evidence for mechanisms other than NF-κB. *J. Biol. Chem.* **271,** 13776–13780.

Perrella, M. A., Pellacani, A., Wiesel, P., Chin, M. T., Foster, L. C., Ibanez, M., Hsieh, C. M., Reeves, R., Yet, S. F., and Lee, M. E. (1999). High mobility group-I(Y) protein facilitates nuclear factor-κB binding and transactivation of the inducible nitric-oxide synthase promoter/enhancer. *J. Biol. Chem.* **274,** 9045–9052.

Pfeilschifter, J., and Vosbeck, K. (1991). Transforming growth factor β 2 inhibits interleukin 1 β- and tumour necrosis factor α-induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. Biophys. Res. Commun.* **175,** 372–379.

Pine, R. (1997). Convergence of TNFα and IFNγ signalling pathways through synergistic induction of IRF-1/ISGF-2 is mediated by a composite GAS/κB promoter element. *Nucleic Acids Res.* **25,** 4346–4354.

Qureshi, S. A., Salditt-Georgieff, M., and Darnell, J. E., Jr. (1995). Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 3829–3833.

Raddassi, K., Berthon, B., Petit, J. F., and Lemaire, G. (1994). Role of calcium in the activation of mouse peritoneal macrophages: Induction of NO synthase by calcium ionophores and thapsigargin. *Cell. Immunol.* **153,** 443–455.

Ram, P. A., and Waxman, D. J. (1997). Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. *J. Biol. Chem.* **272,** 17694–17702.

Ransone, L. J., Kerr, L. D., Schmitt, M. J., Wamsley, P., and Verma, I. M. (1993). The bZIP domains of Fos and Jun mediate a physical association with the TATA box-binding protein. *Gene Express.* **3,** 37–48.

Ravi, R., Mookerjee, B., van Hensbergen, Y., Bedi, G. C., Giordano, A., El-Deiry, W. S., Fuchs, E. J., and Bedi, A. (1998). p53-mediated repression of nuclear factor-κB RelA via the transcriptional integrator p300. *Cancer Res.* **58,** 4531–4536.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998). The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. *Nature* **391,** 79–82.

Roberts, A. B., Vodovotz, Y., Roche, N. S., Sporn, M. B., and Nathan, C. F. (1992). Role of nitric oxide in antagonistic effects of transforming growth factor-β and interleukin-1 β on the beating rate of cultured cardiac myocytes. *Mol. Endocrinol.* **6,** 1921–1930.

Ruocco, M. R., Chen, X., Ambrosino, C., Dragonetti, E., Liu, W., Mallardo, M., De Falco, G., Palmieri, C., Franzoso, G., Quinto, I., Venuta, S., and Scala, G. (1996). Regulation of HIV-1 long terminal repeats by interaction of C/EBP(NF-IL6) and NF-κB/Rel transcription factors. *J. Biol. Chem.* **271,** 22479–22486.

Saavedra, J. E., Billiar, T. R., Williams, D. L., Kim, Y. M., Watkins, S. C., and Keefer, L. K. (1997). Targeting nitric oxide (NO) delivery in vivo. Design of a liver-selective NO donor prodrug that blocks tumor necrosis factor-α-induced apoptosis and toxicity in the liver. *J. Med. Chem.* **40,** 1947–1954.

Sakitani, K., Nishizawa, M., Inoue, K., Masu, Y., Okumura, T., and Ito, S. (1998). Synergistic regulation of inducible nitric oxide synthase gene by CCAAT/enhancer-binding protein β and nuclear factor-κB in hepatocytes. *Genes Cells* **3,** 321–330.

Salkowski, C. A., Barber, S. A., Detore, G. R., and Vogel, S. N. (1996). Differential dysregulation of nitric oxide production in macrophages with targeted disruptions in IFN regulatory factor-1 and -2 genes. *J. Immunol.* **156,** 3107–3110.

Salter, M., Knowles, R. G., and Moncada, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.* **291,** 145–149.

Salvucci, O., Kolb, J. P., Dugas, B., Dugas, N., and Chouaib, S. (1998). The induction of nitric oxide by interleukin-12 and tumor necrosis factor-α in human natural killer cells: Relationship with the regulation of lytic activity. *Blood* **92,** 2093–2102.

Salzman, A., Denenberg, A. G., Ueta, I., O'Connor, M., Linn, S. C., and Szabo, C. (1996). Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *Am. J. Physiol.* **270,** G565–G573.

Sands, W. A., Bulut, V., Severn, A., Xu, D., and Liew, F. Y. (1994). Inhibition of nitric oxide synthesis by interleukin-4 may involve inhibiting the activation of protein kinase C ϵ. *Eur. J. Immunol.* **24,** 2345–2350.

Saura, M., Zaragoza, C., McMillan, A., Quick, R. A., Hohenadl, C., Lowenstein, J. M., and Lowenstein, C. J. (1999). An antiviral mechanism of nitric oxide: Inhibition of a viral protease. *Immunity* **10,** 21–28.

Sawada, T., Falk, L. A., Rao, P., Murphy, W. J., and Pluznik, D. H. (1997). IL-6 induction of protein-DNA complexes via a novel regulatory region of the inducible nitric oxide synthase gene promoter: Role of octamer binding proteins. *J. Immunol.* **158,** 5267–5276.

Schaffer, M. R., Efron, P. A., Thornton, F. J., Klingel, K., Gross, S. S., and Barbul, A. (1997). Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. *J. Immunol.* **158,** 2375–2381.

Schini, V. B., Catovsky, S., Schray-Utz, B., Busse, R., and Vanhoutte, P. M. (1994). Insulin-like growth factor I inhibits induction of nitric oxide synthase in vascular smooth muscle cells. *Circ. Res.* **74,** 24–32.

Schmitz, M. L., Stelzer, G., Altmann, H., Meisterernst, M., and Baeuerle, P. A. (1995). Interaction of the COOH-terminal transactivation domain of p65 NF-κ B with TATA-binding protein, transcription factor IIB, and coactivators. *J. Biol. Chem.* **270,** 7219–7226.

Schroeder, R. A., Gu, J. S., and Kuo, P. C. (1998). Interleukin 1β-stimulated production of nitric oxide in rat hepatocytes is mediated through endogenous synthesis of interferon γ. *Hepatology* **27,** 711–719.

Schulze-Osthoff, K., Ferrari, D., Riehemann, K., and Wesselborg, S. (1997). Regulation of NF-κ B activation by MAP kinase cascades. *Immunobiology* **198,** 35–49.

Schwacha, M. G., Samy, T. S., Catania, R. A., and Chaudry, I. H. (1998). Thermal injury alters macrophage responses to prostaglandin E2: Contribution to the enhancement of inducible nitric oxide synthase activity. *J. Leukocyte Biol.* **64,** 740–746.

Scott-Burden, T., Elizondo, E., Ge, T., Boulanger, C. M., and Vanhoutte, P. M. (1994). Simultaneous activation of adenylyl cyclase and protein kinase C induces production of nitric oxide by vascular smooth muscle cells. *Mol. Pharmacol.* **46,** 274–282.

Sengupta, T. K., Schmitt, E. M., and Ivashkiv, L. B. (1996). Inhibition of cytokines and JAK-STAT activation by distinct signaling pathways. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 9499–9504.

Servidei, T., Aoki, Y., Lewis, S. E., Symes, A., Fink, J. S., and Reeves, S. A. (1998). Coordinate regulation of STAT signaling and c-fos expression by the tyrosine phosphatase SHP-2. *J. Biol. Chem.* **273,** 6233–6241.

Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D'Angelo, D. D., Lynch, K. R., and Peach, M. J. (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J. Biol. Chem.* **267,** 15274–15276.

Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A. J., and Shenk, T. (1992). Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 12028–12032.

Shatrov, V. A., Ratter, F., Gruber, A., Droge, W., and Lehmann, V. (1996). HIV type 1 glycoprotein 120 amplifies tumor necrosis factor-induced NF-κ B activation in Jurkat cells. *AIDS Research & Human Retroviruses* **12,** 1209–1216.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46,** 659–667.

Shen, C. H., and Stavnezer, J. (1998). Interaction of stat6 and NF-κB: Direct association and synergistic activation of interleukin-4-induced transcription. *Mol. Cell. Biol.* **18,** 3395–3404.

Sheppard, K. A., Phelps, K. M., Williams, A. J., Thanos, D., Glass, C. K., Rosenfeld, M. G., Gerritsen, M. E., and Collins, T. (1998). Nuclear integration of glucocorticoid receptor and nuclear factor-κB signaling by CREB-binding protein and steroid receptor coactivator-1. *J. Biol. Chem.* **273,** 29291–29294.

Sherman, M. P., Aeberhard, E. E., Wong, V. Z., Griscavage, J. M., and Ignarro, L. J. (1993). Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **191,** 1301–1308.

Sherman, P. A., Laubach, V. E., Reep, B. R., and Wood, E. R. (1993). Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. *Biochemistry* **32,** 11600–11605.

Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinauer, M., and Nathan, C. (1999). Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* **10,** 29–38.

Shin, W. S., Hong, Y. H., Peng, H. B., De Caterina, R., Libby, P., and Liao, J. K. (1996). Nitric oxide attenuates vascular smooth muscle cell activation by interferon-γ. The role of constitutive NF-κ B activity. *J. Biol. Chem.* **271,** 11317–11324.

Singh, K., Balligand, J. L., Fischer, T. A., Smith, T. W., and Kelly, R. A. (1996). Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells. Role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and STAT1 α. *J. Biol. Chem.* **271,** 1111–1117.

Sizemore, N., Leung, S., and Stark, G. R. (1999). Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-κB p65/RelA subunit. *Mol. Cell. Biol.* **19,** 4798–4805.

Soares, M. P., Muniappan, A., Kaczmarek, E., Koziak, K., Wrighton, C. J., Steinhauslin, F., Ferran, C., Winkler, H., Bach, F. H., and Anrather, J. (1998). Adenovirus-mediated expression of a dominant negative mutant of p65/RelA inhibits proinflammatory gene expression in endothelial cells without sensitizing to apoptosis. *J. Immunol.* **161,** 4572–4582.

Spink, J., Cohen, J., and Evans, T. J. (1995). The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. Activation by nuclear factor-κ B. *J. Biol. Chem.* **270,** 29541–29547.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* **67,** 227–264.

Steger, D. J., and Workman, J. L. (1997). Stable co-occupancy of transcription factors and histones at the HIV-1 enhancer. *EMBO J.* **16,** 2463–2472.

Stein, B., and Baldwin, A. S. (1993). Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. *Mol. Cell. Biol.* **13,** 7191–7198.

Stelzer, G., Goppelt, A., Lottspeich, F., and Meisterernst, M. (1994). Repression of basal transcription by HMG2 is counteracted by TFIIH-associated factors in an ATP-dependent process. *Mol. Cell. Biol.* **14,** 4712–4721.

Stuehr, D. J., and Marletta, M. A. (1985). Mammalian nitrate biosynthesis: Mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7738–7742.

Stuehr, D. J., and Marletta, M. A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *J. Immunol.* **139,** 518–525.

Suschek, C., Rothe, H., Fehsel, K., Enczmann, J., and Kolb-Bachofen, V. (1993). Induction of a macrophage-like nitric oxide synthase in cultured rat aortic endothelial cells. IL-1 β-mediated induction regulated by tumor necrosis factor-α and IFN-γ. *J. Immunol.* **151,** 3283–3291.

Sutrias-Grau, M., Bianchi, M. E., and Bernues, J. (1999). High mobility group protein 1 interacts specifically with the core domain of human TATA box-binding protein and interferes with transcription factor IIB within the pre-initiation complex. *J. Biol. Chem.* **274,** 1628–1634.

Swope, D. L., Mueller, C. L., and Chrivia, J. C. (1996). CREB-binding protein activates transcription through multiple domains. *J. Biol. Chem.* **271,** 28138–28145.

Szabo, C., and Thiemermann, C. (1994). Invited opinion: Role of nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury. *Shock* **2,** 145–155.

Taylor, B. S., Kim, Y. M., Wang, Q., Shapiro, R. A., Billiar, T. R., and Geller, D. A. (1997). Nitric oxide down-regulates hepatocyte-inducible nitric oxide synthase gene expression. *Arch. Surg.* **132,** 1177–1183.

Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1998). Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* **273,** 15148–15156.

Taylor, B. S., Shao, L., Gambotto, A., Ganster, R. W., and Geller, D. A. (1999). Inhibition of cytokine-induced nitric oxide synthase expression by gene transfer of adenoviral I kappa B alpha. *Surgery* **126,** 142–147.

Thanos, D., and Maniatis, T. (1992). The high mobility group protein HMG I(Y) is required for NF-κ B-dependent virus induction of the human IFN-β gene. *Cell* **71,** 777–789.

Thut, C. J., Goodrich, J. A., and Tjian, R. (1997). Repression of p53-mediated transcription by MDM2: A dual mechanism. *Genes Dev.* **11,** 1974–1986.

Toker, A. (1998). Signaling through protein kinase C. *Frontiers Biosci.* **3,** D1134–D1147.

Trepicchio, W. L., Wang, L. L., Bozza, M., and Dorner, A. J. (1997). Il-11 regulates macrophage effector function through the inhibition of nuclear factor-κ-B. *J. Immunol.* **159,** 5661–5670.

Vallance, P., Collier, J., and Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man [see comments]. *Lancet* **2,** 997–1000.

Velasco, M., Diaz-Guerra, M. J., Diaz-Achirica, P., Andreu, D., Rivas, L., and Bosca, L. (1997). Macrophage triggering with cecropin A and melittin-derived peptides induces type II nitric oxide synthase expression. *J. Immunol.* **158,** 4437–4443.

Vieira, L. Q., Goldschmidt, M., Nashleanas, M., Pfeffer, K., Mak, T., and Scott, P. (1996). Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with *Leishmania major,* but control parasite replication. *J. Immunol.* **157,** 827–835.

Vieira, L. Q., Goldschmidt, M., Nashleanas, M., Pfeffer, K., Mak, T., and Scott, P. (1997). Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide in vivo: The roles of macrophages, endogenous IFN-γ, and TNF receptor-1-mediated signaling. *J. Immunol.* **158,** 905–912.

Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q. W., and Nathan, C. (1993). Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. *J. Exp. Med.* **178,** 605–613.

Vodovotz, Y., Geiser, A. G., Chesler, L., Letterio, J. J., Campbell, A., Lucia, M. S., Sporn, M. B., and Roberts, A. B. (1996a). Spontaneously increased production of nitric oxide and aberrant expression of the inducible nitric oxide synthase in vivo in the transforming growth factor β 1 null mouse. *J. Exp. Med.* **183,** 2337–2342.

Vodovotz, Y., Letterio, J. J., Geiser, A. G., Chesler, L., Roberts, A. B., and Sparrow, J. (1996b). Control of nitric oxide production by endogenous TGF-β1 and systemic nitric oxide in retinal pigment epithelial cells and peritoneal macrophages. *J. Leukocyte Biol.* **60,** 261–270.

Vodovotz, Y., Kopp, J. B., Takeguchi, H., Shrivastav, S., Coffin, D., Lucia, M. S., Mitchell, J. B., Webber, R., Letterio, J., Wink, D., and Roberts, A. B. (1998). Increased mortality, blunted production of nitric oxide, and increased production of TNF-α in endotoxemic TGF-β1 transgenic mice. *J. Leukocyte Biol.* **63,** 31–39.

Walker, G., Pfeilschifter, J., and Kunz, D. (1997). Mechanisms of suppression of inducible nitric-oxide synthase (iNOS) expression in interferon (IFN)-γ-stimulated RAW 264.7 cells by dexamethasone. Evidence for glucocorticoid-induced degradation of iNOS protein by calpain as a key step in post-transcriptional regulation. *J. Biol. Chem.* **272,** 16679–16687.

Wathelet, M. G., Lin, C. H., Parekh, B. S., Ronco, L. V., Howley, P. M., and Maniatis, T. (1998). Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo. *Mol. Cell* **1,** 507–518.

Weinberg, J. B., Granger, D. L., Pisetsky, D. S., Seldin, M. F., Misukonis, M. A., Mason, S. N., Pippen, A. M., Ruiz, P., Wood, E. R., and Gilkeson, G. S. (1994). The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: Increased nitric oxide production and nitric oxide synthase expression in MRL-lpr/lpr mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered NG-monomethyl-L-arginine. *J. Exp. Med.* **179,** 651–660.

Welsh, N. (1996). Interleukin-1 β-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. *J. Biol. Chem.* **271,** 8307–8312.

Wenger, R. H., and Gassmann, M. (1997). Oxygen(es) and the hypoxia-inducible factor-1. *Biol. Chem.* **378,** 609–616.

Werner-Felmayer, G., Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G., Schmidt, K., Weiss, G., and Wachter, H. (1993). Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. *J. Biol. Chem.* **268,** 1842–1846.

Whitmarsh, A. J., and Davis, R. J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.* **74,** 589–607.

Widlak, P., Gaynor, R. B., and Garrard, W. T. (1997). In vitro chromatin assembly of the HIV-1 promoter. ATP-dependent polar repositioning of nucleosomes by Sp1 and NFκB. *J. Biol. Chem.* **272,** 17654–17661.

Wilkinson, S. E., and Nixon, J. S. (1998). T-cell signal transduction and the role of protein kinase C. *Cell. Mol. Life Sci.* **54,** 1122–1144.

Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S., and *et al.* (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254,** 1001–1003.

Wright, K., Ward, S. G., Kolios, G., and Westwick, J. (1997). Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric-oxide synthase expression in epithelial cell line HT-29. *J. Biol. Chem.* **272,** 12626–12633.

Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256,** 225–228.

Xie, Q. W., Whisnant, R., and Nathan, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J. Exp. Med.* **177,** 1779–1784.

Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-κ B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269,** 4705–4708.

Xing, L., Gopal, V. K., and Quinn, P. G. (1995). cAMP response element-binding protein (CREB) interacts with transcription factors IIB and IID. *J. Biol. Chem.* **270,** 17488–17493.

Xu, X., Prorock, C., Ishikawa, H., Maldonado, E., Ito, Y., and Gelinas, C. (1993). Functional interaction of the v-Rel and c-Rel oncoproteins with the TATA-binding protein and association with transcription factor IIB. *Mol. Cell. Biol.* **13,** 6733–6741.

Xue, C., Rengasamy, A., Le Cras, T. D., Koberna, P. A., Dailey, G. C., and Johns, R. A. (1994). Distribution of NOS in normoxic vs. hypoxic rat lung: Upregulation of NOS by chronic hypoxia. *Am. J. Physiol.* **267,** L667–L678.

Yamasaki, K., Edington, H. D., McClosky, C., Tzeng, E., Lizonova, A., Kovesdi, I., Steed, D. L., and Billiar, T. R. (1998). Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J. Clin. Invest.* **101,** 967–971.

Yamit-Hezi, A., and Dikstein, R. (1998). TAFII105 mediates activation of anti-apoptotic genes by NF-κB. *EMBO J.* **17,** 5161–5169.

Yin, M. J., Christerson, L. B., Yamamoto, Y., Kwak, Y. T., Xu, S., Mercurio, F., Barbosa, M., Cobb, M. H., and Gaynor, R. B. (1998). HTLV-I Tax protein binds to MEKK1 to stimulate IκB kinase activity and NF-κB activation. *Cell* **93,** 875–884.

Yin, T., Shen, R., Feng, G. S., and Yang, Y. C. (1997). Molecular characterization of specific interactions between SHP-2 phosphatase and JAK tyrosine kinases. *J. Biol. Chem.* **272,** 1032–1037.

Yoon, H. J., Jun, C. D., Kim, J. M., Rim, G. N., Kim, H. M., and Chung, H. T. (1994). Phorbol ester synergistically increases interferon-γ-induced nitric oxide synthesis in murine microglial cells. *Neuroimmunomodulation* **1,** 377–382.

You, M., and Zhao, Z. (1997). Positive effects of SH2 domain-containing tyrosine phosphatase SHP-1 on epidermal growth factor- and interferon-γ-stimulated activation of STAT transcription factors in HeLa cells. *J. Biol. Chem.* **272,** 23376–23381.

You, M., Yu, D. H., and Feng, G. S. (1999). Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway. *Mol. Cell. Biol.* **19,** 2416–2424.

Yu, Z., Su, L., Hoglinger, O., Jaramillo, M. L., Banville, D., and Shen, S. H. (1998). SHP-1 associates with both platelet-derived growth factor receptor and the p85 subunit of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **273,** 3687–3694.

Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M., and Darnell, J. E. (1996). Two contact regions between Stat1 and CBP/p300 in interferon-γ signalling. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15092–15096.

Zhang, X., Laubach, V. E., Alley, E. W., Edwards, K. A., Sherman, P. A., Russell, S. W., and Murphy, W. J. (1996). Transcriptional basis for hyporesponsiveness of the human inducible nitric oxide synthase gene to lipopolysaccharide/interferon-γ. *J. Leukocyte Biol.* **59,** 575–585.

Zhong, H., Voll, R. E., and Ghosh, S. (1998). Phosphorylation of NF-κ B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell* **1,** 661–671.

Zhou, Q., and Engel, D. A. (1995). Adenovirus E1A243 disrupts the ATF/CREB-YY1 complex at the mouse c-fos promoter. *J. Virol.* **69,** 7402–7409.

CHAPTER 9

Molecular Control of Endothelial Derived Nitric Oxide

A New Paradigm for Endothelial NOS Regulation by Posttranslational Modifications

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REGULATION OF ENDOTHELIUM-DERIVED NITRIC OXIDE (NO) PRODUCTION IS AN IMPORTANT AREA OF RESEARCH RELEVANT TO VARIOUS ASPECTS OF VASCULAR BIOLOGY INCLUDING VASOMOTOR CONTROL, THROMBOSIS, INFLAMMATION, VASCULAR REMODELING, AND ANGIOGENESIS. THE PURPOSE OF THIS CHAPTER IS TO HIGHLIGHT RECENT DEVELOPMENTS IN THE FIELD OF ENDOTHELIAL NITRIC OXIDE SYNTHASE REGULATION BY POSTTRANSLATIONAL CONTROL MECHANSIMS AS THEY PERTAIN TO BOTH BASAL AND STIMULATED PRODUCTION OF NO.

Introduction

The physiological production of nitric oxide (NO) by the vascular endothelium is important for cardiovascular homeostasis. NO synthesized by the nitric oxide synthase (NOS) family member, endothelial NOS (eNOS or NOS3), is the enzyme that produces endothelium-derived relaxing factor, identified as NO as originally demonstrated by the classic studies of Furchgott, Ignarro, and Moncada (Furchgott and Zawadski, 1981; Ignarro *et al.,* 1987; Palmer *et al.,* 1987). eNOS-derived NO is important for blood pressure control, angiogenesis, and vascular remodeling as demonstrated by the profound perturbations in these processes in mice deficient in this NOS isoform (Huang *et al.,* 1995; Murohara *et al.,* 1998; Rudic *et al.,* 1998). The purpose of this chapter is to highlight recent developments emerging in the area of eNOS regulation by posttranslational control mechanisms including fatty acylation, protein–protein interactions, and phosphorylation.

eNOS Fatty Acylation

N-Myristoylation

eNOS is unique among the NOS family of proteins due to the presence of an N-myristoylation consensus sequence elucidated from the cloning of its cDNA (Janssens *et al.,* 1992; Lamas *et al.,* 1992; Marsden *et al.,* 1992; Nishida *et*

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al., 1992; Sessa *et al.,* 1992). The presence of glycine 2 (Gly-2) and serine 6 (Ser-6) in bovine and human eNOS conforms to the preferred substrate specificity for *N*-myristoyltransferase (Gordon *et al.,* 1991). Different studies investigating this premise, through metabolic labeling and site-directed mutagenesis of Gly-2, confirmed that native eNOS is able to incorporates [^{3}H]myristic acid and that mutation of Gly-2 to an alanine (G2A mutation) converts the membrane-associated eNOS to a cytosolic form (Busconi and Michel, 1993; Liu and Sessa, 1994; Pollock *et al.,* 1992; Sessa *et al.,* 1993). The precise membrane compartments in which eNOS resides are controversial, but a majority of studies by several independent groups have shown that a large portion of membrane-associated eNOS is found on the Golgi complex in endothelial cells or cells stably transfected with the eNOS cDNA (Garcia-Cardena *et al.,* 1996a; Rizzo *et al.,* 1998; Sessa *et al.,* 1995; Stanboli and Morin, 1994). In transfected HEK 293 cells, wild-type eNOS localizes to the Golgi complex and produces ample quantities of NO, whereas cell lines expressing G2AeNOS, which is cytoplasmically distributed, are poor releasers of NO (Sessa *et al.,* 1995). This study was the first to suggest that compartmentalization of eNOS is important for the fidelity of stimulus-dependent NO release from cells. Interestingly, the kinetics of wild-type and G2A eNOS are identical both in cell lysates (Sessa *et al.,* 1993, 1995) and after purification to homogeneity (Venema *et al.,* 1995), in support of the idea that N-myristoylation is important for targeting but not the catalytic function of the enzyme.

Palmitoylation

On the basis of models with N-myristoylated peptides and certain lipids, it appears as if N-myristoylation per se is not sufficient to stably anchor proteins to biological membranes (Peitzsch and McLaughlin, 1993). This is supported by the observations that several N-myristoylated proteins are found in the cytosolic fractions of cells (Gordon *et al.,* 1991). High-salt treatment or membrane denaturation failed to release eNOS from the membrane fraction of endothelial cells (Busconi and Michel, 1994; Pollock *et al.,* 1991), suggesting that the association of eNOS with membranes be mainly due to strong hydrophobic interactions. Therefore, we and others suspected that additional modifications must be involved to keep eNOS tightly embedded in membranes. Posttranslational palmitate incorporation on cysteine residues of proteins, by the enzyme palmitoyltransferase, increases the hydrophobic properties of some proteins, thus facilitating stronger interactions with lipid bilayers (Resh, 1994). Using metabolic labeling and V8 protease digestion of labeled eNOS, we demonstrated that eNOS is palmitoylated on cysteine-15 and cysteine-26 (Liu *et al.,* 1995), and mutation of either cysteines reduced [^{3}H]palmitic acid incorporation (by 95%) into eNOS, suggesting that palmitoylation of either cysteine is highly dependent on the presence of the other. However, it is controversial whether palmitoylation of eNOS will influence its overall ability to interact with membranes. In our laboratory, palmitate incorporation is not necessary for membrane association of eNOS as the enzyme localizes in the membrane fractions following mutation of both cysteine-15 and cysteine-26 to serine (C15/26S eNOS). In addition, treatment of wild type versus palmitoylation mutants of eNOS with high ionic strength, high pH, or phase solubilization with Triton X-114 (a way to examine protein hydrophobicity) does not change the association of eNOS with membranes. However, another group has demonstrated that palmitoylation mutants of eNOS can partition slightly better into the cytosol, suggesting that palmitate did influence the overall hydrophobic character of the protein (Robinson and Michel, 1995). Regardless of these inconsistencies, both groups have shown that N-myristoylation must precede cysteine palmitoylation. Utilizing a different approach with various deletion mutants of eNOS coupled to the green fluorescent protein (GFP), N-myristoylation without palmitoylation does permit eNOS to interact with biological membranes and results in a diffuse perinuclear membrane appearance of the protein (Liu *et al.,* 1997). Identical results were obtained with C15/26S palmitoylation deficient eNOS-GFP and mutations that change the pentameric glycine–leucine repeat between the palmitoylation sites.

Palmitoylation is a reversible process, unlike N-myristoylation (Gordon *et al.,* 1991). The basal turnover of palmitate on eNOS is 45 min, while the turnover of myristate and the protein are close to 18–20 hours (Liu *et al.,* 1995). There is evidence that palmitoylation and depalmitoylation of proteins can be regulated by extracellular signals providing a mechanism for the dynamic regulation of protein localization (Degtyarev *et al.,* 1993; James and Olson, 1989; Mumby *et al.,* 1994; Wedegaertner and Bourne, 1994) however, this is a controversial area due to the lack of consensus demonstrating that palmitoylation (of G-protein α subunits, and/or src family members) really influences protein hydrophobicity (Huang *et al.,* 1999). The controversy spills into the NOS field since different groups have reported discrepant findings. One group has demonstrated that bradykinin and calcium ionophore stimulation of endothelial cells induces the depalmitoylation of eNOS (Robinson *et al.,* 1995), resulting in its translocation from the membrane to the cytosol; however this result was not shown by other groups who have documented that agonists cause an increase in calcium, NO release, and eNOS phosphorylation (Liu *et al.,* 1995; D. G. Harrison, personal communication) with no effect on the rate of palmitoylation and depalmitoylation. So if palmitoylation of eNOS does not influence the catalytic activity or overall hydrophobicity, then what is its role? Two groups independently reported that palmitoylation of eNOS is necessary for targeting of eNOS to specific plasmalemmal domains called caveolae (Garcia-Cardena *et al.,* 1996a; Shaul *et al.,* 1996). We will discuss in the following sections the importance of eNOS localization into caveolae microdomains.

eNOS Trafficking into Caveolae

Caveolin and Caveolae

Caveolae are small, specialized cholesterol- and sphingolipid-rich invaginations of the plasma membrane. They are similar in composition to lipid rafts, which can be a target for GPI linked or fatty acylated proteins. Caveolae are present in most eukaryotic cell types and are abundantly found in capillary endothelium, vascular smooth muscle, fibroblasts, and adipocytes (Okamoto *et al.,* 1998). These specialized membrane domains have been implicated in transcytosis of macromolecules (Schnitzer *et al.,* 1994), in potocytosis (Anderson, 1993), and in signal transduction (Lisanti *et al.,* 1994).

The major structural protein of caveolae is caveolin, a 21- to 24-kDa integral membrane protein which, at first, received attention as a tyrosine phosphorylated substrate of the Rous sarcoma viral oncogene on cellular transformation and was subsequently shown to be associated with cell caveolae (Glenney and Soppet, 1992; Kurzchalia *et al.,* 1992; Rothberg *et al.,* 1992). Caveolin expression corresponds to some extent with caveola formation, furthermore cell lines that do not express caveolin, such as lymphocytes and neuroblastoma cells, appear to function without detectable number of caveolae presumably due to lipid rafts which may subserve a similar functional role (Fra *et al.,* 1994; Gorodinsky and Harris, 1995). The caveolin family comprises three isoforms, caveolin-1, -2, and -3, that show general similarity in structure and function but differ in specific properties and tissue distribution. Two isoforms of caveolin-1, α and β, are derived from alternate initiator codons during translation. Caveolin-3 shows a muscle specific tissue distribution (e.g., skeletal muscle and myocytes) and may replace caveolin-1 as the major caveolar protein of differentiated muscle cells (Way and Parton, 1995). Caveolin-1 and caveolin-2 are abundantly expressed in adipocytes, endothelial cells, and fibroblastic cell types. They also colocalize in the cell and may form a stable hetero-oligomeric complex *in vivo* (Scherer *et al.,* 1997).

The amino and carboxyl termini of caveolin are cytoplasmic and are flanked by an unusual 33-amino acid putative transmembrane domain. Caveolin also possess multiple palmitoylation sites at the carboxyl-terminal end. The role of caveolin in caveolar formation may be related to the self-assembly properties of caveolin-1. Firstly, in the endoplasmic reticulum, caveolin-1 monomers assemble into homo-oligomers (14–16 monomers per oligomers). Subsequently, these oligomers can interact with each other to form clusters of particles of 25–50 nm in diameter. Finally, these oligomers can specifically interact with glycosphingolipids and cholesterol, and they require a high cholesterol content to insert themselves into lipid membranes. Thus, through the interaction of caveolin-1 with itself and the selectivity of caveolin for the endogenous lipid component of the membrane, a caveola vesicle may be generated (Li *et al.,* 1996).

eNOS Localization in Caveolae

A fraction of eNOS has been shown to reside in a Triton X-100 insoluble fraction of endothelial cell lysates. Triton X-100 insolubility of proteins has been considered as a marker of both cytoskeletal proteins and, more recently, proteins residing in caveolae and other glycolipid-rich domains (Garcia-Cardena *et al.,* 1996a). Using the low buoyant properties of these microdomains, purification of caveolae has been achieved by different fractionation techniques. This has allowed different laboratories to show that the caveolae are enriched by many signaling molecules, including components of the Ras-extracellular signal-related kinase (ERK) pathway (Anderson, 1998; Oh and Schnitzer, 1999; Smart *et al.,* 1995). Interestingly, many of the proteins shown to be present in plasmalemmal caveolae are, like eNOS, dually acylated. Using the previously mentioned techniques and purification of lumenal caveolae with colloidal silica, it was shown that eNOS is enriched in the caveola fractions of endothelial cells (Garcia-Cardena *et al.,* 1996a; Shaul *et al.,* 1996). Furthermore, immunolabeling and electron microscopy studies have shown that some of eNOS colocalizes with caveolin-1 and in caveolae (Garcia-Cardena *et al.,* 1996a; Rizzo *et al.,* 1998; Shaul *et al.,* 1996). The absolute amount of eNOS in caveolae may vary within vascular beds (Andries *et al.,* 1998), between species, or change with the passage or confluency state of the endothelial cells in culture (Sowa *et al.,* 1999).

The targeting of eNOS to caveolae is dependent on cysteine palmitoylation of eNOS. Mutation of the cysteine palmitoylation sites markedly reduces its targeting to the plasma membrane and caveolae (Garcia-Cardena *et al.,* 1996a; Shaul *et al.,* 1996; Sowa *et al.,* 1999) and stimulated NO release from stably transfected HEK cells (Liu *et al.,* 1996), suggesting that palmitoylation is a ''molecular zip code'' for the trafficking of eNOS into glycolipid-rich microdomains of the plasmalemma and for efficient NO release. Interestingly, a study using fluorescent recovery after photobleaching (FRAP) of eNOS-GFP in living cells has demonstrated that the trafficking rates of eNOS-GFP differ in Golgi versus plasma membrane suggesting that each pool of enzyme may uniquely interact organelle specific proteins that determine its rate of movement (Sowa *et al.,* 1999). In cells expressing palmitoylation mutant eNOS-GFP, the protein is diffusely distributed in the perinuclear region of cells, does not move into intercellular apical junctions, and has a much faster rate of movement compared to wild-type NOS in the Golgi and plasma membrane. These data support the ideas that (1) palmitoylation is a targeting motif for eNOS and (2) that palmitoylation can kinetically slow down the rate of protein movement in cellular microdomains.

eNOS Interactions with Caveolin

Caveolin, being the major coat protein of caveolae, has the capacity to directly interact with other intracellular pro-

teins such as c-Src and Ha-Ras through amino acids 82–101, the putative scaffolding domain (Okamoto *et al.,* 1998). Indeed, three groups have independently demonstrated that eNOS could directly interact with caveolin-1 or caveolin-3 (Feron *et al.,* 1996; Garcia-Cardena *et al.,* 1996b, 1997; Ju *et al.,* 1997). Mapping of the eNOS binding site in caveolin was studied by the usage of recombinant GST–caveolin fusion proteins. The primary binding region of caveolin-1 for eNOS is within amino acids 60–101 and to a lesser extent within amino acids 135–178 (Garcia-Cardena *et al.,* 1997; Ju *et al.,* 1997). These results were also obtained in a yeast two-hybrid system where the different domains of eNOS and caveolin-1 were expressed (Ju *et al.,* 1997). Furthermore, the caveolin–eNOS immunocomplex is disrupted in presence of caveolin scaffolding peptides (Michel *et al.,* 1997a).

In order to determine what is the preferred and specific binding motif for proteins that can interact with caveolin-1, Couet *et al.* (1997) used a phage display library and found a consensus caveolin binding sequence. This sequence is rich in aromatic residues with specific spacing between each of them, and the motifs $\phi\chi\chi\phi\chi\chi\chi\phi$, $\phi\chi\chi\chi\chi\phi\chi\chi\phi$, and $\phi\chi\phi\chi\chi\chi\chi\phi\chi\chi\phi$ (where ϕ represents either Trp, Phe, or Tyr and χ represents any amino acid) were shown to bind the caveolin scaffolding domain. Interestingly, these motifs are present in most known caveolin-interacting proteins and were shown to be important for specific caveolin interactions (Couet *et al.,* 1997). eNOS contains one of these particular caveolin-binding sequences (**Phe**-Ser-Ala-Ala-Pro-**Phe**-Ser-Glu-**Trp**) located within amino acids 350–358.

Importance of the caveolin interaction with eNOS has been most reproducibly demonstrated by the effects of caveolin scaffolding peptides and GST–caveolin on NOS activity. Incubation of pure eNOS with peptides derived from the scaffolding domains of caveolin-1 and -3 resulted in inhibition of eNOS activity (Garcia-Cardena *et al.,* 1997). In cotransfection experiments, caveolin overexpression in COS-7 cells resulted in a reduction of eNOS activity (Michel *et al.,* 1997b), and a reduction in NO release was also observed (Garcia-Cardena *et al.,* 1997). Furthermore, site-directed mutagenesis of the predicted caveolin binding motif within eNOS blocked the ability of caveolin to suppress NO release in the latter experiments (Garcia-Cardena *et al.,* 1997). The reduction of eNOS activity by caveolin peptides, or overexpressed caveolin, is reversed by exogenous addition of calmodulin, suggesting a reciprocal regulation of eNOS by calmodulin, an activator, and caveolin, an inhibitor (Feron *et al.,* 1998a,b; Michel *et al.,* 1997a,b). This dual regulation of eNOS by these two proteins has also been shown in terms of protein interactions *in vitro.* The coimmunoprecipitation of the eNOS/caveolin-1 complex is favored in absence of calcium and reduced by addition of calcium–calmodulin. Stimulation of cells by calcium-dependent agonists and calcium ionophores results in a reduced eNOS–caveolin interaction. However, the prolonged time frame used to demonstrate these reciprocal interactions are not consistent with NO release from cells.

Collectively, these overall results suggest that NO production is negatively regulated by interactions with caveolin and that for NO release to occur, the inhibitory clamp by caveolin must be overcome. Calmodulin (CaM) has been proposed to be solely responsible for the dissociation of eNOS from caveolin. However, the releationship between caveolin as an inhibitor of eNOS and CaM as its allosteric modulator have not been examined in light of new findings demonstrating a role for heat-shock protein 90 (hsp90) and protein phosphorylation as regulator of eNOS activation (see below). It remains to be confirmed if activation of eNOS implies dissociation from caveolin; that is, the inhibitory action might be overcome without any physical dissociation of the eNOS from caveolin or caveolae, particularly during calcium-independent stimulation of NO release elicited by prolonged shear stress (Rizzo *et al.,* 1998). There is no direct evidence to date showing that disruption of the eNOS–caveolin complex can lead to increased NO release from cells, a fundamental experiment if caveolin-1 truly regulates eNOS and NO release. The observed dissociation of caveolin and eNOS following stimulation of endothelial cells by calcium mobilizing agonists, may be a consequence of eNOS activation rather than removal of an inhibitory clamp on eNOS activity. However, there is a recent report demonstrating that loading of cells with low density lipoprotein (LDL)–cholesterol increases caveolin-1 expression and its interaction with eNOS, thereby dampening agonist stimulated NO release (Feron *et al.,* 1999) but another report shows no effects of cholesterol loading on caveolin-1 levels, but increases in eNOS protein levels (Peterson *et al.,* 1999). In this latter study, the fractionation of eNOS into caveolin-enriched domains is enhanced whereas agonist-induced NO release is not effected (Peterson *et al.,* 1999). The interactions of eNOS with other activating proteins such as hsp90 or after phosphorylation might allow us to better understand the regulatory cycle of basal versus stimulated NO release and the additional roles of caveolin in NO biosynthesis.

hsp90 and eNOS

As discussed previously, eNOS is negatively regulated by a direct interaction with caveolin. However, more recent work has identified a positive regulator of eNOS, hsp90 (Garcia-Cardena *et al.,* 1998).

hsp90

The hsp90 family is a group of highly conserved stress proteins that are expressed in all eukaryotic cells (Pratt, 1997). Two genes encode hsp90, with the human gene products hsp90α and hsp90β having 86% sequence homology. Hsp90 is highly abundant in cells, accounting for 1–2% of cytosolic protein, and it is localized to the cytoplasm, with a small amount found in the nucleus (Czar *et al.,* 1996; Pratt, 1997). The main function of hsp90 has been its involvement

in a multicomponent chaperone system that is responsible for the proper folding of proteins such as steroid receptors and cell-cycle dependent kinases (Buchner, 1999). However, the abundance of hsp90 associated with signaling proteins suggests that this may not be its only function. There is increasing evidence that hsp90 may be an integral part of signal transduction in all cells.

hsp90 Involvement in Signal Transduction

A variety of peripheral membrane signaling proteins including v-Src (Oppermann *et al.,* 1981), Raf-1 (Stancato *et al.,* 1993), MEK (Stancato *et al.,* 1997), and G-protein βγ (Inanobe *et al.,* 1994) can exist in heteromeric complexes with hsp90. Genetic studies in both *Drosophila melanogaster* and *Saccharomyces cerevisiae* have demonstrated that hsp90 homologs are essential genes (Cutforth and Rubin, 1994; Nathan and Lindquist, 1995). In *Drosophila melanogaster* (Cutforth and Rubin 1994), the hsp90 ortholog hsp83 is involved in mediating receptor tyrosine kinase signaling pathways, whereas in *Saccharomyces cerevisiae,* hsp82 is an integral component of reconstituted steroid receptor and v-Src-dependent signal transduction events (Nathan and Lindquist, 1995). Potential mechanisms to account for these effects include hsp90-mediated actions on protein folding, stability, and targeting of signaling proteins.

hsp90–eNOS Interaction

Previously we had shown that eNOS coprecipitated with a 90-kDa tyrosine phosphorylated protein (Garcia-Cardena *et al.,* 1998) and work by Venema *et al.* (1996) has demonstrated that bradykinin induced the recruitment of a 90-kDa protein to eNOS (Venema *et al.,* 1996). In metabolic labeling studies in our laboratory, immunoprecipitation of ^{35}S-labeled eNOS resulted in the coprecipitation of a 90-kDa protein (J.-P. Gratton, J. Fontana, and W. C. Sessa, unpublished). These findings, coupled with the cadre of exciting information linking hsp90 to signal transduction, prompted our investigation into whether hsp90 was associated with eNOS. Indeed, hsp90 was associated with eNOS in resting endothelial cells, and treatment of cells with three distinct stimuli that cause NO release [vascular endothelial growth factor (VEGF), histamine, and fluid shear stress] all enhanced the interaction between hsp90 and eNOS (Garcia-Cardena *et al.,* 1998). More importantly, VEGF or histamine stimulated the rapid recruitment of hsp90 to eNOS in endothelial cells in a time frame consistent with NO release. Fluid shear stress also caused the recruitment of hsp90 to eNOS, but the kinetics of the recruitment were slower with maximal recruitment at 1 hour.

To further study the relationship between hsp90-mediated signaling and NO production, a specific inhibitor of hsp90, the ansamycin antibiotic geldanamycin (GA), was used. Geldanamycin binds to the ATP site of hsp90 and influences the conformational stability of hsp90 binding to its substrates (Stebbins *et al.,* 1997). Activation of protein kinases such as Raf-1 (Schulte *et al.,* 1995; Stancato *et al.,* 1997) and v-Src (Whitesell *et al.,* 1994) and facilitation of steroid hormone binding to nuclear receptors (Sullivan and Toft, 1993) are all signaling events dependent on hsp90 and are blocked by GA. Treatment of endothelial cells with GA attenuated histamine- and VEGF-stimulated cGMP production but did not influence the ability of the NO donor sodium nitroprusside to increase cGMP production, indicating that inhibition of agonist-induced NO release was due to inhibition of hsp90 signaling. To test the involvement of hsp90 in endothelium-dependent relaxations of isolated blood vessels, the influence of GA on acetylcholine (ACh)-induced relaxations of the rat aorta was examined. GA blocked ACh-induced vasorelaxation of rat aortic rings by greater than 70%, and these effects were reversible, as washout of GA restored ACh-induced vasorelaxation (Garcia-Cardena *et al.,* 1998). Collectively these data supported the idea that the rapid activation of hsp90 (perhaps via phosphorylation) and the subsequent binding to eNOS occurred on a time frame consistent with NO release.

Further support for the relevance of hsp90–eNOS interactions was demonstrated in a model of portal vein ligation (PVL) in rats (Shah *et al.,* 1999). In this study, the participation of hsp90 in the eNOS-dependent hyperresponsiveness in rat mesentery was examined. In normal animals eNOS and hsp90 were coassociated in extracts prepared from the mesenteric microcirculation and both eNOS and hsp90 localized to the endothelial lining of mesenteric vessels. In the perfused mesenteric vasculature of normal animals, GA attenuated ACh-dependent vasodilatation to the same extent as conventional NOS inhibitors. In portal hypertensive rats, eNOS protein levels are not changed compared to control rats, but NOS activity is markedly enhanced in the mesenteric tissue of hypertensive rats. The enhanced activity correlated with hyporesponsiveness to the vasoconstrictor methoxamine (MTX) in perfusion studies (Sieber *et al.,* 1992). Interestingly, GA potentiated the MTX-induced vasoconstriction after PVL, partially reversing the hyporeactivity to this agent, indicating that hsp90 can act as a signaling component leading to NO-dependent responses in the mesenteric microcirculation.

Potential Mechanisms for hsp90 Recruitment and Activation of eNOS

The evidence discussed earlier provides a great deal of support to the hypothesis that hsp90 associates with eNOS in a stimulus-dependent manner, and it suggests that hsp90 is necessary for both NO release and endothelium-dependent responses within intact blood vessels. However, the intracellular signaling pathways that lead to association of hsp90 and eNOS and the mechanism by which hsp90 is able to activate eNOS remain unresolved. The rapid stimulus-dependent formation of the hsp90–eNOS heteromeric complexes suggests that it occurs simultaneously with other

signaling events, such as the mobilization of intracellular calcium and/or protein phosphorylation. Calcium mobilization will lead to activation of calmodulin, a known NOS-interacting protein that may, in turn, influence the association of hsp90 and eNOS. The change in the state of phosphorylation of proteins in the VEGF signaling pathway may lead to activation of downstream effectors required for the hsp90–eNOS association. Also, a change in the phosphorylation state of hsp90 and eNOS themselves may result in a conformational change leading to increased association of the two. These possibilities are currently being examined.

When hsp 90 interacts with eNOS, how does this influence NO release? Previously we showed that hsp90 can directly activate eNOS *in vitro* (Garcia-Cardena *et al.,* 1998) and that coexpression of eNOS with hsp90 in COS cells increased NOS activity. These results suggest that hsp90 may act as an allosteric modulator of eNOS by inducing a conformational change in the enzyme, which results in increased activity or possibly stabilizes the complex. A paper by Bender *et al.* (1999) demonstrated that neuronal NOS (nNOS) could also interact with hsp90. In that paper, hsp90 is associated with nNOS in cells in which the nNOS protein is overexpressed, and the interaction was detected using lower stringency immunoprecipitation conditions than those used for eNOS/hsp90. Short-term treatment with GA of cells expressing nNOS decreased NO release, an effect not associated with a change in nNOS levels, whereas long-term treatment with GA reduced nNOS protein levels. In these experiments the authors could not demonstrate an effect of hsp90 on apo-nNOS reconstituted with heme; however, hsp90 did increase heme incorporation. Overall the authors concluded that if heme is rate limiting, hsp90 is required for sustained opening of the heme binding cleft in NOS; on the other hand, when heme is not the limiting factor, as in a cell-free system, hsp90 is not required (Bender *et al.,* 1999). This is an interesting idea and may be one of the ways hsp90 activates eNOS, but the findings do not completely coincide with the studies on hsp90 and eNOS. First, the kinetics of exogenous heme incorporation into nNOS is much slower than that of eNOS activation in response to VEGF. Second, hsp90 does influence eNOS conformation in order to increase its activity, but it remains to be seen if heme binding or stabilization is the only action that hsp90 exerts on eNOS.

eNOS Regulation by Protein Phosphorylation

Endothelial NOS can be phosphorylated on serine, tyrosine, and threonine residues. eNOS serine phosphorylation was originally described by Michel *et al.,* who demonstrated that different forms of stimuli such as bradykinin, ionomycin, and sodium nitroprusside can increase eNOS serine phosphorylation (Michel *et al.,* 1993). It was subsequently demonstrated that activation of protein kinase C increases eNOS serine phosphorylation (Hirata *et al.,* 1995; Ohara *et al.,* 1995). In addition, we demonstrated that eNOS is phosphorylated on both tyrosine and serine, an effect which is markedly enhanced by the tyrosine phosphatase inhibitor vandium (Garcia-Cardena *et al.,* 1996b). In these experiments the documented increase in tyrosine phosphorylation was associated with a decrease in eNOS activity in immunoprecipitates. Phosphorylation of eNOS on serine, tyrosine, and threonine was demonstrated by Flemming *et al.* (1998). These authors also showed that fluid shear stress enhanced eNOS serine phosphorylation but reduced tyrosine phosphorylation, whereas Corson *et al.* (1996) showed that shear stress increased serine phosphorylation only, with no detectable levels of tyrosine phosphorylation. However, in all the above cases, the site(s) of phosphorylation was not described, nor was a direct physiological link between phosphorylation and eNOS activation established.

Previously it was shown that insulin- and VEGF-driven NO release from endothelial cells was partially attenuated by structurally distinct inhibitors of phosphatidylinositol 3-kinase (PI-3 kinase) (Papapetropoulos *et al.,* 1997; Zeng and Quon, 1996). These findings sparked our interest in examining the possible participation of downstream kinases in the activation of eNOS. One downstream effector of PI-3 kinase is the serine/threonine kinase, called protein kinase B or Akt (Downward, 1998; Franke *et al.,* 1997). On activation of growth factor receptors, G-protein-coupled receptors, or mechanotransduction, Akt is recruited to the plasma membrane (via its pleckstrin homology domain) and binds to PI-3-kinase generated inositol phospholipids {phosphatidylinositol 1,4,5-trisphosphate (PIP_3), phosphatidylinositol 3,4-bisphosphate [PI $(3,4)P_2$]}. Once in the plasma membrane, Akt is phosphorylated on threonine-308 (by the serine/threonine kinase PDK-1) and/or calmodulin dependent protein kinase kinase (CaMKK) and on serine-473 (by the serine/threonine kinase PDK2) resulting in its ability to phosphorylate and inhibit effectors such as the proapoptotic proteins, BAD and caspase-9 or influence cell metabolic pathways by phosphorylating phosphofructokinase (PFK) and glycogen synthase kinase (GSK).

To examine the relationship between Akt and eNOS, we examined if Akt overexpression could stimulate eNOS-derived NO production in a heterologous expression system (Fulton *et al.,* 1999). Indeed, this did occur by direct phosphorylation of serine-1179, *in vitro* and *in vivo.* Mutation of serine-1179 to alanine attenuated Akt-stimulated NO production, whereas mutation of serine-1179 to aspartate, mimicking the negative charge afforded by the phosphate, results in constitutive activation of eNOS. More importantly, adenoviral transduction of endothelial cells with activated Akt increased basal NO production, whereas transduction with activation-deficient Akt prevented VEGF-driven NO release. Thus, eNOS is the first plasma membrane-associated substrate of Akt, coupling the release of the potent vasodilator NO to the known vasculoprotective actions of Akt. Two additional groups reported identical results using fluid shear stress and VEGF as ways to activate Akt and eNOS, building a strong consensus that eNOS phosphorylation exerts a

major influence on stimulus-dependent NOS activation (Dimmeler *et al.,* 1999; Michell *et al.,* 1999).

Summary and Model of eNOS Activation/Inactivation

The literature on eNOS subcellular trafficking, studies on protein–protein interactions with caveolin and hsp90, and recent evidence supporting a role of protein phosphorylation have been briefly overviewed. Taking into account the complex interplay of signaling systems, we propose the following working model for eNOS activation in endothelial cells. In cells where eNOS is in caveolae, it is likely that binding to caveolin-1 may negatively regulate its function. On stimulation with calcium-mobilizing agonists such as bradykinin, acetylcholine, or VEGF, the simultaneous recruitment of hsp90 and CaM to eNOS may loosen the eNOS–caveolin inhibitory interaction, thus permitting Akt-induced phosphorylation and NO release. In the context of a stimulus that does not elevate intracellular calcium, such as fluid shear stress (Ayajiki *et al.,* 1996; Kuchan and Frangos, 1994; O'Neill, 1995), estrogen (Caulin-Glaser *et al.,* 1997), and insulin (Tsukahara *et al.,* 1994), the recruitment of hsp90 to eNOS may weaken the eNOS–caveolin interaction. Again phosphorylation by Akt occurs, resulting in NO release. Interestingly, overexpression of dominant negative Akt blocks both VEGF- and shear-induced NO release, suggesting that phosphorylation may be the common denominator for both forms of stimulation. In terms of eNOS inactivation, a decrease in cytoplasmic calcium will facilitate CaM and hsp90 dissociation, and, presumably, eNOS dephosphorylation will re-equilibrate the system back to baseline levels. The temporal and spatial nature of this model is not known. Definitive evidence showing that this mechanism occurs in caveolae is lacking, and such complex interactions may also take place with eNOS localized on the Golgi and in the cytosol. Further dissection of this model utilizing structural biology of protein complexes, reconstituted systems, and dominant negative strategies will permit a clearer picture of eNOS activation to emerge in the not-too-distant future.

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References

Anderson, R. G. W. (1993). Potocytosis of small molecules and ions by caveolae. *Trends Cell Biol* **3,** 69–72.

Anderson, R. G. (1998). The caveolae membrane system. *Annu. Rev. Biochem.* **67,** 199–225.

Andries, L. J., Brutsaert, D. L., and Sys, S. U. (1998). Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. *Circ. Res.* **82,** 195–203.

Ayajiki, K., Kindermann, M., Hecker, M., Fleming, I., and Busse, R. (1996). Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells [see comments]. *Circ. Res.* **78,** 750–8.

Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B., and Osawa, Y. (1999). Neuronal nitric-oxide synthase is regulated by the Hsp90-based chaperone system in vivo. *J. Biol. Chem.* **274,** 1472–1478.

Buchner, J. (1999). Hsp90 & Co.—a holding for folding. [Review] [57 refs.]. *Trends Biochem. Sci.* **24,** 136–141.

Busconi, L., and Michel, T. (1993). Endothelial nitric oxide synthase. N-terminal myristoylation determines subcellular localization. *J. Biol. Chem.* **268,** 8410–8413.

Busconi, L., and Michel, T. (1994). Endothelial nitric oxide synthase membrane targeting. Evidence against involvement of a specific myristate receptor. *J. Biol. Chem.* **269,** 25016–25020.

Caulin-Glaser, T., Garcia-Cardena, G., Sarrel, P., Sessa, W. C., and Bender, J. R. (1997). 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ. Res.* **81,** 885–892.

Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C., and Harrison, D. G. (1996). Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ. Res.* **79,** 984–991.

Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997). Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *J. Biol. Chem.* **272,** 6525–6533.

Cutforth, T., and Rubin, G. M. (1994). Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in *Drosophila. Cell* **77,** 1027–1036.

Czar, M. J., Welsh, M. J., and Pratt, W. B. (1996). Immunofluorescence localization of the 90-kDa heat-shock protein to cytoskeleton. *Eur. J. Cell Biol.* **70,** 322–330.

Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. (1993). Increased palmitoylation of the Gs protein alpha subunit after activation by the beta-adrenergic receptor or cholera toxin. *J. Biol. Chem.* **268,** 23769–23772.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399,** 601–605.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.* **10,** 262–267.

Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996). Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *J. Biol. Chem.* **271,** 22810–22814.

Feron, O., Michel, J. B., Sase, K., and Michel, T. (1998a). Dynamic regulation of endothelial nitric oxide synthase: Complementary roles of dual acylation and caveolin interactions. *Biochemistry* **37,** 193–200.

Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998b). The endothelial nitric-oxide synthase–caveolin regulatory cycle. *J. Biol. Chem.* **273,** 3125–3128.

Feron, O., Dessy, C., Moniotte, S., Desager, J. P., and Balligand, J. L. (1999). Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J. Clin. Invest.* **103,** 897–905.

Fleming, I., Bauersachs, J., Fisslthaler, B., and Busse, R. (1998). Ca^{2+}-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ. Res.* **82,** 686–695.

Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994). Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem.* **269,** 30745–30748.

Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997). P13K: Downstream AKTion blocks apoptosis. *Cell* **88,** 435–437.

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399,** 597–601.

Furchgott, R. F., and Zawadski, J. V. (1981). The obligatory role of the endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E., and Sessa, W. C. (1996a). Targeting of nitroc oxide synthase to endothelial cell caveolae via palmitoylation: Implications for nitric oxide signaling. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6448–6453.

Garcia-Cardena, G., Fan, R., Stern, D. F., Liu, J., and Sessa, W. C. (1996b). Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.* **271,** 27237–27240.

Garcia-Cardena, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S., Lisanti, M. P., and Sessa, W. C. (1997). Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J. Biol. Chem.* **272,** 25437–25440.

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392,** 821–824.

Glenney, J. J., and Soppet, D. (1992). Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10517–10521.

Gordon, J. I., Duronio, R. J., Rudnick, D. A., Adams, S. P., and Gokel, G. W. (1991). Protein N-myristoylation. [Review] [49 refs.]. *J. Biol. Chem.* **266,** 8647–8650.

Gorodinsky, A., and Harris, D. A. (1995). Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. *J. Cell Biol.* **129,** 619–627.

Hirata, K., Kuroda, R., Sakoda, T., Katayama, M., Inoue, N., Suematsu, M., Kawashima, S., and Yokoyama, M. (1995). Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension* **25,** 180–185.

Huang, C., Duncan, J. A., Gilman, A. G., and Mumby, S. M. (1999). Persistent membrane association of activated and depalmitoylated G protein alpha subunits. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 412–417.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase [see comments]. *Nature* **377,** 239–242.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein in nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Inanobe, A., Takahashi, K., and Katada, T. (1994). Association of the beta gamma subunits of trimeric GTP-binding proteins with 90-kDa heat shock protein, hsp90. *J. Biochm.* **115,** 486–492.

James, G., and Olson, E. N. (1989). Identification of a novel fatty acylated protein that partitions between the plasma membrane and cytosol and is deacylated in response to serum and growth factor stimulation. *J. Biol. Chem.* **264,** 20998–21006.

Janssens, S. P., Shimouchi, a., Quertermous, T., Bloch, D. B., and Bloch, K. D. (1992). Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase [published erratum appears in *J. Biol. Chem.* (1992) Nov. 5;**267**31), 22694]. *J. Biol. Chem.* **267,** 14519–14522.

Ju, H., Zou, R., Venema, V. J., and Venema, R. C. (1997). Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J. Biol. Chem.* **272,** 18522–18525.

Kuchan, M. J., and Frangos, J. A. (1994). Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am. J. Physiol.* **266,** C628–C636.

Kurzchalia, T. V., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. (1992). VIP21, a 21-kD membrane protein is an integral component of trans-Golgi- network-derived transport vesicles. *J. Cell Biol.* **118,** 1003–1014.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6348–6352.

Li, S., Song, K. S., and Lisanti, M. P. (1996). Expression and characterization of recombinant caveolin. Purification by polyhistidine tagging and cholesterol-dependent incorporation into defined lipid membranes. *J. Biol. Chem.* **271,** 568–573.

Lisanti, M. P., Scherer, P. E., Zang, Z.-L., and Sargiacomo, M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: A signalling hypothesis. *Trends Cell Biol.* **4,** 231–235.

Liu, J., and Sessa, W. C. (1994). Identification of covalently bound amino-terminal myristic acid in endothelial nitric oxide synthase. *J. Biol. Chem.* **269,** 11691–11694.

Liu, J., Garcia, C. G., and Sessa, W. C. (1995). Biosynthesis and palmitoylation of endothelial nitric oxide synthase: Mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry* **34,** 12333–12340.

Liu, J., Garcia. C. G., and Sessa, W. C. (1996). Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: Implications for caveolae localization. *Biochemistry* **35,** 13277–13281.

Liu, J., Hughes, T. E., and Sessa, W. C. (1997). The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: A green fluorescent protein study. *J. Cell Biol.* **137,** 1525–1535.

Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S., and Michel, T. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett.* **307,** 287–293.

Michel, J. B., Feron, O., Sase, K., Prabhakar, P., and Michel, T. (1997a). Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J. Biol. Chem.* **272,** 25907–25912.

Michel, J. B., Feron, O., Sacks, D., and Michel, T. (1997b). Reciprocal regulation of endothelial nitric-oxide synthase by Ca^{2+}-calmodulin and caveolin. *J. Biol. Chem.* **272,** 15583–15586.

Michel, T., Li, G. K., and Busconi, L. (1993). Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6252–6256.

Michell, B. J., Griffiths, J. E., Mitchelhill, K. I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., de Montellano, P. R., Kemp, B. E., and Pearson, R. B. (1999). The Art kinase signals directly to endothelial nitric oxide synthase. *Curr. Biol.* **12,** 845–848.

Mumby, S. M., Kleuss, C., and Gilman, A. G. (1994). Receptor regulation of G-protein palmitoylation. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2800–2804.

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J. F., Fishman, M. C., Huang, P. L., and Isner, J. M. (1998). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101,** 2567–2578.

Nathan, D. F., and Lindquist, S. (1995). Mutational analysis of Hsp90 function: Interactions with a steroid receptor and a protein kinase. *Mol. Cell. Biol.* **15,** 3917–3925.

Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W., and Murphy, T. J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.* **90,** 2092–2096.

O'Neill, W. C. (1995). Flow-mediated NO release from endothelial cells is independent of K^+ channel activation or intracellular Ca^{2+}. *Am. J. Physiol.* **269,** C863–C869.

Oh, P., and Schnitzer, J. E. (1999). Immunoisolation of caveolae with high affinity antibody binding to the oligomeric caveolin cage. Toward understanding the basis of purification [In Process Citation]. *J. Biol. Chem.* **274,** 23144–23154.

Ohara, Y., Sayegh, H. S., Yamin, J. J., and Harrison, D. G. (1995). Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. *Hypertension* **25,** 415–420.

Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998). Caveolins, a family of scaffolding proteins for organizing ''preassembled signaling complexes'' at the plasma membrane. *J. Biol. Chem.* **273,** 5419–5422.

Opperman, H., Levinson, W., and Bishop, J. M. (1981). A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1067–1071.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Papapetropoulous, A., Garcia-Cardena, G., Madri, J. A., and Sessa, W. C. (1997). Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100,** 3131–3139.

Peitzsch, R. M., and McLaughlin, S. (1993). Binding of acylated peptides and fatty acids to phospholipid vesicles: Pertinence to myristoylated proteins. *Biochemistry* **32,** 10436–10443.

Peterson, T. E., Poppa, V., Ueba, H., Wu, A., Yan, C., and Berk, B. C. (1999). Opposing effects of reactive oxygen species and cholesterol on endothelial nitric oxide synthase and endothelial cell caveolae. *Circ. Res.* **85,** 29–37.

Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H., Nakane, M., and Murad, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10480–10484.

Pollock, J. S., Klinghofer, V., Forstermann, U., and Murad, F. (1992). Endothelial nitric oxide synthase is myristylated. *FEBS Lett.* **309,** 402–404.

Pratt, W. B. (1997). The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. [Review][183 refs.]. *Annu. Rev. Pharmacol. Toxicol.* **37,** 297–326.

Resh, M. D. (1994). Myristylation and palmitylation of Src family members: The fats of the matter. [Review]. *Cell* **76,** 411–413.

Rizzo, V., McIntosh, D. P., Oh, P., and Schnitzer, J. E. (1998). In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. *J. Biol. Chem.* **273,** 34724–34729.

Robinson, L. J., and Michel, T. (1995). Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targeting. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11776–11780.

Robinson, L. J., Busconi, L., and Michel, T. (1995). Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J. Biol. Chem.* **270,** 995–998.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell* **68,** 673–682.

Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O., Segal, S. S., and Sessa, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J. Clin. Invest.* **101,** 731–736.

Scherer, P. E., Lewis, R. Y., Volonte, D., Engelman, J. A., Galbiati, F., Couet, J., Kohtz, D. S., Van, D. E., Peters, P., and Lisanti, M. P. (1997). Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *J. Biol. Chem.* **272,** 29337–29346.

Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: Reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **127,** 1217–1232.

Schulte, T. W., Blagosklonny, M. V., Ingui, C., and Neckers, L. (1995). Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J. Biol. Chem.* **270,** 24585–24588.

Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D'Angelo, D. D., Lynch, K. R., and Peach, M. J. (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J. Biol. Chem.* **267,** 15274–15276.

Sessa, W. C., Barber, C. M., and Lynch, K. R. (1993). Mutation of N-myristoylation site converts endothelial cell nitric oxide synthase from a membrane to a cytosolic protein. *Circ. Res.* **72,** 921–924.

Sessa, W. C., Garcia, C. G., Liu, J., Keh, A., Pollock, J. S., Bradley, J., Thiru, S., Braverman, I. M., and Desai, K. M. (1995). The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J. Biol. Chem.* **270,** 17641–17644.

Shah, V., Wiest, R., Garcia-Cardena, G., Cadelina, G., Groszmann, R. J., and Sessa, W. C. (1999). Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. *Am. J. Physiol.* **277,** G463–G468.

Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G., and Michel, T. (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271,** 6518–6522.

Sieber, C., Beglinger, C., Jager, K., and Stalder, G. A. (1992). Intestinal phase of superior mesenteric artery blood flow in man [see comments]. *Gut* **33,** 497–501.

Smart, E. J., Ying, Y. S., Mineo, C., and Anderson, R. G. (1995). A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10104–10108.

Sowa, G., Liu, J., Papapetropoulos, A., Rex-Haffner, M., Hughes, T. E., and Sessa, W. C. (1999). Trafficking of endothelial nitric-oxide synthase in living cells. Quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *J. Biol. Chem.* **274,** 22524–22531.

Stanboli, A., and Morin, A. M. (1994). Nitric oxide synthase in cerebrovascular endothelial cells is inhibited by brefeldin A. *Neurosci. Lett.* **171,** 209–212.

Stancato, L. F., Chow, Y. H., Hutchison, K. A., Perdew, G. H., Jove, R., and Pratt, W. B. (1993). Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *J. Biol. Chem.* **268,** 21711–21716.

Stancato, L. F., Silverstein, A. M., Owens, G. J., Chow, Y. H., Jove, R., and Pratt, W. B. (1997). The hsp90-binding antibiotic geldanamycin decreases Raf levels and epidermal growth factor signaling without disrupting formation of signaling complexes or reducing the specific enzymatic activity of Raf kinase. *J. Biol. Chem.* **272,** 4013–4020.

Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, Hartl, F. U., and Pavletich, N. P. (1997). Crystal structure of an Hsp90-geldanamycin complex: Targeting of a protein chaperone by an antitumor agent. *Cell* **89,** 239–250.

Sullivan, W. P., and Toft, D. O. (1993). Mutational analysis of hsp90 binding to the progesterone receptor. *J. Biol. Chem.* **268,** 20373–20379.

Tsukahara, H., Gordienko, D. V., Tonshoff, B., Gelato, M. C., and Goligorsky, M. S. (1994). Direct demonstration of insulin-like growth factor-I-induced nitric oxide production by endothelial cells. *Kidney Int.* **45,** 598–604.

Venema, R. C., Sayegh, H. S., Arnal, J. F., and Harrison, D. G. (1995). Role of the enzyme calmodulin-binding domain in membrane association and phospholipid inhibition of endothelial nitric oxide synthase. *J. Biol. Chem.* **270,** 104705–14711.

Venema, V. J., Marrero, M. B., and Venema, R. C. (1996). Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. *Biochem. Biophys. Res. Commun.* **226,** 703–710.

Way, M., and Parton, R. G. (1995). M-caveolin, a muscle-specific caveolin-related protein [corrected and republished in *FEBS Lett.* (1996) Jan. 2; **378**(1), 108–112]. *FEBS Lett.* **376,** 108–112.

Wedegaertner, P. B., and Bourne, H. R. (1994). Activation and depalmitoylation of Gs alpha. *Cell* **77,** 1063–1070.

Whitesell, L., Mimnaugh, E. G., De, C. B., Myers, C. E., and Neckers, L. M. (1994). Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: Essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8324–8328.

Zeng, G., and Quon, M. J. (1996). Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J. Clin. Invest.* **98,** 894–898.

CHAPTER 10

Tetrahydrobiopterin: An Essential Cofactor of Nitric Oxide Synthase with an Elusive Role

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NITRIC OXIDE SYNTHASES (NOSs) ARE THE MOST RECENT ADDITION TO A GROUP OF ENZYMES, NOW TOTALING FIVE, THAT RELY ON THE NATURALLY OCCURRING COFACTOR (6*R*)-5,6,7,8-TETRAHYDRO-L-BIOPTERIN (BH_4) FOR CATALYTIC ACTIVITY. INTRACELLULAR LEVELS OF BH_4 DICTATE THE RATE OF NITRIC OXIDE (NO) SYNTHESIS BY NOSs AND EVEN DETERMINE THE END PRODUCT [NO, PEROXYNITRITE ($OONO^-$) or (O_2^-)]. WE NOW APPRECIATE THAT DIVERSE MECHANISMS REGULATE BH_4 EXPRESSION AND, ACCORDINGLY, SERVE AS VERIFIED OR POTENTIAL CONTROL POINTS FOR MODULATING NOS FUNCTION IN PHYSIOLOGY AND PATHOPHYSIOLOGY. INCLUDED IN THE GROUP OF BH_4-DEPENDENT ENZYMES ARE THE THREE AROMATIC AMINO ACID HYDROXYLASES (AAHs; PHENYLALANINE, TYROSINE, AND TRYPTOPHAN HYDROXYLASES), WHERE A REDOX FUNCTION OF BH_4 IN CATALYSIS IS WELL ESTABLISHED AND UNDERSTOOD. DESPITE THE ABSOLUTE DEPENDENCE OF NOS ON BH_4 FOR CATALYTIC FUNCTION, IT IS REMARKABLE THAT PINPOINTING THE MOLECULAR BASIS FOR THIS REQUIREMENT HAS BEEN AN ELUSIVE GOAL. INDEED, THE MECHANISM BY WHICH BH_4 FUNCTIONS IN NO SYNTHESIS IS ARGUABLY THE MOST POORLY UNDERSTOOD ASPECT OF NOS ENZYMOLOGY. THIS GAP IN OUR KNOWLEDGE EXISTS DESPITE RIGOROUS STRUCTURAL AND FUNCTIONAL STUDIES ATTEMPTING TO RESOLVE THE QUESTION OF BH_4 FUNCTION. ONE CERTAIN CONCLUSION IS THAT NOS USES BH_4 IN A MANNER THAT DIFFERS FROM ALL OTHER KNOWN BH_4-DEPENDENT ENZYMES. IN THE FIRST PART OF THIS CHAPTER, WE SURVEY THE ROLES OF PTERINS IN BIOLOGY AND PRESENT OUR CURRENT UNDERSTANDING OF BIOCHEMICAL PATHWAYS THAT DETERMINE BH_4 LEVELS IN MAMMALIAN CELLS AND HOW THEY ARE REGULATED. THIS INCLUDES A DISCUSSION OF GENETIC DEFECTS IN BH_4 METABOLISM. EVIDENCE IS THEN PRESENTED TO SUPPORT THE VIEW THAT BH_4 AVAILABILITY IS A MAJOR DETERMINANT OF MAMMALIAN NO SYNTHESIS RATE IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL SETTINGS. FINALLY, WE CONSIDER THE FUNCTION OF BH_4 IN NOS CATALYSIS. ALTHOUGH CONTROVERSIAL, INSIGHTS INTO BH_4 FUNCTION ARE GLEANED BY CONSIDERING THE APPARENTLY UNIQUE ATTRIBUTES OF STRUCTURAL AND FUNCTIONAL INTERACTIONS BETWEEN BH_4 AND NOS ISOFORMS, WHILE JUDICIOUSLY BORROWING FROM OUR KNOWLEDGE BASE OF BH_4 BINDING AND FUNCTION DERIVED FROM STUDIES OF AAHs. THE AUTHORS APOLOGIZE FOR THE SURVEY FORMAT OF THIS VOLUME THAT PRECLUDES SPECIFIC RECOGNITION OF THE MANY IMPORTANT SCIENTIFIC CONTRIBUTORS WHOSE WORK IS SURVEYED HEREIN.

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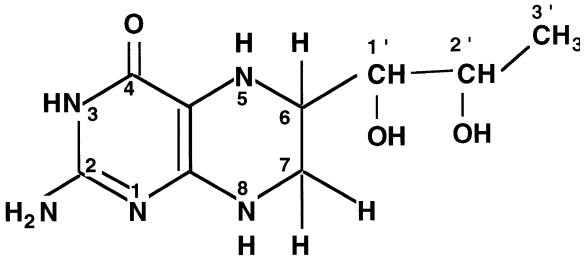
BH_4 Synthesis in Biological Systems

From the Wings of Butterflies to Hydroxylating Cofactor: An Overview of Pteridine Chemistry and Function in Biology

BH_4 is a pteridine, a chemical classification defined by a unique heterocyclic ring structure. The chemical structure of BH_4 is provided in Fig. 1, along with the convention for numbering pterin ring positions. It is notable that biopterin is defined as the pteridine analog wherein the heterocyclic ring is substituted with amino at the 2 position, carbonyl oxygen at the 4 position, and 1,2-dihydroxypropyl at the 6 position. This substituted ring can either by oxidized (biopterin), partially reduced (dihydrobiopterin; BH_2) or fully reduced (BH_4); changes in ring redox status have profound consequences for chemical and biological activity. Assymetry at C-6 endows biopterin with a stereochemical center; the (6*R*) isomer is naturally occurring. Biopterin devoid of its 6-dihydroxypropyl side chain is termed *pterin,* a heterocycle common to biopterin, folate, and molybdopterin cofactors. We now appreciate that pteridines have a broad range of functions in biology, ranging from eye pigments to light-gathering molecules to enzyme cofactors for redox and one-carbon transfers.

The first studies of what proved to be pteridines in biology can be traced to 1889, when Sir Frederick Gowland Hopkins reported attempts to isolate and purify the yellow pigments from wings of English butterflies (Hopkins, 1889). Elucidation of structures comprising three of these wing pigments was achieved by Robert Purrmann in the early 1940s (xanthopterin, leucopterin, and isoxanthopterin), revealing that all share a novel pyrazine [2,3-D]pyrimidine ring system that is the defining feature of the pteridine class of compounds. The name *pterin* originates from the Greek word *ptera* for wing, in recognition of the biological source from which members of this chemical class were first identified.

The field of pteridine chemistry advanced significantly in 1957, when Max Viscontini's group reported the isolation of two blue fluorescent eye pigments from *Drosophila melanogaster* (Viscontini and Karrer, 1957); these novel structures



(6R) 5,6,7,8-tetrahydro-L-biopterin

Figure 1 Structure of BH_4, the naturally occurring pterin cofactor of NOSs and AAHs. The standard nomenclature for numbering the positions of the pteridine ring and the biopterin side chain is indicated.

termed HB_1 and HB_2 (for the German *Himmel Blau,* HB) proved to be pterin and biopterin. At a similar time in another field, the protazoan *Crithidia fasiculata* was found to require high levels of folic acid for growth. Biopterin was shown to substitute for folic acid in supporting growth. Further studies lead to the elucidation of a pteridine-dependent reaction unique to *Crithidia* and related microorganisms, involving conversion of dihydroorotic acid to orotic acid. These early studies gave a first glimpse at the cofactor roles that would be revealed for redox cycling pterins.

A seminal report by Seymour Kaufman in the early 1960s (Kaufman, 1963) revealed that 5,6,7,8-tetrahydrobiopterin is the identity of the essential cofactor for metabolism of phenylalanine to tyrosine by phenylalanine hydroxylase in liver. BH_4 was later established as the reducing cofactor for two other mammalian AAHs, namely, tyrosine hydroxylase and tryptophan hydroxylase. Thus, BH_4 was established to play an essential role in biosynthesis of the hormone epinephrine and monoaminergic neurotransmitters: dopamine, norepinephrine, and serotonin. Although it is clear that the mechanistic role of BH_4 in AAH-mediated catalysis is for activation of molecular oxygen, additional regulatory functions of AAHs are subserved by BH_4. For example, BH_4 binding elicits allosteric effects on phenylalanine hydroxylase that modulate the binding of substrate and phosphorylation by A-kinase. Another role for BH_4 is as the hydroxylating cofactor in the oxidative cleavage of acyl glyceryl ethers, catalyzed by the enzyme glyceryl ether monooxygenase.

The observation that high levels of pterin are present in blood, spleen, and lung, tissues that contain negligible levels of AAHs, hinted at additional roles for BH_4. One putative function of BH_4 is in hematopoetic cell proliferation and differentiation. Although the molecular mechanisms and physiological importance remain uncertain, it is intriguing that BH_4 appears specifically during the differentiation of reticulocytes to erythrocytes and undergos cell cycle-dependent expression in diverse species. Notably, in both mammalian thymocytes and the acellular slime mold *Physarum,* BH_4 levels are observed to peak during the S phase (Zhuo *et al.,* 1996; Werner-Felmeyer *et al.,* 1994). A role for BH_4 in the cell cycle is further suggested from studies in *Drosophila* that demonstrate mitotic asynchrony, persisting chromatin bridges, and death of embryos treated with an inhibitor of BH_4 synthesis or possessing a mutation that incapacitates *de novo* BH_4 synthesis (Chen *et al.,* 1994).

An aspect of BH_4 function that remained mysterious for many years was a dramatic and well-documented induction of BH_4 synthesis in mammalian cells by cytokines, in the absence of a known enzyme that was both cytokine inducible and BH_4 dependent. A role for immunostimulant-evoked BH_4 synthesis was clarified with the key discovery that extracts of cytokine-activated macrophages produce NO from L-arginine in a BH_4-dependent manner (Kwon *et al.,* 1989; Tayeh and Marletta, 1989). Although it had been considered that this BH_4-dependence might be a unique feature of cytokine-activated NO synthesis, inasmuch as NO synthesis

by brain extracts was not similarly increased after addition of excess BH_4, the suggested isoform dissimilarity proved incorrect. Indeed, the demonstration of a BH_4 dependence from nNOS was hindered by the high affinity it possesses for BH_4 binding, causing nNOS to be BH_4 replete as isolated. We now know that each of the three mammalian NOS gene products require BH_4 for activity, as do NOS homologs from each of the diverse species yet examined. The universal requirement of NOSs for BH_4 is not surprising; indeed, given the intricacies of NOS enzymology, it is unlikely that nature would ever invent the chemistry twice! Inasmuch as NOS performs two successive monooxygenation reactions on the substrate arginine, once it was revealed that BH_4 functions as NOS cofactor, it became obvious that one or both NOS reactions would be catalyzed by a mechanism analogous to that for AAHs, wherein BH_4 serves to activate molecular oxygen. Remarkably, NOSs have failed to live up this prophesy. Although a redox function for BH_4 in NOS catalysis remains possible, if not likely, pterin mechanisms and intermediates are assuredly unique. The role of BH_4 in NOS catalysis is addressed later.

De Novo Synthesis of Tetrahydrobiopterin: General Features of the Pathway

BH_4 is synthesized from guanosine 5'-triphosphate (GTP) in four enzymatic steps, requiring three distinct enzymes (see Fig. 2). The first and rate-limiting reaction is catalyzed by GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), an enzyme that converts GTP to 7,8-dihydroneopterin triphosphate

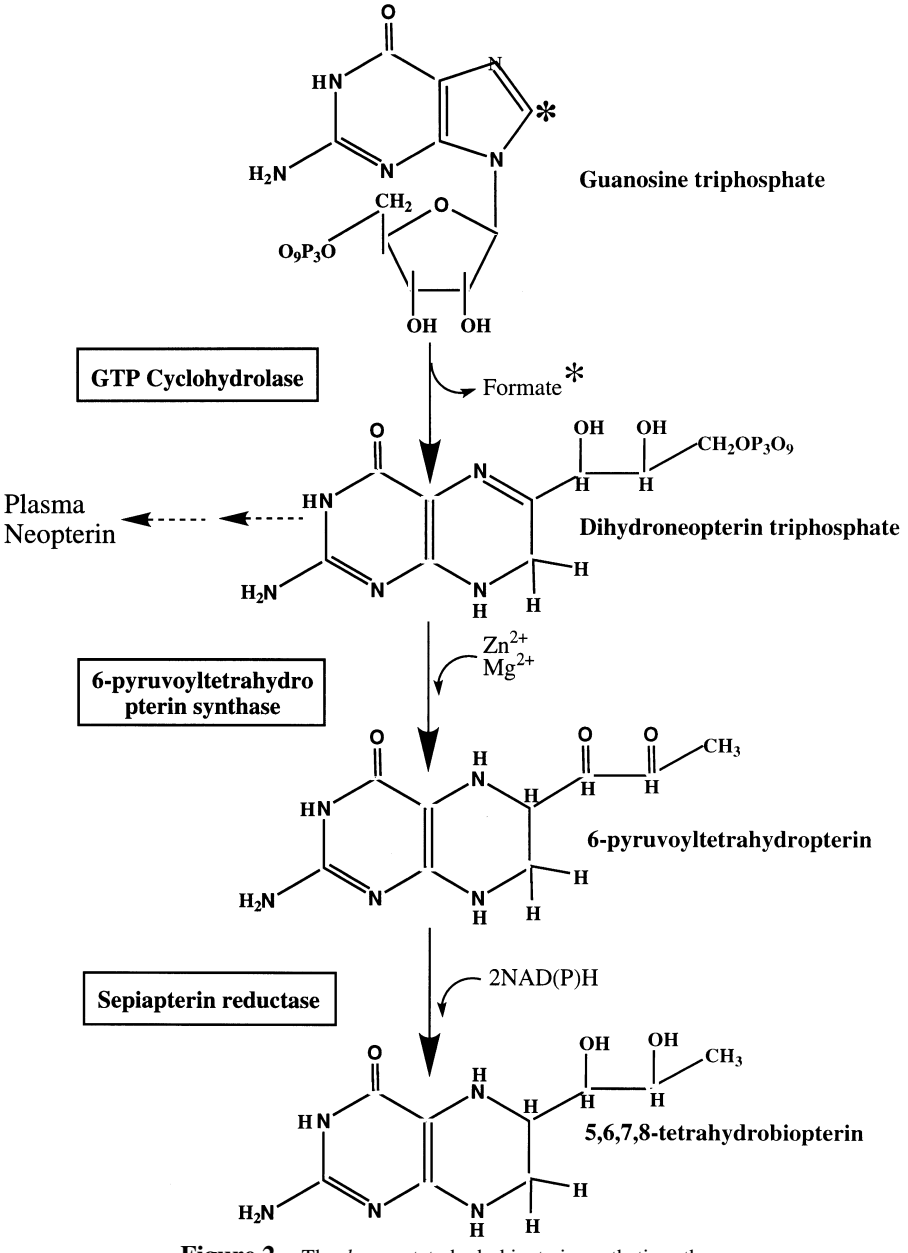


Figure 2 The *de novo* tetrahydrobiopterin synthetic pathway.

(NTP). In this complex but pivotal step, GTPCH acts in a concerted reaction to open the imidazole ring of GTP, releasing C-8 as formate, triggering an Amadori rearrangement of the ribose ring carbons, and creating a cyclic six-membered pyrazine ring. GTPCH-catalyzed formation of NTP is a common initial step in the biosynthesis of unconjugated pterins, folates, and riboflavin, but not molybdopterin (a cofactor of sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase in humans). GTPCH (actually GTPCH I) should not be confused with the unrelated enzyme GTP cyclohydrolase II that catalyzes the second step in riboflavin biosynthesis by microorganisms. As discussed later, GTPCH provides the major target for pathway regulation; GTPCH activity is regulated by diverse mechanisms, including transcriptional, translational, and posttranslational levels. A recently elucidated physiological mechanism for posttranslation control of GTPCH activity involves feedback inhibition by BH_4 and other pterin analogs. Notably, feedback inhibition results from BH_4-induced complex formation of GTPCH with a regulatory protein known as GTPCH feedback regulatory protein, a species whose sequence was first inferred from cDNA cloning in 1997 (Milstien *et al.,* 1996). A prototypic inhibitor of GTPCH activity is 2,4-diamino-6-hydroxypyrimidine (DAHP); this agent mediates GTPCH inhibition both by triggering BH_4-like feedback inhibition and by competing for the binding of substrate GTP (Xie *et al.,* 1998).

In the second step of BH_4 synthesis, 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.6.1.10) catalyzes the conversion of NTP to 6-pyruvoyltetrahydropterin. The PTPS reaction is magnesium and zinc dependent, involving intermolecular redox rearrangement of N-5, C-6, and C-1 of NTP, followed by nonenzymatic triphosphate elimination from the three-carbon side chain on C-6. Although GTPCH is rate limiting to BH_4 synthesis in most cells and circumstances, PTPS has been suggested to limit BH_4 synthesis specifically in human hepatocytes, and PTPS may become the rate-limiting enzyme in other cell types after exposure to immunological stimuli that trigger upregulation of GTPCH activity (discussed below). Although it has been reported that PTPS requires phosphorylation to be fully active, this posttranslational modification has not been affirmed as a site for regulation of enzyme activity *in vivo.* No inhibitor of PTPS has yet been described.

The final step in the *de novo* synthesis involves two successive NADPH-dependent reductions of the C-1' and C-2' oxo groups of 6-pyruvoyltetrahydropterin to form BH_4; both reactions are catalyzed by the enzyme sepiapterin reductase (SR, EC 1.1.1.153). SR catalysis involves proton transfer to the C-1' and C-2' carbonyl groups, accompanied by a stereospecific side-chain isomerization. The C-1' position appears to be the predominant initial site of reduction, resulting in formation of the unstable intermediate 1-hydroxy-2'-oxopropyltetrahydropterin. Inasmuch as SR activity is abundant in all BH_4-producing cells studied to date, it does not appear to be a generic site for regulation of BH_4 synthesis. Nonetheless, SR is potently inhibited by the serotonin metabolite *N*-acetylserotonin, presenting the possibility for a highly specific feedback regulatory mechanism between indole amines and pterins *in vivo. N*-Acetylserotonin (NAS) and *N*-acetyldopamine are potent and selective inhibitors of SR that have been used to assess the role of SR in biological systems (Smith *et al.,* 1992).

Regeneration of Tetrahydrobiopterin for Sustained AAH Activity

Although BH_4 may be redox active in catalysis by NOS isoforms, a dihydropterin product is certainly not released as a stoichiometric product with NO. Indeed, if an oxidized derivative is produced from BH_4 in the course of NOS catalysis, this intermediate is most certainly regenerated to BH_4 *in situ,* while bound to NOS. In contrast, for aromatic amino acid hydroxylases (AAHs) BH_4 is oxidized and then released as the unstable species 4a-hydroxytetrahydrobiopterin[1] (pterin-4a-carbinolamine), leading to potential accumulation of the dehydration product, 5,6-dihydrobiopterin (also known as quinonoid dihydrobiopterin or q-BH_2). If q-BH_2 accumulated without its regeneration to BH_4, the function of BH_4 in aromatic amino acid hydroxylation would be as a stoichiometric substrate, rather than the catalytic cofactor that it is. Thus, a two-enzyme pathway exists for efficient recycling of q-BH_2 to BH_4, thereby enabling BH_4 to function as a catalytic cofactor of AAHs. This pterin cycling pathway is depicted in Fig. 3.

The oxidant required for aromatic amino acid hydroxylation is an unstable pterin species in which dioxygen is transferred from nonheme iron, in the active site of AAHs, to the C-4a position of the bound BH_4 cofactor. From the resulting 4a-hydroperoxytetrahydrobioterin, one atom of oxygen is inserted into an aromatic amino acid substrate, and a second oxygen atom is retained as the hydroxyl group in the pterin product, pterin-4a-carbinolamine. Pterin-4a-carbinolamine is subsequently dehydrated to q-BH_2. Although the dehydration to q-BH_2 does occur efficiently nonenzymatically, dehydration is markedly accelerated by the enzyme 4α-hydroxytetrahydropterin dehydratase (PCD). PCD was first identified as a contaminant in a purified phenylalanine hydroxylase preparation that enhanced BH_4-stimulated hydroxylating activity (Kaufman, 1970). The critical importance of PCD to BH_4-mediated catalytic function is implicit in the identification of patients with BH_4 deficiency (manifest as atypical phenylketonuria) arising from mutation of the gene encoding PCD. The major function of PCD may be to prevent the accumulation of 7-BH_4, an AAH inhibitory species that arises during nonenzymatic dehydration of pterin-4α-carbinolamine that is formed as a result of dihydroxypropyl side-chain migration from the 6 position to the 7 position of the pterin ring. Knowledge of the amino acid sequence of

[1] Although this is a tetrahydropterin, considering the number of double bonds in the system, the term quinonid dihydrobiopterin c4a-hydrate provides a better appreciation of redox state and chemical properties. In any event, a commonly used alternative name is pterin-4a-carbinolamine, which does not comment on the issue of ring redox state.

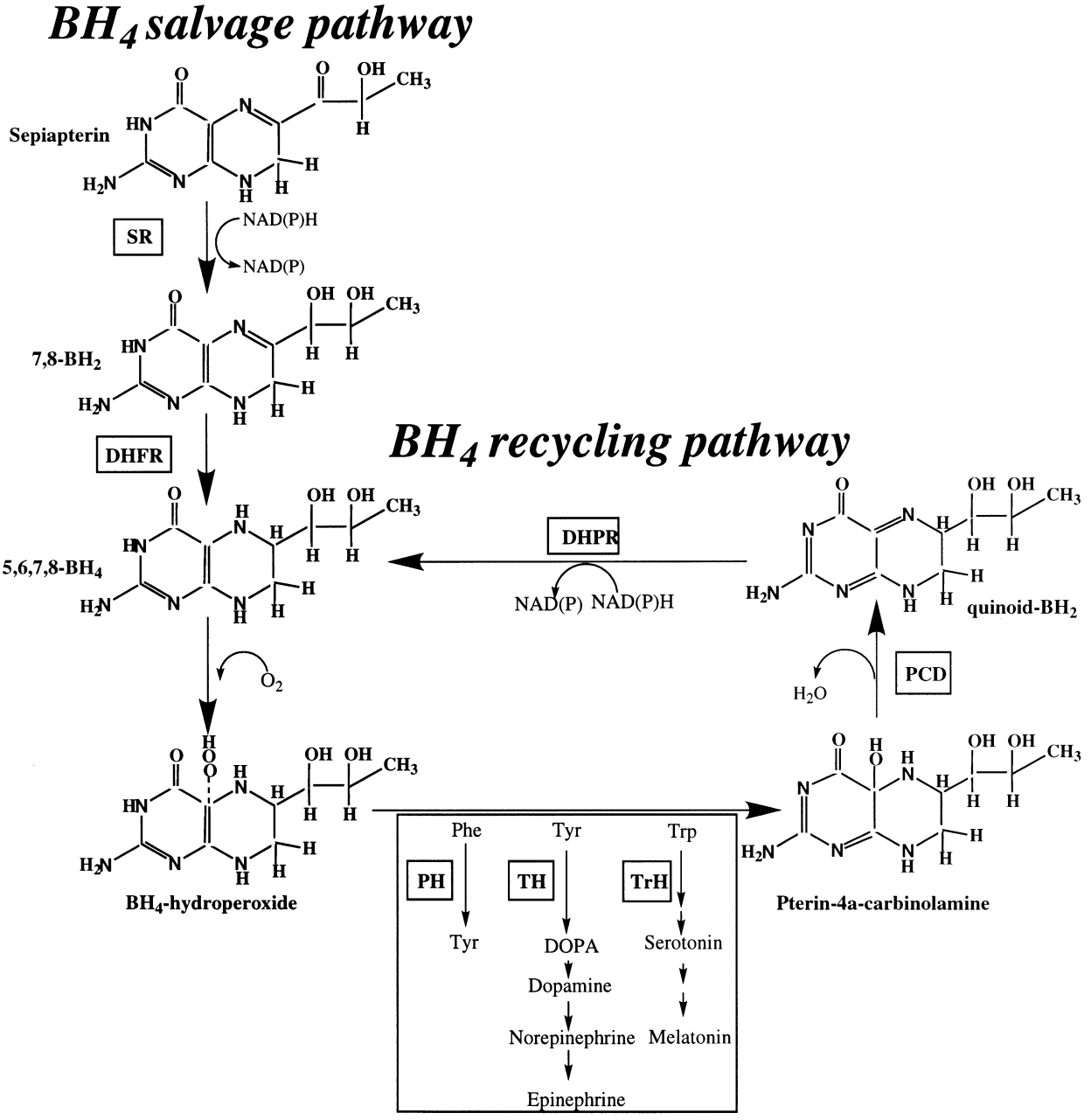


Figure 3 Pathways for salvage and recycling of BH_4. Abbreviations: 7,8-BH_2, 7,8-dihydrobiopterin; SR, sepiapterin reductase; DHFR, dihydrofolate reductase; PH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TrH, tryptophan hydroxylase; PCD, pterin-4a-carbinolamine dehydratase; DHPR, dihydropteridine reductase.

PCD revealed it to be a protein with a remarkable dual function—PCD is identical to a previously identified transcription factor, identified in mammalian cell nuclei, that regulates dimerization of a homeodomain protein (Citron *et al.,* 1992). In this other identity, PCD was known as *dimerization cofactor of hepatocyte nuclear factor 1*α (DCoH). The two activities of PCD/DCoH are thought to be completely unrelated, explained by coevolution of independent protein functions.

The final step in BH_4 recycling from q-BH_2 is mediated by the NADH- or NADPH-dependent enzyme dihydropteridine reductase (DHPR). DHPR is a ubiquitous enzyme that functions to reduce q-BH_2 and not 7,8-dihydropterins.

The Pterin Salvage Pathway

Independent of the BH_4 regeneration pathway described above, a pathway exists to recycle BH_4 after nonenzymatic ring oxidation to its dihydropterin counterpart. Thus, 7,8-dihydrobiopterin (BH_2) is produced nonenzymatically, when BH_4 is exposed to an oxidative environment, and can be salvaged as BH_4, at the expense of NADPH (see Fig. 3). The

enzyme that catalyzes this reaction is dihydrofolate reductase (DHFR), well known for its role in regenerating reduced 5,6,7,8-tetrahydrofolates from 7,8-dihydrofolates. Notably, DHFR does not reduce q-BH_2, and thus does not have DHPR activity. Methotrexate (MTX) and related cytotoxic drugs are potent and selective inhibitors of DHFR, although these agents also inhibit DHPR, albeit at higher drug concentrations. Thus, clinical use of MTX effectively blocks the regeneration of tetrahydrofolates and tetrahydropterins. Although it is assumed that the clinical efficacy of MTX in chronic inflammatory conditions (e.g., rheumatoid arthritis) is a consequence of reduced tetrahydrofolate depletion, a therapeutic effect arising from BH_4 depletion cannot be discounted. Indeed, NO production and toxicity have been demonstrated in joints of arthritic patients, and BH_4 depletion can limit the production of cytotoxic quantities of NO (or NO-derived species). Further work is needed to define the extent to which anti-inflammatory effects of DHFR inhibitors arise specifically through BH_4 depletion. As indicated in Fig. 3, SR extends the BH_4 salvage pathway by catalyzing the reduction of sepiapterin to 7,8-BH_2; this activity is distinct from the role of SR in the *de novo* synthesis of BH_4 from GTP. Intracellular pools of sepiapterin can arise by oxidation of tetrahydropterin intermediates from the *de novo* BH_4 pathway (e.g., 6-lactoyltetrahydropterin).

Plasma Neopterin as a Marker for BH_4 Synthesis in Humans

The concentration of neopterin in human plasma is a convenient indicator of *de novo* BH_4 synthesis. Neopterin arises as a metabolite of GTPCH-produced NTP. When GTPCH becomes markedly activated in human cells, NTP accumulates due to a relative insufficiency of the subsequent enzyme, PTPS. Intracellular NTP is then subject to the action of phosphatase, which is released from cells into the circulation, and it is nonenzymatically oxidized from dihydroneopterin to yield plasma neopterin. Compared with humans, rodents possess significantly greater levels of PTPS, explaining the relative lack of neopterin in murine cells, tissues, and body fluids (Werner *et al.,* 1991).

Before any clear function could be ascribed, plasma neopterin levels had been recognized as a sensitive indicator of immune cell activation for clinical diagnosis (Wachter *et al.,* 1989). Since the only presently known function of immunologically evoked BH_4 synthesis is to support coinduced NO synthesis, elevated neopterin levels presumably signal inducible NOS (iNOS) activation. In this regard, it is notable that neopterin concentrations correlate with the extent and the activity of disorders in which cell-mediated immune stimulation play a role. In allograft recipients, neopterin concentrations rise early during the course of rejection episodes. Increasing neopterin is among the first signs of infection by virus. In inflammatory conditions such as rheumatoid arthritis, neopterin levels correlate with the extent and activity of the disease. Neopterin also has long been known to be a potent marker for HIV progression. It is tempting to consider that neopterin informs us of iNOS induction in each of these clinical circumstances.

Genetic Diseases Arising from BH_4 Deficiency

Although rare, numerous inborn errors of BH_4 metabolism have been documented in human patients. Given that knockout of a single BH_4-dependent enzyme (tyrosine hydroxylase) is embryonic lethal in mice, inactivating all BH_4-dependent enzymes should similarly be incompatible with life. Accordingly, observed patient mutations are likely to arise from severe, but incomplete, deficiencies in BH_4 expression. Failure to identify specific symptoms that derive from NOS deficit in these patients may reflect in part the overall severity of phenotype; additionally, a much lower level of BH_4 is required to support activity of NOSs, compared with AAHs (~1/1000). Thus, a deficiency in AAH activity is predicted to manifest with BH_4 levels that are fully sufficient for maximal NOS activity. An international database of mutations causing BH_4 deficiency, along with predicted amino acid sequences of the cognate proteins, is available (www.ivr.unizh.ch/blau/biodef1.html).

Atypical Phenylketonuria

BH_4 deficiency gives rise to a variant of hyperphenylalaninemia, termed atypical phenylketonuria. This condition presents phenotypically with hyperphenylalaninemia associated with depletion of the neurotransmitters dopamine and serotonin. Atypical phenylketonuria has been found to arise from mutations in any of four genes. These include genes encoding for two of three enzymes required for *de novo* BH_4 synthesis (GTPCH and PTPS) and each of the two enzymes required for BH_4 regeneration from pterin-4a-carbinolamine (PCD and DHPR). Inherited deficiencies of GTPCH and PTPS are clinically characterized by severe neurological symptoms unresponsive to the classic low-phenylalanine diet. Out of several hundred patients registered in the international data base, 57% suffer from PTPS deficiency and 4% from GTPCH deficiency. The failure to identify SR mutations that manifest as a atypical phenylketonuria suggests that one or more other enzymes may be capable of substituting for SR in catalyzing the final reductive step in *de novo* BH_4 synthesis.

Parkinsonism and DOPA-Responsive Dystonia

Catecholamine biosynthesis is regulated by tyrosine hydroxylase (TH) and available BH_4 cofactor. Total biopterin (essentially all BH_4) is highly concentrated in the striatum and specifically enriched in nigrostriatal dopaminergic nerve terminals. Throughout the human brain, there is a positive correlation between total biopterin content and TH activity. In patients with classical Parkinsonism, typified by degeneration of nigrostriatal dopaminergic neurons, neuronal cell death is associated with decreased biopterin levels in cerebrospinal fluid (CSF). The extent of biopterin lowering in CSF may reflect the degree of dopaminergic neuron loss in these patients.

Genetic deficiency in GTPCH activity, inherited in an autosomal dominant pattern, was shown to correlate with dopamine depletion and progressive development of dystonia (Ichinose *et al.,* 1994). Dystonia was characterized by marked diurnal fluctuations in dopamine metabolites, with an increased incidence in females. Patients responded to relatively low doses of L-3,4-dihydroxyphenylalanine (DOPA); thus, this form of GTPCH deficiency was termed DOPA-responsive dystonia (DRD). When GTPCH activity is decreased to less than 20% of normal, activity of TH in the nigrostriatal dopaminergic neurons declines to a level that apparently triggers symptoms of DRD. The candidate gene for DRD was mapped to chromosome 14q by linkage analysis. Subsequently, the first mutations in the GTPCH gene were identified (Ichinose *et al.,* 1995), confirming that it must be a gene for DRD. In contrast to DRD, juvenile Parkinsonism patients have normal GTPCH activity. In Parkinson's disease, GTPCH, TH, and dopamine in the striatum decrease in parallel, secondary to death of dopaminergic neurons. Autosomal dominance of GTPCH mutations may be understood from the obligate decameric structure of GTPCH. Inasmuch as each GTPCH active site is formed at the interface of three subunits (see later for structural details), a single functionally inactive GTPCH gene product would predictably result in 87.5% of enzyme active sites containing at least one mutant subunit. This would predict autosomal dominance of the trait (further assuming that decameric assembly of mutant subunits is not impaired and each of the parental GTPCH gene products are equivalently expressed). On the other hand, if the mutant GTPCH subunit were incapable of decamerization, but expressed at levels equal to the wild type, all GTPCH decamers would have full activity, but the enzyme concentration would be diminished by 50%—such a scenario would anticipate a recessive pattern of inheritance.

VITILIGO AND PIGMENTATION DISORDERS

The importance of BH_4 in the human epidermis is well recognized. Both epidermal melanocytes and keratinocytes have a basal capacity for *de novo* synthesis and recycling of BH_4, thereby enabling PAH activity. While both cell types need PAH to produce tyrosine, the fate of tyrosine is markedly different in each case. Notably, keratinocytes use tyrosine as a precursor of catecholamine biosynthesis via TH, whereas melanocytes use tyrosine for melanin biosynthesis, via the action of tyrosinase. In accord with the finding that tyrosinase mRNA levels are identical in white and black skin, enzyme activity is thought to be regulated by substrate supply or another mode of moment-to-moment regulation. Patients with the skin depigmentation disorder vitiligo suffer from dysregulation in melanin biosynthesis. In vitiligo, the ability of PDC in skin to dehydrate pterin-4a-carbinaolamine was shown to be low or absent, resulting in a reduced rate of BH_4 regeneration and accumulation in epidermis of the nonenzymatic dehydration product, 7-BH_4. Importantly, 7-BH_4 is a potent competitive inhibitor of the PAH reaction. Thus, 6-BH_4 seems to control melanin biosynthesis in the human epidermis, whereas 7-BH_4 may initiate depigmentation in patients with vitiligo. It is notable that 6-BH_4 and 7-BH_4 also specifically bind and inhibit tyrosinase activity; this binding and inhibition are reversed by UVB light. Thus, BH_4s may serve as a photoswitch to regulate *de novo* melanogenesis. Tyrosinase complexes formed with 7-BH_4 are significantly less photolabile than those with 6-BH_4, offering another mechanism by which 7-BH_4 attenuates melanin biosynthesis.

A MOUSE MODEL OF BH_4 DEFICIENCY

The *hph-1* mouse was created by chemical mutagenesis of spermatogonial stem cells and by a breeding scheme that selects for recessive mutations giving rise to hyperphenylalaninemia (McDonald and Bode, 1988). Analysis of the *hph-1* mouse phenotype revealed a BH_4 deficiency, arising from mutation in the GTPCH gene. The mutation does not alter the sequence within the reading frame of GTPCH but apparently diminishes steady-state levels of GTPCH mRNA. It is therefore presumed that the defect resides in the regulatory region of the GTPCH gene. The mutation leads to greatly reduced levels of BH_4 in liver and brain, hyperphenylalaninemia, and impaired monoamine transmitter synthesis. Since DRD results from mutations in the gene for GTPCH, the mouse model may be useful to assess the pathophysiology of DRD and investigate novel therapeutic approaches. Indeed, *hph-1* mice and patients with DRD are very similar, neurochemically. Although standard therapy for DRD involves monoamine replacement therapy, this approach should not alleviate a deficit in NOS activity. Thus, it is significant that the *hph-1* mouse has an apparent impairment of brain NOS activity, indicated by low levels of cGMP, and this deficit can be corrected with peripherally administered BH_4. This observation suggests that patients with DRD may have a previously unrecognized NOS deficiency, owing to diminished availability of BH_4 cofactor in brain. Accordingly, consideration should be given to the development and use of agents that can reactivate NOS in DRD patients.

Regulation of BH_4 Synthesis in Mammalian Cells

GTPCH Structure and Localization: An Overview

GTPCH is a homodecamer of 27- to 29-kDA subunits that is remarkably conserved throughout the phylogenetic kingdom; *Escherichia coli* and rat enzymes are 73% identical. High-resolution crystal structures of the *E. coli.* GTPCH reveals two face-to-face pentameric torroids of 100 Å diameter and 65 Å in depth. Ten substrate binding pockets are created around the outer torroid circumference, each 10 Å deep, formed by surfaces of three adjacent subunits (Nar *et al.,* 1995). Although a high-resolution structure is not yet available for a mammalian GTPCH, the rat homolog was confirmed to resemble the *E. coli* enzyme in having a homodecameric structure (Steinmetz *et al.,* 1998). Given that an active site is formed at the interface of three subunits,

contributed by face-to-face pentamers, it is anticipated that the decameric structure of GTPCH will prove to be obligate for function. A distinct feature of mammalian GTPCH subunits is the presence of an EF-hand motif that binds Ca^{2+} with high affinity. The functional significance of Ca^{2+} binding by GTPCH is unknown; however, it is notable that GTPCH activity has recently been reported to increase with calcium influx into cells (Hwang *et al.,* 1999).

The structural homology of GTPCH with PTPS and two related enzymes (dihydroneopterin aldolase and dihydroneopterin epimerase) reveals an evolutionarily conserved protein fold common to pterin biosynthetic enzymes. Although the only GTPCH structure yet solved is from *E. coli,* homology-based three-dimensional computer modeling suggests the rat homolog is extremely similar (S. Gross, unpublished finding). Indeed, the degree of sequence homology and goodness of fit ensure the overall accuracy of this model. The only indeterminate region of significant length is the N-terminal 54 amino acids of rat GTPCH; this sequence is unique to mammalian GTPCHs and could not be fit, as there is no structural homolog in bacterial GTPCHs. It is notable that the N-terminal sequence of GTPCH is conserved among mammalian species and is not required for catalysis; thus, it is anticipated to serve regulatory functions unique to mammals.

In situ hybridization and immunohistochemical staining reveal that GTPCH is predominantly localized to the adrenal medulla and tyrosine hydroxlase-expressing cells of the substantia nigra, ventral tegamental area, hypothalamus, and locus ceruleus (Hwang *et al.,* 1998; Nagatsu *et al.,* 1995). Among tryptophan hydroxylase-containing cells, GTPCH is particularly abundant in the dorsal raphe nucleus and pineal gland. Neither GTPCH mRNA nor protein expression is pronounced in neurons or endothelial cells that constitutively express NOS isoforms; this may be explained by the low levels of low levels of BH_4 utilized by NOSs compared with AAHs. Alternatively, it is conceivable that BH_4 is produced in AAH-containing neurons and delivered to NOS-containing neurons (presumably as 7,8-BH_2).

Staining and electron microscopy indicate that GTPCH protein in neurons resides in cytosol and also in association with microtubules in axonal processes (Nagatsu *et al.,* 1995). GTPCH expression is induced by immunostimulants in many cell types in which iNOS is coinduced (see below). Although cytokine-induced GTPCH is largely cytosolic, a distinct membrane-associated component is observed with unknown function (Xie *et al.,* 1998).

Induction of GTPCH Activity

The first evidence of GTPCH induction was provided in 1981, when it was shown that administration of either reserpine or insulin to rats elicits an increase in adrenal medullary GTPCH activity (Viveros *et al.,* 1981). A requirement for *de novo* protein synthesis in GTPCH induction was implied by the finding that these effects were attenuated by coadministration of cycloheximide. Subsequently, GTPCH mRNA levels were also shown to increase in the adrenal medulla following cortisol treatment, suggesting but not proving steroid-induced transcriptional upregulation of the GTPCH gene. This mode of GTPCH regulation may be physiologically relevant to the regulation of epinephrine biosynthesis but apparently not to that of NO.

Several years later it was shown that GTPCH activity was also induced by immunostimulants (for review, see Werner *et al.,* 1998). Thus, *in vitro* studies of human peripheral blood mononuclear cells revealed that γ-interferon (IFN-γ) stimulated production of the GTPCH-derived product, neopterin. This finding established a connection between GTPCH activation and the elevated neopterin levels observed in plasma patients with viral infections and tumors. Inasmuch as a function for immunologically evoked BH_4 synthesis was unknown, the finding of cytokine-induced GTPCH activity was initially perplexing. We now appreciate that the pivotal function of immunostimulant-induced GTPCH is to provide the BH_4 cofactor needed for support of coinduced iNOS. Interestingly, coinduction of GTPCH and NOS is a phenomenon that is not restricted to mammals; it also occurs in other eukaryotes such as the slime mold *Physarum polycephalum* (Werner-Felmeyer *et al.,* 1994).

GTPCH induction by immunostimulants and mitogens has been observed in most cells and tissues studied, although specific activating signals vary with cell type. Stimuli that induce GTPCH in a given tissue have generally been observed either to enhance the activity of basally expressed NOS isoforms or to be essential for the activity of coinduced iNOS protein, or both. Various cell types have been used to study the mechanistic basis for GTPCH induction.

In mononuclear cells and macrophages, GTPCH activity is induced by stimuli such as IFN-α, -β, -γ, interleukin 2 (IL-2), kit-ligand, bacterial endotoxins such as lipopolysaccharide (LPS), phytohemagglutinin, and pokeweed mitogen. The effects of these stimuli tend to be synergistic with one another. Many of these agents have also been found to induce GTPCH expression in numerous other cell types, including thymocytes, dermal fibroblasts, mast cells, vascular smooth muscle cells and endothelial cells, and glomerular mesangial cells. The ubiquitous induction of GTPCH is not a cell culture artifact and has been observed *in vivo.* For example, LPS administration to rats elicits a substantial increase in GTPCH activity in muscle, lung, heart, kidney, and aorta (Hattori *et al.,* 1996; Hussain *et al.,* 1997).

Induction of GTPH activity by immunostimulants and mitogens has been associated with significant increases in the expression of GTPCH mRNA, and it is presumed to occur as a result of transcriptional upregulation of the GTPCH gene; nonetheless, definitive proof is lacking from the literature. In preliminary studies of reporter gene constructs, stably transfected into rat aortic smooth muscle cells, we have found that the initial 1 kb of 5'-upstream DNA sequence to the rat GTPCH gene confers threefold inducibility by LPS (S. S. Gross and C. L. Jones, unpublished finding). It is unclear whether this level of transcriptional upregulation is sufficient to explain the more substantial (>30-fold) induction of GTPCH mRNA and activity observed in these cells with LPS. Additional DNA sequence

may mediate more profound transcriptional upregulation by LPS, although 1 kb and 6 kb of 5'-upstream GTPCH gene fragments gave indistinguishable results. Given that LPS-induced transcription of the iNOS gene has been linked to an NF-κB response element that resides within 80 bp of the transcription start site, it is notable that the rat GTPCH gene also displays an NF-κB consensus element immediately upstream of transcription start. Function of the NF-κB element in mediating LPS-induced GTPCH expression has not been established but is suggested from the finding that GTPCH induction is blocked by the NF-κB inhibitor pyrroline dithiocarbomate (Hattori *et al.,* 1996).

Cytokines, growth factors, and immunosuppressants have been identified that attenuate GTPCH activity or its induction. For example, in cultured human endothelial cells IL-4, IL-10, and transforming growth factor β (TGF-β) deactivate GTPCH. In rat smooth muscle cells TGF-β, thrombin, ciliary neurotrophic factor, and leukemia inhibitory factor downregulate LPS-induced GTPCH expression. In macrophages, endothelial cells, cardiac myocytes, and some other cell types, cyclosporin, FK506, and glucocorticoids elicit GTPCH downregulation. Although these latter agents downregulate immunostimulant-induced NO synthesis, this effect is not exclusively mediated by BH_4 depletion, an iNOS induction is also attenuated. Upregulation of GTPCH has been observed with NGF and EGF in neuronal-like cell lines; given that these cells express both NOS and AAHs, the function of resulting BH_4 may conceivably be to support both catecholamine and NO biosynthesis.

Cyclic AMP may also be an important regulator of GTPCH. Cell-permeant analogs of cAMP and the adenylate cyclase activator forskolin have been found to either increase GTPCH expression directly in some cell types (mesangial cells, vascular smooth muscle cells, adrenal chromaffin cells, neuronal cells, and neuronal-like cell lines) or potentiate GTPCH induction by IL-1β and other immunostimulants (most cell types). Increases in cAMP apparently mediate GTPCH induction via the action of several hormones in tissues containing cognate receptors that trigger A-cyclase activation; these hormones include vasopressin, adrenomedullin, and adrenocorticotrophin. Although a putative cAMP response element binding protein (CREB) consensus element is identified within the rat GTPCH promoter, the function has not been verified.

Posttranslational Regulation of GTPCH

Phosphorylation of GTPCH has been reported to occur in PC12 cells after KCl-induced depolarization. Although neither the precise site nor the functional consequences of this phosphorylation on enzyme activity have been evaluated, it was shown to be coincident with a protein–protein interaction between GTPCH and the monomeric G protein rabphillin 3A (Rab3A). It is yet unclear whether the reported GTPCH–Rab3A interaction is direct or involves additional protein components. Since Rab3A is a component of synaptic vesicle membranes, playing a role in vesicle translocation and transmitter release, it is conceivable that subcellular localization of GTPCH is modulated by phosphorylation, perhaps controlling intracellular compartmentalization of BH_4 synthesis.

Phorbol esters that activate protein kinase C (PKC) have been reported to increase the neopterin and biopterin content in T lymphocytes in culture. In cultured dopaminergic neurons, adenylate cyclase activation and membrane depolarization affect GTPCH expression and increase BH_4 biosynthesis. Similarly, reagents that increase intracellular levels of cAMP have been shown to cause an elevation of GTPCH activity in some experimental settings. Such evidence suggests that in addition to induction of GTPCH, posttranslational modifications such as phosphorylation may play a role in GTPCH regulation. Indeed, GTPCH appears to be basally phosphorylated in mast cells, and both phosphorylation and activity transiently increase on IgE receptor activation (Hesslinger *et al.,* 1998). Although phosphorylation of GTPCH has been demonstrated *in vitro* with casein kinase II and PKC, the regulatory significance of these modifications *in vivo* are unknown.

Feedback Inhibition of GTPCH by BH_4/GFRP

It was appreciated for many years that GTPCH is inhibited by BH_4; thus *de novo* BH_4 synthesis is regulated by end product inhibition. The capacity to inhibit GTPCH is not restricted to BH_4; it is observed with many pterins, most potently with reduced pterins (Ballahsene *et al.,* 1984; Shen *et al.,* 1988). It was a surprise when studies of rat GTPCH, purified from a bacterial expression system, revealed no inhibition of activity by BH_4 *in vitro.* This led to the identification of a novel protein in liver that bound to purified recombinant GTPCH in the presence of BH_4 and reconstituted the ability of BH_4 to inhibit purified recombinant GTPCH (Harada *et al.,* 1993). Initially named p35 based on an apparent molecular mass of 35 kDa, this protein was cloned and found to encode an 84-amino-acid polypeptide, comprising only 9.5 kDa (Milstien *et al.,* 1996). p35 was renamed GFRP, for GTPCH feedback regulatory protein. In the presence of bacterially expressed rat GFRP, BH_4 was found to elicit a concentration-dependent and complete inhibition of recombinant GTPCH. This finding affirmed that the 9.5-kDa GFRP was both necessary and sufficient for BH_4-mediated feedback inhibition of GTPCH. GFRP appears to be a homopentamer, and it is speculated that one GFRP pentamer interacts with each of the two pentameric faces of GTPCH decamer (Yoneyama and Hatakeyama, 1998). GFRP-mediated inhibition of GTPCH is specifically reversed by phenylalanine, explaining the clinical observation that a Phe-rich meal increases circulating levels of total biopterin. Although phenylalanine reverses inhibition of GTPCH imposed by GFRP/BH_4, it does not dissociate the protein assembly. A model that summarizes this mechanism for feedback inhibition of GTPCH by pterins is depicted in Fig. 4.

Given that the K_m of BH_4 for support of NOS activity is extremely low (30–100 n*M*) in comparison with that reported for feedback inhibition (1–10 μ*M*), it was not

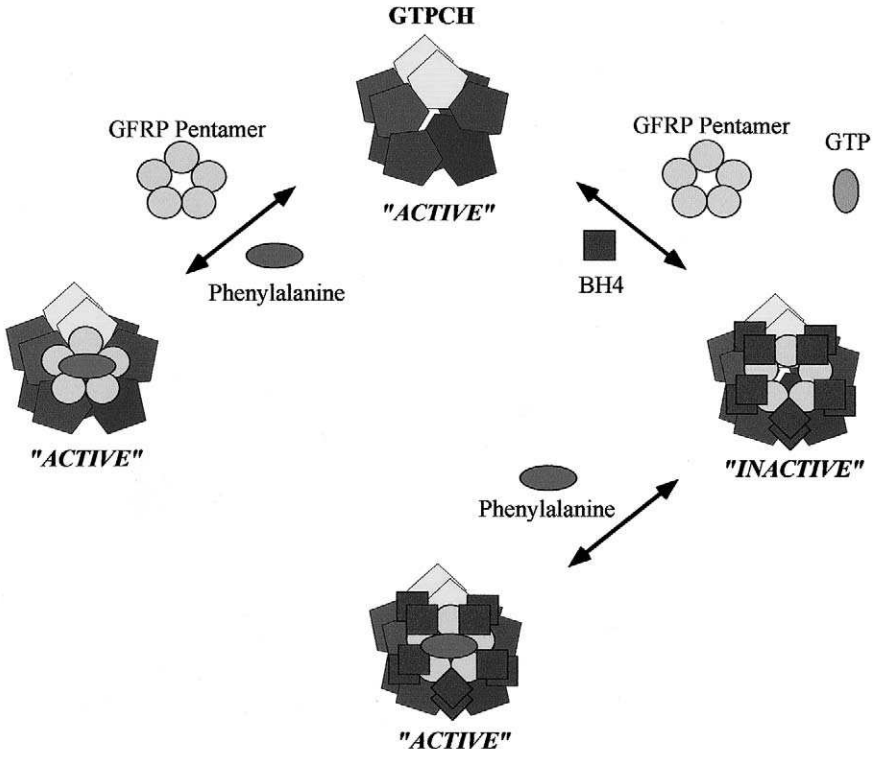


Figure 4 Schematic to depict the role of GFRP as a mediator of GTPCH feedback inhibition by BH_4. When either BH_4 or phenylalanine are present in sufficiently high concentration, one GFRP pentamer is bound to each free GTPCH decamer. Whereas phenylalanine-bound GFRP promotes GTPCH activity, BH_4 binding inhibits GTPCH activity. See color insert.

anticipated that feedback inhibition of GTPCH could influence NOS activity. Nonetheless, GFRP mRNA is detectable in vascular smooth muscle cells and inducible by immunostimulants, and GFRP mediates tonic inhibition of GTPCH (in the absence of Phe) when BH_4 synthesis is induced by immunostimulants (Xie *et al.,* 1998). Whether this system significantly impacts on immunostimulant-induced NOS activity *in vivo* remains to be ascertained.

Other Sites for Regulation of BH_4 Synthesis

PTPS

6-Pyruvoyltetrahydropterin synthase (PTPs), the enzyme catalyzing the second step in BH_4 biosynthesis, has been purified from human liver and rat brain. PTPS has homology to aldose reductase, which catalyzes the reduction of a wide variety of carbonyl compounds to their corresponding alcohols in an NADPH-dependent reaction. Based on knowledge of the broad range of catalytic efficiencies for aldose reductase, it is conceivable that PTPS might also function as a reductase, especially in circumstances where SR is rate limiting or genetically impaired.

In humans, levels of PTPS become rate limiting after GTPCH upregulation. Full activity of PTPS depends on phosphorylation by cGMP-dependent kinase (Ser-19 in human PTPS; Scherer-Opplinger *et al.,* 1999). Since NO activates soluble guanylate cyclase, triggering increases in cGMP and cGMP-dependent kinase activity, PTPS activation should occur as a consequence. Phosphoregulation of PTPS and upregulation of BH_4 production potentially endow NO with the capacity to enhance its own synthesis.

In some cell types, PTPS has been demonstrated to be cytokine-inducible, as is almost uniformly observed for GTPCH. For example, PTPS mRNA and protein activity in human umbilical vein endothelial cells are significantly induced (10-fold and threefold, respectively) by the combination of tumor necrosis factor alpha (TNF-α) and IFN-γ, although the extent of this induction is an order of magnitude less than that for GTPCH (Linscheid *et al.,* 1998).

GTP AVAILABILITY

Intracellular GTP levels in hepatocytes and other cells are reported to be in the range of 100–200 μM, approximately the K_m for utilization by GTPCH. Moreover, GTP elicits positive cooperativity in a concentration-dependent manner for increasing the GTPCH reaction velocity. Since GTPCH activity is rate limiting in *de novo* BH_4 synthesis, these findings imply that an increase in availability of GTP substrate would enhance the rate of intracellular BH_4 accumulation. Thus, it is notable that GTP levels in cytokine-activated cells have been found to significantly increase in many cell types, concurrently with GTPCH activity. The mechanism of GTP upregulation by cytokines is not known.

Inosine monophosphate (IMP) dehydrogenase is the rate-limiting enzyme for *de novo* GTP biosynthesis and can be specifically inhibited by mycophenolic acid and 2-amino-

1,3,4-thiadiazole. When these GTP synthesis inhibitors were applied to neuronal-like cell lines that produce catecholamines, BH_4 production was inhibited. Addition of guanine or guanosine (which is converted to GTP by hypoxanthine guanine phosphoribosyltransferase through the purine salvage pathway) restored BH_4 levels and reversed the effects on IMP dehydrogenase inhibition. Inhibition of *de novo* purine synthesis was also found to inhibit NO production in cytokine-activated vascular cells, specifically by preventing the otherwise observed increase in levels of GTP and BH_4.

Biopterin Uptake by Cells

Despite the finding that immunostimulant-activated vascular smooth muscle cells *in vitro* produce BH_4 in quantities which support a rate of NO synthesis that is less than half-maximal (Gross and Levi, 1992), these cells secrete more that 95% of the BH_4 that they produce. Similarly, endothelial cells in culture have been shown to secrete large quantities of BH_4 (Walter *et al.,* 1994). Thus, in cell culture, BH_4 efflux dominates over cell retention. Cell efflux and uptake of BH_2/BH_4 were also indicated by the finding that iNOS-transfected cells produce barely detectable levels of NO when cultured alone, but substantial amounts of NO when cocultured with BH_4-producing cells (Tzeng *et al.,* 1996). Thus, BH_4 may engage in a transcellular metabolism, wherein BH_4 efflux from producing cells provides BH_2 as substrate for uptake and reduction by pterin synthesis-deficient cells. The relatively low amounts of BH_4 needed to support NOSs makes such a cycle feasible for NOS-containing cells, whereas it is unlikely that sufficient BH_4 can be provided to influence AAH activities. Anatomic evidence consistent with intercellular BH_4 transfer is provided by the observation that iNOS-containing hypothalamic neurons, containing undetectable levels of GTPCH protein or mRNA, make direct contact with GTPCH-rich dopaminergic neurons (Hwang *et al.,* 1998). Cellular transport mechanisms for reduced pterins have not been described.

The BH_4 Requirement for NO Synthesis

BH_4 Availability Determines the Rate of NO Synthesis and Product of NOS

Most cells in culture secrete copious quantities of NO after cytokine activation. The observation that BH_4 synthesis is induced simultaneously with NO synthesis immediately raised the possibility that the function of cytokine-induced BH_4 is to support iNOS. Using selective inhibitors of GTPCH, SR, and DHFR, it was indeed verified that *de novo* synthesis of BH_4 is essential for induction of NO production by bacterial LPS in endothelial cells (Gross *et al.,* 1991), fibroblasts (Werner *et al.,* 1991), and vascular smooth muscle cells (Gross and Levi, 1992). This dependence of NO synthesis on BH_4 was extended to cardiac myocytes (Kasai *et al.,* 1997) and to numerous other cell types, to varying extents. In contrast, macrophage cell lines appear to have sufficient constitutive BH_4 to support maximal NOS activity (Kwon *et al.,* 1989). Thus a dependence of NOS on induced BH_4 may be cell-type specific. Importantly, the dependence of cytokine-induced NO synthesis on *de novo* BH_4 production has been confirmed in isolated vessels and animals. Depletion of BH_4 elicits a dramatic decrease in NO synthesis not only from iNOS, but also eNOS in endothelial cells and blood vessels (Kinoshita *et al.,* 1997).

In contrast to the inhibition of immunostimulant-evoked NO synthesis observed with BH_4 synthesis blockers, BH_4 repletion typically elicits an increased rate of NO synthesis by cells in culture (Gross and Levi, 1992), including human endothelial cells (Rosenkranz-Weiss *et al.,* 1997). This implies that induced levels of BH_4 remain rate limiting for NO production. Loading of some cells with exogenous BH_4 (by treatment with either BH_4 or sepiapterin) also results in earlier detection of LPS-induced NOS activity, implying that LPS-induced NOS enzyme may appear prior to the BH_4 that is required for expression of NOS catalytic activity (Gross and Levi, 1992).

BH_4 also limits LPS-induced killing and vascular dysfunction *in vivo.* Pretreatment of rats with a combination of inhibitors of BH_4 synthesis (DAHP) and pterin salvage (MTX) prevented the profound reduction in aortic contractility that arises from LPS-induced NO overproduction in the vascular wall (Gross and Levi, 1995). In another study, a GTPCH inhibitor alone was shown to attenuate LPS-induced NO synthesis and hypotension in rats (Bune *et al.,* 1996). Pretreatment with BH_4 converted a nonlethal dose of LPS in mice to a lethal dose (Gross and Levi, 1995). In the absence of LPS, BH_4 alone was without significant effect on vascular tone or animal viability. Using a greater dose of LPS that kills all mice within a 24-hour period, we observed that combined treatment with DAHP and MTX offered significant protection against killing. A BH_4 synthesis inhibitor was also shown to protect against ischemia-induced NO synthesis and hippocampal cell death in rats subjected to transient forebrain ischemia (Cho *et al.,* 1999). Accordingly, disruption of pterin synthesis may be a useful target for pharmacological interventions intended to limit NO synthesis in pathophysiological settings associated with NO overproduction.

BH_4 Binding and Activation of NOS Catalysis

BH_4 is essential for each of the two oxygenation reactions in NOS catalysis: conversion of L-arginine (L-Arg) to hydroxy-L-arginine (OH-Arg) and conversion of OH-Arg to NO and L-citrulline. The molecular basis for the BH_4 dependence of these reactions has been controversial although clarity is in view.

To recap, for AAHs catalytic recycling of BH_4 permits the use of substrate quantities of pterin cofactor, rather than stoichiometric quantities. An essential feature of this system is that q-BH_2 is recycled to BH_4 by a distinct DHPR. Kaufman and co-workers initially rejected the view that NOS emulate AAHs in using BH_4 as a direct redox-active participant in catalysis, arguing instead for an allosteric or struc-

ture-stabilizing role for BH_4 (Giovanelli *et al.,* 1991). The principal reason for this rejection was that for each mole of BH_4 added to pure brain NOS, more than 15 mol of NO were formed in the absence of added DHPR. Dissociation of BH_4 from NOSs during catalysis is also contraindicated by the finding that NOSs bind BH_4 with three orders of magnitude greater affinity (10–100 μM) than AAHs.

Radioligand binding experiments reveal that BH_4 binding to NOS elicits an increase in affinity for arginine-based ligands (for review, see Liu and Gross, 1996); this is consistent with an allosteric effect of pterin binding. An allosteric effect of pterin binding is also suggested from spectrophotometric and EPR studies, which show that BH_4 binding to NOS converts the heme iron from low-spin to a high-spin state (Wang *et al.,* 1995; Gorren *et al.,* 1996). A structural role for BH_4 was also indicated by the finding of an apparently important role in NOS dimer assembly (Ghosh and Stuehr, 1995). However, given that all three recombinant NOS isoforms are isolated as dimers in the absence of BH_4, it is obvious that BH_4 is not essential for dimerization. Nonetheless, stability of the NOS dimer is enhanced by bound pterin cofactor. SDS-elicited dimer dissociation was investigated to quantify the BH_4 contribution to NOS dimer stabilization (Klatt *et al.,* 1995). Although nNOS dimer is significantly stabilized by bound BH_4, this effect is not so apparent for eNOS. The crystal structure of iNOS and eNOS heme domains revealed that bound BH_4 contributes to the active site channel (Crane *et al.,* 1997; Raman *et al.,* 1998).

NOS consumption of NADPH can become uncoupled from NO synthesis, resulting in the production of superoxide and hydrogen peroxide. A unique role for BH_4 has been demonstrated in providing efficient coupling of nNOS and eNOS to NO production. Depletion of BH_4 has been shown to cause uncoupling of oxygen reduction and arginine oxidation, thereby generating superoxide and subsequently hydrogen peroxide (Pou *et al.,* 1992). Reciprocally, addition of BH_4 to eNOS was shown to increase NO production and diminish superoxide generation (Wever *et al.,* 1997). These findings were subsequently confirmed by EPR studies which revealed that recombinant nNOS and eNOS produce robust quantities of superoxide anion in the absence of BH_4 (Vivar-Vasquez *et al.,* 1998; Xia *et al.,* 1998). Production of superoxide by recombinant eNOS was blocked by heme ligands, indicating that heme, rather than FMN or FAD, is the source of superoxide. Thus, BH_4 may serve an important catalytic function for eNOS by preventing premature release of heme iron-bound dioxygen, as superoxide anion. Reasonable mechanisms whereby BH_4 might preserve bound oxygen on eNOS include a direct or allosteric-induced modification in heme-iron redox potential.

Despite a potential allosteric role of BH_4 as a NOS cofactor, a redox function for BH_4 is appealing, and it is consistent with several findings. First, although various tetrahydropterins have been reported to possess NOS cofactor activity (Riethmüller *et al.,* 1999), a dihydropterin has never been shown to support catalysis. Moreover, tetrahydropterins with modifications that would predictably cause them to be redox-silent, such as 5-deazatetrahydropterin (Hevel and Marletta, 1992) and 4-aminotetrahydropterin (Werner *et al.,* 1996), are devoid of NOS cofactor activity. Finally, redox-inactive pterin analogs can mimic the effects of BH_4 on NOS dimerization, substrate affinity, and heme spin state but fail to support NOS catalysis. It is also notable that BH_4-deficient NOS is largely uncoupled, producing superoxide rather than NO. This may reflect the premature release of heme-bound O_2 under conditions were BH_4 is unavailable to donate a catalytically required electron. Thus, putative allosteric effects of pterins may be necessary for NOS function, but they are clearly insufficient to explain cofactor function. Importantly, the NOS oxygenase domain crystal structure reveals that bound BH_4 is positioned in a manner which could accommodate electron donation from the pterin to heme iron, through pterin N-3/O-4 contact with the heme pyrrole A propionate group (Crane *et al.,* 1998; Raman *et al.,* 1998). Additional structural features of the NOS–pterin interaction and recent spectral findings strongly infer a redox function of BH_4; these findings are discussed below.

Structural Interactions of Pterin Cofactor with NOSs

Crystal structures of the heme domains of iNOS (Crane *et al.,* 1998; Fischmann *et al.,* 1999), eNOS (Raman *et al.,* 1998; Fischmann *et al.,* 1999), and nNOS (Raman *et al.,* 2000) all reveal a nearly identical mode of pteridine cofactor binding. Three structural features stand out as hallmarks in describing the binding mode of BH_4 with NOS (Fig. 5): (i) an extensive network of hydrogen bonds and van der Waals contacts hold the pterin cofactor in place; (ii) the heme propionate and pterin pyrimadone (amine at C-2 and N-3) make direct interactions; and (iii) aromatic side chains stack around the pterin ring. These features of pterin binding are now considered in more detail.

First, the large number of hydrogen-bonding (>12) and nonbonded contacts between the protein and the cofactor illustrates why BH_4 is bound with high affinity ($K_d \approx 30$ nM) to NOS (Werner-Felmayer and Gross, 1996). In addition, the bound cofactor, for the most part, is sequestered from the bulk solvent. This mode of binding is quite different from that observed in the case of TH (Goodwill *et al.,* 1998), in which BH_4 is expected to largely retain solvent accessibility and in which binding affinity is orders of magnitude weaker than with NOSs. The structure of TH complexed with 7,8-dihydrobiopterin (Fig. 6A) provides insight into how pterins are recognized by enzymes other than NOS, and it also illustrates the reasons for the relatively poor binding affinity.

Second, of the two rings that comprise the pteridine, namely, pyrimidine and pyrazine, it appears that the pyrmidine moiety incorporates the signature for molecular recognition by NOS. This conclusion is based on the observation that a heme propionate is used to perform the recognition in NOS instead of the protein carboxylates (Glu and Asp) typically utilized by other pterin-binding enzymes. The use of protein carboxylates in pyrimidine recognition has a rich his-

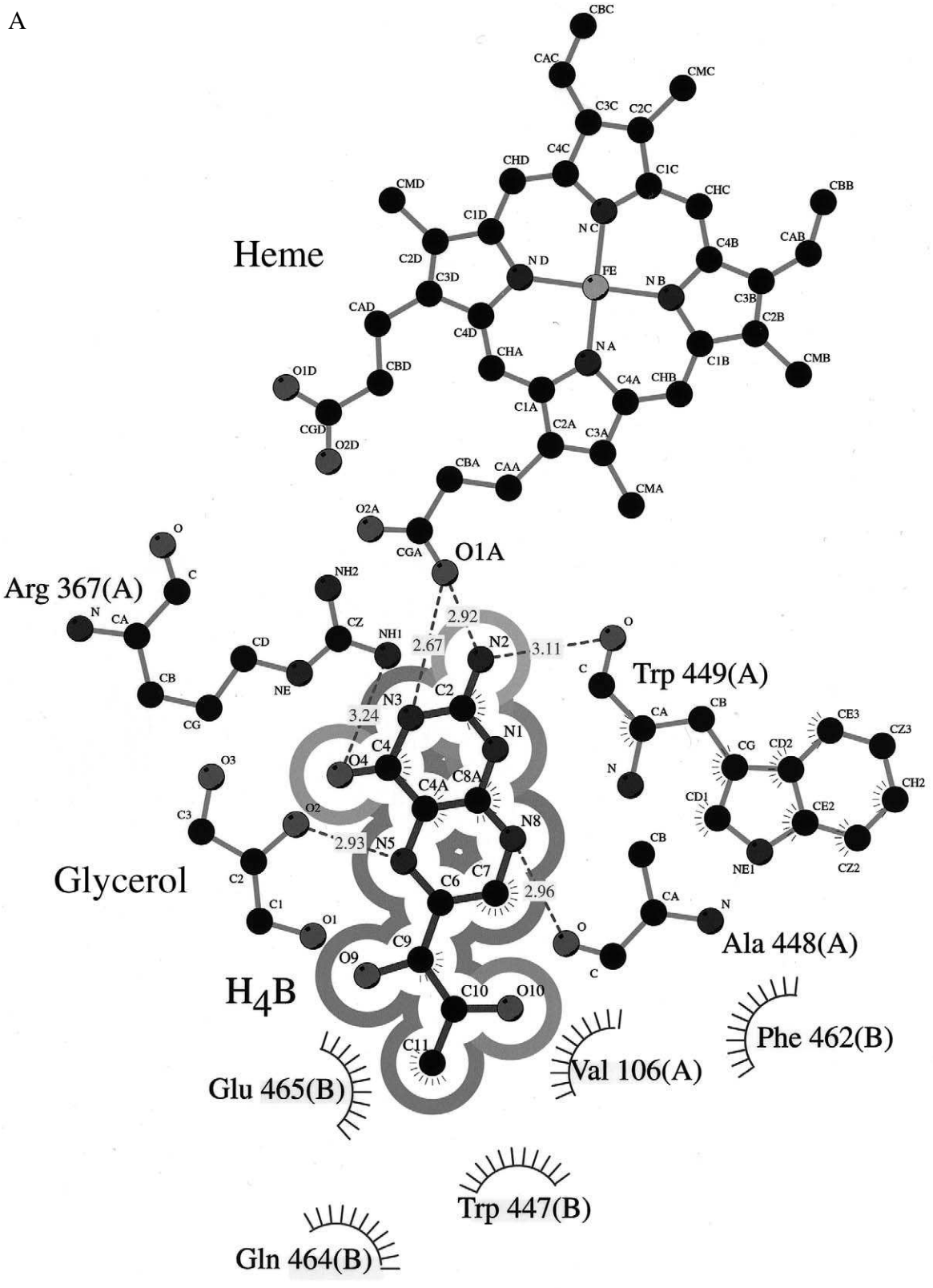


Figure 5 High-resolution structures of BH_4 bound to eNOS. (A) Key hydrogen-bonded (dashed lines) and van der Waals contacts between the cofactor and the protein are depicted. Light shading around the cofactor corresponds to the solvent-accessible surface. Darker shading represents the region of poor solvent accessibility. The glycerol bound next to the cofactor derives from the cryoprotectant cocktail used during data collection. Solvent molecules are expected to replace glycerol in the native state of the enzyme. An (A) or (B) next to the label corresponds to the subunit with which a given residue is associated. (B) A schematic representation of the cross talk between the active and cofactor sites in NOS. Both stacking (at the BH_4 site) and hydrogen-bonding interactions entertained by the substrate and cofactor are shown. The ''B'' next to F462 identifies this residue to be part of the second subunit in the NOS dimer. See color insert.
(Continues)

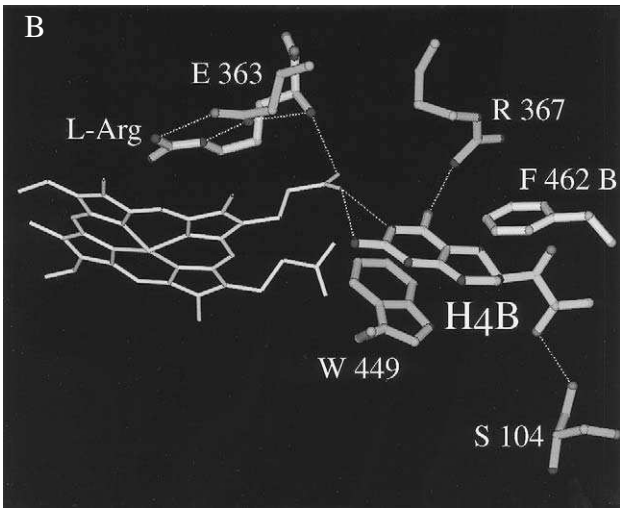


Figure 5 *(continued)*

tory. The GTPase superfamily (Bourne *et al.,* 1991; Varnum *et al.,* 1995) is a prime example in which a conserved protein carboxylate mediates recognition of GTP via its pyrimidine component. Interestingly, each of the three enzymes of the *de novo* BH_4 synthetic pathway—GTPCH, PTPS, and SR—bind to their substrates—GTP, dihydroneopterin triphosphate, and 6-pyruvoyltetrahydropterin, respectively—via a conserved protein carboxylate (Auerbach *et al.,* 1997) (see Fig. 6B). The key role of a conserved protein carboxylate in recognizing pterin is also observed in DHFR (Bolin *et al.,* 1982), thymidylate synthase (Birdsall *et al.,* 1996), 7,8-dihydroneopterin aldolase (Hennig *et al.,* 1998), and dihydropteroate synthase (Hampele *et al.,* 1997). There are clear exceptions to this widespread scheme of molecular recognition; however, TH, which requires BH_4 as an obligatory cofactor, does not incorporate a protein carboxylate to recognize the guanidinium function. A similar case is observed for PCD (Cronk *et al.,* 1996) and DHPR (Varughese *et al.,* 1992). An interesting question is why TH, PCD, and DHPR do not utilize a protein carboxylate to lock the pyrimadone in place; plausible functional reasons are offered in the next section. The ability of an enzyme to utilize a carboxylate to perform recognition of pterin extends even to enzymes dependent on molybdopterin cofactor, for example, aldehyde ferredoxin oxidoreductase (Chan *et al.,* 1995). It is also worth pointing out that the affinity of carboxylic acids toward the pyrimadone has been characterized in detail by direct chemical investigations (Etter and Adsmond, 1990).

Third, aromatic amino acids stack on either side of the pterin cofactor in NOS. Interestingly, one of the stacking aromatic side chains (e.g., Phe-462 in bovine eNOS) stems from the second subunit. Such a flanking arrangement of aromatics around a pterin is not unique to NOS and has been observed in other systems, including thymidylate synthase (Birdsall *et al.,* 1996) and ricin (Yan *et al.,* 1997). Considering that the high N/C ratio afford the pteridine ring system greatly reduced aromaticity, and therefore a weakened π-electron layer, it is intriguing that aromatics stack around pteridine compounds.

Structural Insights into the Role of Pterin Cofactor in NOS Catalysis

What is the role of BH_4 in NO biosynthesis? Does it play a structural or functional role? That BH_4 plays a catalytic function is implicit in the structure of eNOS. Raman *et al.* (1998) compared pterin-bound and pterin-free crystal structures, revealing that cofactor binding is not essential for maintaining the quaternary structure of NOS. In addition, there were no detectable conformational differences in the pterin binding site with and without bound cofactor. If BH_4 does not play a dominant structural role, it should play a catalytic role, and it is worth reiterating the conditions under which NOS catalysis is supported by pterin cofactor: (a) NO generation does not occur in the absence of BH_4; (b) only the four-electron reduced pterin, BH_4, will, *in toto,* function as a cofactor; (c) the two-electron oxidized pterin species (BH_2) is not a cofactor, although it is capable of binding to the pterin cofactor site; and (d) whereas the dihydroxypropyl side-chain substitution at the 6 position on the pteridine dictates the stereospecificity of BH_4 recognition, it is not essential for cofactor function.

In approaching the problem of understanding the catalytic function of BH_4 in NO biosynthesis, it is informative to consider similarities with other enzymes where reduced pterin cofactors are a *sine qua non* for catalysis. As summarized earlier, pteridines function as cofactors in a wide variety of enzyme-catalyzed reactions. In particular, tetrahydrofolate (bacterial and eukaryotes) and tetrahydromethanopterin (archaea) mediate transfer of one-carbon fragments. BH_4 is utilized as a cofactor by alkylglyceryl monooxygenases and in the hydroxylation of aromatic amino acids; molybdopterin is a cofactor in a wide variety of redox enzymes. Of the BH_4-utilizing enzymes, AAHs have been most extensively studied with respect to cofactor function, and they serve as a useful reference to compare and contrast the role of pterin in NOSs. AAH requires an enzyme-bound nonheme Fe^{2+} for activating molecular oxygen and hydroxylating the aromatic ring of the amino acid substrate. Molecular oxygen, however, oxidizes the Fe^{2+} to Fe^{3+}. BH_4 can reduce the inactive Fe^{3+} enzyme back to the catalytically competent Fe^{2+} state, facilitating catalysis. During this process BH_4 is oxidized to 4a-hydroxytetrahydrobiopterin and released from the enzyme. The carbinolamine is then dehydrated by PCD (to qBH_2, see earlier), which is then reduced by NADH-dependent DHPR to regenerate BH_4. The crystal structure of TH (Goodwill *et al.,* 1998) complexed with 7,8-BH_2 reveals the BH_4 binding site in the enzyme. The crystal structure of TH also reveals that a nonheme iron is located within 5.6 Å of the 4a site of BH_4, which is the position of oxidation. When the pterin binding sites of TH and NOS are compared, the dissimilarities are most conspicuous. First, the distance between the heme iron and 4a of BH_4 in NOS is markedly greater in NOS (12.4 Å). Second, the pyrimadone in TH is

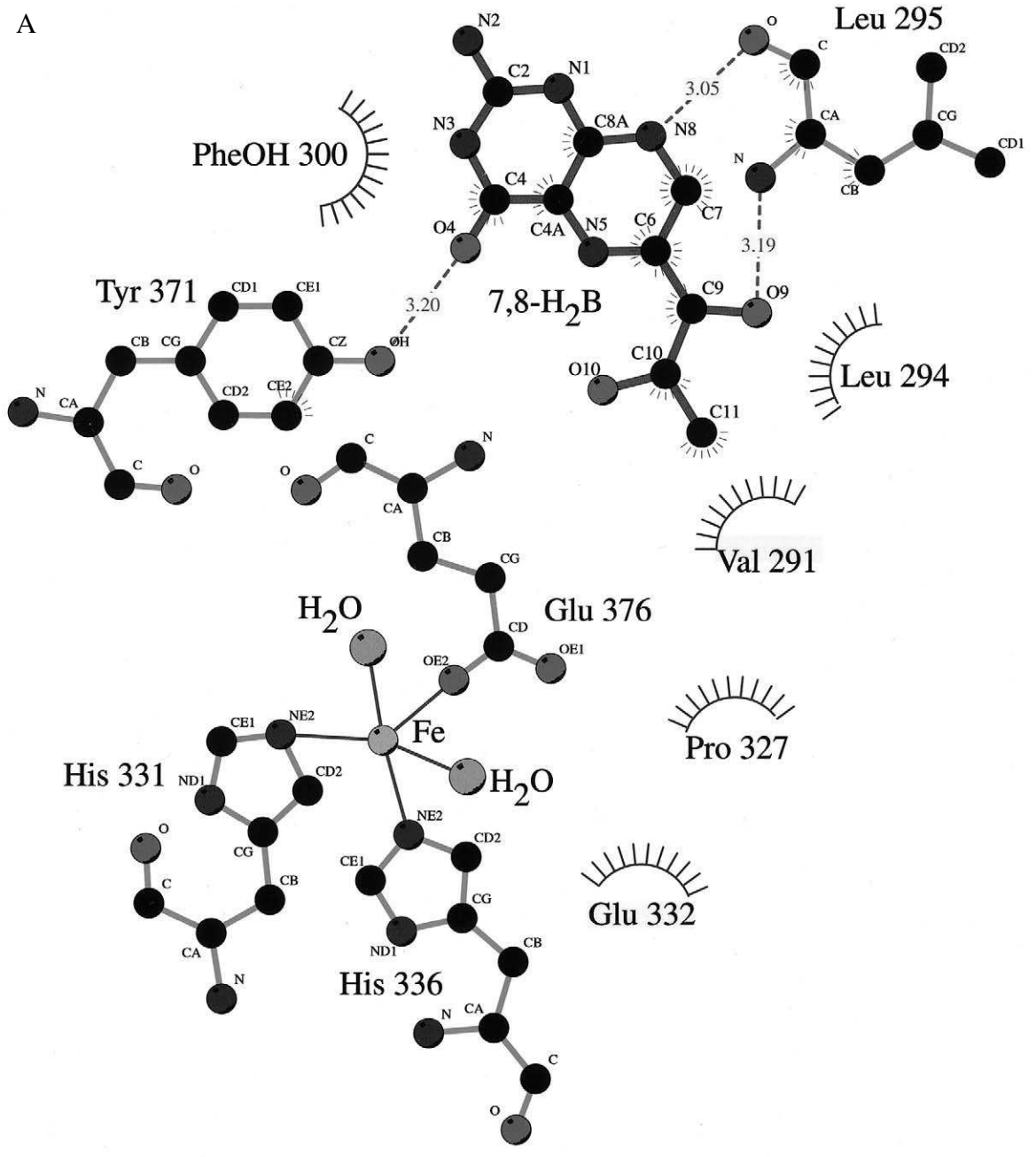


Figure 6 Structures of tyrosine hydroxylase and sepiapterin reductase with bound pterin. (A) The cofactor site of TH is depicted in complex with 7,8-BH_2. Notice that the amines at C-2 and N-3 do not interact with the enzyme, in contrast to the case for SR, shown in (B). The distance of separation between the catalytic Fe(II) and N-5 of the cofactor is 5.6 Å. A nonheme iron, however, is not part of the catalytic NOS heme domain structure. (B) The substrate binding site of SR is depicted in complex with biopterin. Notice the key Asp-258 side-chain carboxylate mediating the specific recognition of the pyrimadone. See color insert. *(Continues)*

not protected by hydrogen bonds as it is in NOS, and an extensive hydrogen-bonding network of pterin contacts is absent from TH. It has been known for some time that the highly activated pyrmidine nucleus is responsible for facile oxidation of BH_4. Therefore, it is reasonable that N-3 and N-5 of pterin are not tied up in strong hydrogen-bonding interactions in TH, but rather are available for promoting the pterin ring oxidation essential to catalysis. A similar scenario is seen in the crystal structure of BH_2 complexed PCD (Cronk *et al.,* 1996). The crystal structure of DHPR—albeit uncompleded with q-BH_2—and associated biochemical studies suggest that the pyrimadone is free in the pterin-bound enzyme (Varughese *et al.,* 1992). When these observations in related enzyme systems are compared with NOS, three key differences emerge: (a) a nonheme iron is absent in NOS, and therefore BH_4 does not undergo oxidation to

B

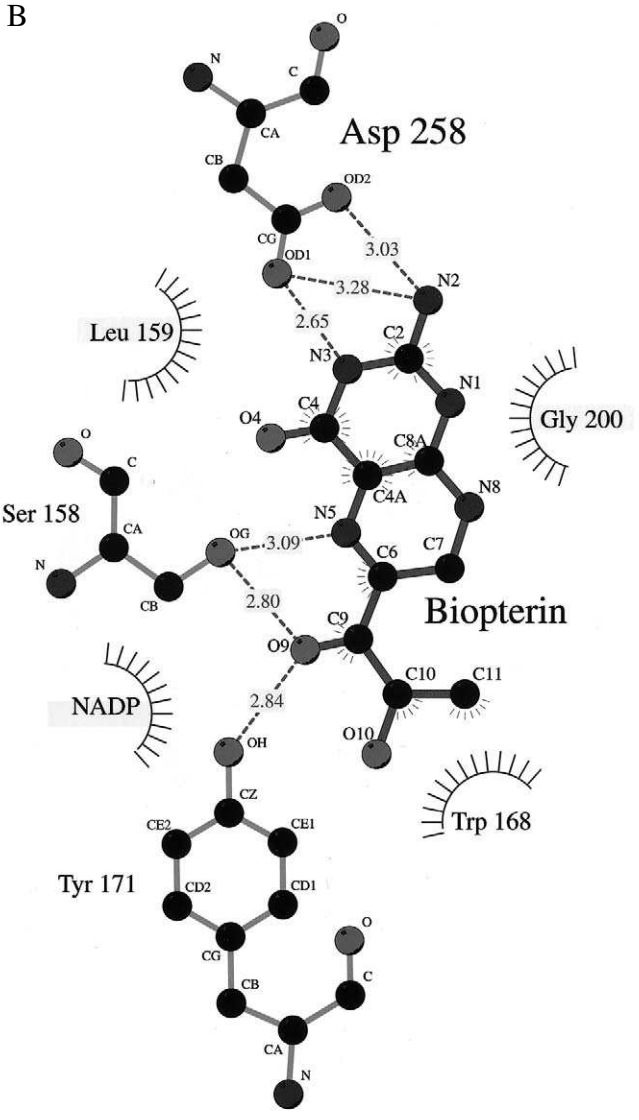


Figure 6 *(continued)*

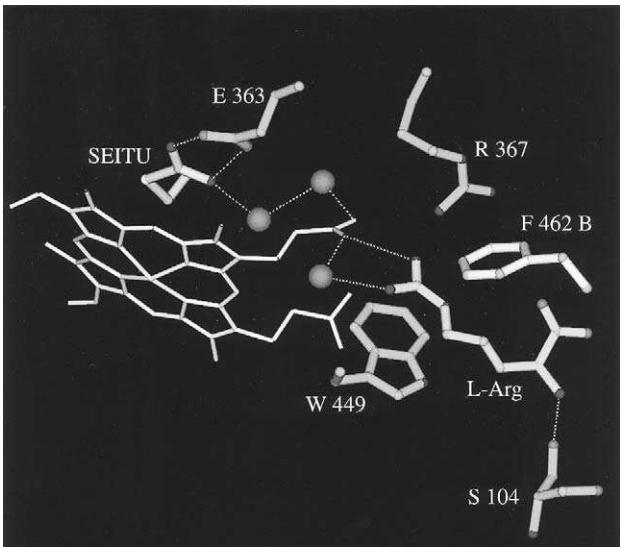


Figure 7 Cofactor mimicry by substrate L-Arg, when *S*-ethylisothiourea (SEITU) occupies the substrate site. Hydrogen bonds are shown as dotted lines, and red spheres correspond to water molecules. The mode of L-Arg binding at the pterin site exquisitely mimics that of BH_4 and serves as the basis for the structure-based proposal for the involvement of pterin radical in NOS catalysis. See color insert.

the 4a-hydroxy-BH_4 state; (b) the pterin cofactor of NOS, unlike that of AAH, is nondissociable during catalysis, as evidenced by the plethora of interactions it entertains and its observed high affinity for enzyme binding; and (c) the indispensable requirement for a tetrahydropterin cofactor argues in favor of a novel redox mechanism for the participation of BH_4 in the catalytic steps leading to NO biosynthesis.

The precise mechanism by which BH_4 functions in NOS catalysis is uncertain, although the crystal structure of pterin-free eNOS has supported a mechanistic proposal that could be experimentally tested. Raman *et al.* (1998) had solved two pterin-free eNOS heme domain crystal structures: (a) a structure with substrate bound at the active site and (b) a structure with a potent inhibitor, *S*-ethylisothiourea (SEITU), bound at the active site. When the substrate is bound at the active site, the pterin site was empty with the exception of solvent molecules present. In the SEITU-complexed state, however, the substrate itself was bound to the BH_4 site (see Fig. 7). This is interesting because the ability of the pterin site to bind other molecules, particularly the substrate, was unknown from earlier biochemical studies. L-Arginine bound at the pterin site closely mimics the BH_4 binding mode, with the guanidinium of the substrate making hydrogen-bonding contacts with the heme propionate. The pyrimidine ring of BH_4 has a built-in guanidinium function (see earlier), and the molecular recognition of an L-Arg guanidinium at the pterin binding site reveals that occupancy by a strong base can be accommodated. The recruitment of L-Arg (pKa of the guanidinium 12.48) to the pterin site further suggests that the BH_4 binding site may be poised to stabilize a positively charged state of the pterin ring. Thus, a fully protonated pterin can potentially bind to the pterin site of NOS. Complete protonation of BH_4 would restrict the lone pair reactivity on N-5 of the pyrazine ring and favor the ability to generate a trihydropterin radical via one-electron oxidation. On the basis of rapid reaction kinetics, Bec and colleagues (1998) proposed the formation of a pterin radical in nNOS, which is in complete agreement with the structure-based proposal presented here. More recently, Hurshman *et al.* (1999) have trapped the trihydropterin radical and conclude that BH_4 indeed participates in the electron transfer reactions of NOS catalysis. Details on the role of pterin radical in NOS function are discussed elsewhere (Raman *et al.,* 2000).

Does the identification of a pterin radical provide sufficient information to describe the NO biosynthetic machinery? The simple answer to this question is no! There are, however, structural features that are intriguing. First, the heme propionate that hydrogen bonds to the pyrimadone of BH_4 is also hydrogen-bonded to the primary amino group of substrate, L-Arg. Second, the primary amino group of L-Arg

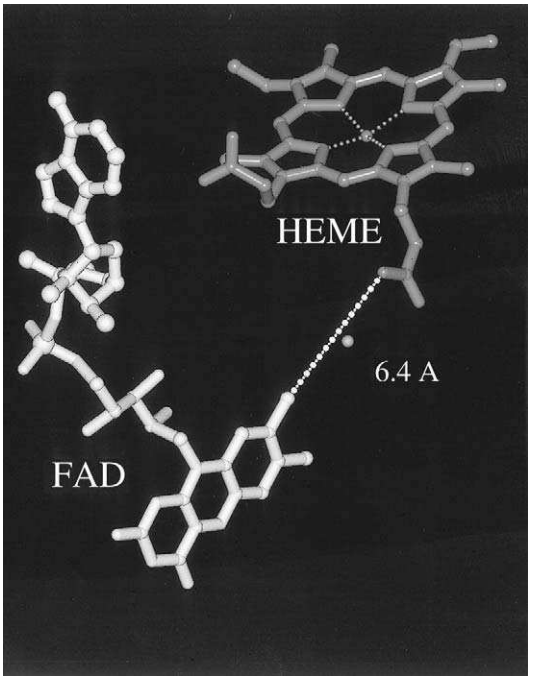


Figure 8 Close proximity between flavin adenine dinucleotide and the heme prosthetic group in bacterial flavohemoglobin. The dashed line corresponds to the distance of separation between FAD and the propionate. A water molecule is equidistant between the two. See color insert.

is hydrogen bonded to Glu-363 (bovine eNOS), which also holds the substrate guanidinium in place. Thus, electron transfer can be facilitated by the direct link between the cofactor, heme, and the substrate. Although direct interaction between a pterin cofactor and heme prosthetic group is without precedent, function in electron transfer is in accord with knowledge of bacterial flavohemoglobins (Ermler *et al.,* 1995). Notably, electron transfer from FAD to heme in flavohemoglobin is effective in a configuration in which FAD is less than 6 Å distant from a heme propionate group (Fig. 8). Thus, both circumstantial and direct lines of evidence implicate an electron transfer role for pterin in NO biosynthesis.

Despite the fundamental role of BH_4 in the regulation and mediation of NO synthesis, the function of BH_4 in NOS catalysis remains largely an enigma. Nonetheless, the elusive role of BH_4 will surely be defined in the near future, promising cardinal advance in our understanding of NOS enzymology.

References

Auerbach, G., Herrmann, A., Gutlich, M., Fischer, M., Jacob, U. Bacher, A., and Huber, R. (1997). The 1.25 Å crystal structure of sepiapterin reductase reveals its binding mode to pterins and brain neurotransmitters. *EMBO J.* **16,** 7219–7230.

Ballahsene, Z., Dhondt, J.-L., and Farriaux (1984). Guanosine triphosphate cyclohydrolase activity in rat tissues. *Biochem. J.* **217,** 59–65.

Bec, N., Gorren, A. C., Voelker, C., Mayer, B., and Lange, R. (1998). Reaction of neuronal nitric-oxide synthase with oxygen at low temperature. Evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin. *J. Biol. Chem.* **273,** 13502–13508.

Birdsall, D. L., Finer-Moore, J., and Stroud, R. M. (1996). Entropy in bisubstrate enzymes: Proposed role of an alternate site in chaperoning substrate into, and products out of thymidylate synthase. *J. Mol. Biol.* **255,** 522–535.

Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C., and Kraut, J. (1982). Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 Å resolution. General features and binding of methotrexate. *J. Biol. Chem.* **257,** 13650–13662.

Bourne, H. R., Sanders, D. A., and McCormick, F. (1991). The GTPase superfamily: Conserved structure and molecular mechanism. *Nature* **349,** 117–127.

Bune, A. J., Brand, M. P., Heales, S. J., Shergill, J. K., Cammack, R., and Cook, H. T. (1996). Inhibition of tetrahydrobiopterin synthesis reduces in vivo nitric oxide production in experimental endotoxic shock. *Biochem. Biophys. Res. Commun.* **220,** 13–19.

Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W. W., and Rees, D. C. (1995). Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science* **267,** 1463–1469.

Chen, X., Reynolds, E. R., Ranganayakulu, G., and O'Donnell, J. M. (1994). A maternal product of the Punch locus of *Drosophila melanogaster* is required for precellular blastoderm nuclear divisions. *J. Cell Sci.* **107,** 3501–3513.

Cho, S., Volpe, B. T., Bae, Y., Hwang, O., Choi, H. J., Gal, J., Park, L. C., Chu, C. K., Du, J., and Joh, T. H. (1999). Blockade of tetrahydrobiopterin synthesis protects neurons after transient forebrain ischemia in rat: A novel role for the cofactor. *J. Neurosci.* **19,** 878–889.

Citron, B. A., Davis, M. D., Milstien, S., Gutierrez, J., Mendel, D. B., Crabtree, G. R., and Kaufman, S. (1992). Identity of 4a-carbinolamine dehydratase, a component of the phenylalanine hydroxylation system, and DCoH, a transregulator of homeodomain proteins. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11891–11894.

Crane, B. R., Arvai, A. S., Gachhui, R., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1997). The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **278,** 425–431.

Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* **279,** 2121–2126.

Cronk, J. D., Endrizzi, J. A., and Alber, T. A. (1996). High-resolution structures of the bifunctional enzyme and transcriptional coactivator DCoH and its complex with a product analogue. *Protein Sci.* **5,** 1963–1972.

Ermler, U., Siddiqui, R. A., Cramm, R., and Friedrich, B. (1995). Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 A resolution. *EMBO J.* **14,** 6067–6077.

Etter, M. C., and Adsmond, D. A. (1990). *J. Chem. Soc. Commun.* **8,** 589–591.

Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999). Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat. Struct. Biol.* **6,** 233–242.

Ghosh, D. K., and Stuehr, D. J. (1995). Macrophage NO synthase: Characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. *Biochemistry* **34,** 801–807.

Giovanelli, J., Campos, K. L., and Kaufman, S. (1991). Tetrahydrobiopterin, a cofactor for cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7091–7095.

Goodwill, K. F., Sabatier, C., and Steven, R. C. (1998). Crystal structure of tyrosine hydroxylase with bound cofactor analogue and iron at 2.3 A resolution: Self-hydroxylation of Phe300 and the pterin-binding site *Biochemistry* **37,** 13437–13445.

Gorren, A. C., List, B. M., Schrammel, A., Pitters, E., Hemmens, B., Werner, E. R., Schmidt, K., and Mayer, B. (1996). Tetrahydrobiopterin-free neuronal nitric oxide synthase: Evidence for two identical highly anticooperative pteridine binding sites. *Biochemistry* **35,** 16735–16745.

Gross, S. S., and Levi, R. (1992). Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J. Biol. Chem.* **267,** 25722–25729.

Gross, S. S., and Levi, R. (1995). Tetrahydrobiopterin availability limits LPS-induced killing and NO synthesis in rats. *4th Annual Meeting Biology of Nitric Oxide, Endothelium* **3**(Suppl.), s118.

Gross, S. S., Jaffe, E. A., Levi, R., and Kilbourn, R. G. (1991). Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine and analogs with a rank-order of potency characteristics of activated macrophages. *Biochem. Biophys. Res. Commun.* **178,** 823–829.

Hampele, I. C., D'Arcy, A., Dale, G. E., Kostrewa, D., Nielsen, J., Oefner, C., Page, M. G., Schonfeld, H. J., Stuber, D., and Then, R. L. (1997). Structure and function of the dihydropteroate synthase from *Staphylococcus aureus. J. Mol. Biol.* **268,** 21–30.

Harada, T., Kagamiyama, H., and Hatakeyama, K. (1993). Feedback regulation mechanisms for the control of GTP cyclohydrolase I activity. *Science* **260,** 1507–1510.

Hattori, Y., Nakanishi, N., Kasai, K., Murakami, Y., and Shimoda, S. (1996). Tetrahydrobiopterin and GTP cyclohydrolase I in a rat model of endotoxic shock: Relation to nitric oxide synthesis. *Exp. Physiol.* **81,** 665–671.

Hennig, M., D'Arcy, A., Hampele, I. C., Page, M. G. P., Oefner, C., and Dale, G. E. (1998). Crystal structure and reaction mechanism of 7,8-dihydroneopterin aldolase from *Staphylococcus aureus. Nat. Struct. Biol.* **5,** 357–362.

Hesslinger, C., Kremmer, E., Hultner, L., Ueffing, M., and Ziegler, I. (1998). Phosphorylation of GTP cyclohydrolase I and modulation of its activity in rodent mast cells. GTP cyclohydrolase I hyperphosphorylation is coupled to high affinity IgE receptor signaling and involves protein kinase C. *J. Biol. Chem.* **273,** 21616–21622.

Hevel, J. M., and Marletta, M. A. (1992). Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity *Biochemistry* **31,** 7160–7165.

Hopkins, F. G. (1889). Note on yellow pigments in butterflies. *Nature* **40,** 335.

Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A. (1999). Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. *Biochemistry* **38,** 15689–15696.

Hussain, S. N., Giaid, A., El Dawiri, Q., Sakkal, D., Hattori, R., and Guo, Y. (1997). Expression of nitric oxide synthases and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. *Am. J. Respir. Cell Mol. Biol.* **17,** 173–80.

Hwang, O., Baker, H., Gross, S., and Joh, T. H. (1998). Localization of GTP cyclohydrolase in monoaminergic but not nitric oxide-producing cells. *Synapse* **28,** 140–153.

Hwang, O., C. H., and Park, S. Y. (1999). Up-regulation of GTP cyclohydrolase I and tetrahydrobiopterin by calcium influx. *Neuroreport* **10,** 3611–3614.

Ichinose, H., Ohye, T., Tokahashi, E., Seki, N., Hori, T., Segawa, M., Nomura, Y., Eudo, K., Tanatia, H., Tsuji, S., *et al.* (1994). Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase 1 gene. *Nature Genet.* **8,** 236–242.

Ichinose, H., Ohye, T., Yokochi, M., Fujita, K., and Nagatsu, T. (1995). GTP cyclohydrolase I activity in mononuclear blood cells in juvenile Parkinsonism. *Neurosci Lett.* **190,** 140–142.

Kasai, K., Hattori, Y., Banba, N., Hattori, S., Motohashi, S., Shimoda, S., Nakanishi, N., and Gross, S. S. (1997). Induction of tetrahydrobiopterin synthesis in rat cardiac myocytes: Impact on cytokine-induced NO generation. *Am. J. Physiol.* **273,** H665–H672.

Kaufman, S. (1963). The structure of the primary oxidation product formed from tetrahydropteridines during phenylalanine hydroxylation. *J. Biol. Chem.* **239,** 332–338.

Kaufman, S. (1970). A protein that stimulates rat liver phenylalanine hydroxylase. *J. Biol. Chem.* **254,** 4751–4759.

Kinoshita, H., Milstien, S., Wambi, C., and Katusic, Z. S. (1997). Inhibition of tetrahydrobiopterin biosynthesis impairs endothelium-dependent relaxations in canine basilar artery. *Am. J. Physiol.* **273,** H718–H724.

Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P., and Mayer, B. (1995). Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J.* **14,** 3687–3695.

Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* **264,** 20496–20501.

Linscheid, P., Schaffner, A., and Schoedon, G. (1998). Modulation of inducible nitric oxide synthase mRNA stability by tetrahydrobiopterin in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **243,** 137–141.

Liu, Q., and Gross, S. S. (1996). Binding sites of nitric oxide synthases. *Methods Enzymol.* **268,** 311–324.

McDonald, J. D., and Bode, V. C. (1988). Hyperphenylalaninemia in the hph-1 mouse mutant. *Pediatr. Res.* **23,** 63–67.

Milstien, S., Jaffe, H., Kowlessur, D., and Bonner, T. L. (1996). Purification and cloning of the GTP cyclohydrolase I feedback regulatory protein. *J. Biol. Chem.* **271,** 19743–19751.

Nagatsu, I., Ichinose, H., Sakai, M., Titani, K., Suzuki, M., and Nagatsu, T. (1995). Immunocytochemical localization of GTP cyclohydrolase I in the brain, adrenal gland, and liver of mice. *J. Neural Transm. Gen. Sect.* **102,** 175–188.

Nar, H., Huber, R., Meining, W., Schmid, C., Weinkauf, S., and Bacher, A. (1995). Atomic structure of GTP cyclohydrolase I. *Structure* **3,** 459–466.

Pou, S., Pou, W. S., Bredt, D. S., Synder, S. H., and Rosen, G. M. (1992). Generation of superoxide by purified brain nitric oxide synthase. *J. Biol. Chem.* **267,** 24173–24176.

Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L. (1998). Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell* **95,** 939–950.

Raman, C. S., Martasek, P., and Masters, B. S. S. (2000). Structural themes determining function in nitric oxide synthases. *In* ''The Porphyrin Handbook'' (K. M. Kadish, K. M. Smith, and R. Guilard, eds.), Vol. 4, pp. 293–339.

Riethmüller, C., Gorren, A. C. F., Pitters, E., Hemmens, B., Habisch, H.-J., Heales, S. J. R., Schmidt, K., Werner, E. R., and Mayer, B. (1999). Activation of neuronal nitric oxide synthase by the 5-methyl analog of tetrahydrobiopterin. *J. Biol. Chem.* **274,** 16047–16051.

Rosenkranz-Weiss, P., Sessa, W. C., Milstien, S., Kaufman, S., Watson, C. A., and Pober, J. S. (1994). Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J. Clin. Invest.* **93,** 2236–2243.

Scherer-Oppliger, T., Leimbacher, W., Blau, N., and Thony, B. (1999). Serine I9 of human 6-pyruvoyltetrahydropterin synthase is phosphorylated by cGMP protein kinase II. *J. Biol. Chem.* **274,** 31341–31348.

Shen, R.-S., Alam, A., and Zhang, Y. X. (1988). Inhibition of GTP cyclohydrolase I by pterins. *Biochim. BIophys. Acta* **965,** 9–15.

Smith, G. K., Duch, D. S., Edelstein, M. P., and Bigham, E. C. (1992). New inhibitors of sepiapterin reductase. Lack of an effect of intracellular tetrahydrobiopterin depletion upon in vitro proliferation of two human cell lines. *J. Biol. Chem.* **267,** 5599–5607.

Steinmetz, M. O., Pluss, C., Christen, U., Wolpensinger, B., Lustig, A., Werner, E. R., Wachter, H., Engel, A., Aebi, U., Pfeilschifter, J., and Kammerer, R. A. (1998). Rat GTP cyclohydrolase I is a homodecameric protein complex containing high-affinity calcium-binding sites. *J. Mol. Biol.* **279,** 189–199.

Tayeh, M. A., and Marletta, M. A. (1989). Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. *J. Biol. Chem.* **264,** 19654–19658.

Tzeng, E., Yoneyama, T., Hatakeyama, K., Shears, L. L., and Billiar, T. R. (1996). Vascular inducible nitric oxide synthase gene therapy: Re-

quirement for guanosine triphosphate cyclohydrolase 1. *Surgery* **120,** 315–321.

Varnum, M. D., Black, K. D., and Zagotta, W. N. (1995). Molecular mechanism for ligand discrimination of cyclic nucleotide-gated channels. *Neuron* **15,** 619–625.

Varughese, K. I., Skinner, M. M., Whiteley, J. M., Matthews, D. A., and Xuong, N. H. (1992). Crystal structure of rat liver dihydropteridine reductase. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6080–6084.

Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc. Natl. Acad. Sci. USA* **95,** 9220–9225.

Visconti, M., and Karrer, P. (1957). Fluoreszierende Stoffe aus *Drosophila melanogaster. Helv. Chim. Acta* **40,** 986.

Viveros, O. H., Lee, C. L., Abou-Donia, M. M., Nixon, J. C., and Nichol, C. A. (1981). Biopterin cofactor biosynthesis: Independent regulation of GTP cyclohydrolase in adrenal medulla and cortex. *Science* **213,** 349–350.

Wachter, H., Fuchs, D., Hausen, A., Reibnegger, G., and Werner, E. R. (1989). Neopterin as marker for activation of cellular immunity: Immunologic basis and clinical application. *Adv. Clin. Chem.* **27,** 81–141.

Walter, R., Schaffner, S., Blau, N., Kierat, L., and Schoedon, G. (1994). Tetrahydrobiopterin is a secretory product of murine vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **203,** 1522–1526.

Wang, J., Stuehr, D. J., and Rousseau, D. L. (1995). Tetrahydrobiopterin-deficient nitric oxide synthase has a modified heme environment and forms a cytochrome P-420 analogue. *Biochemistry* **34,** 7080–7087.

Werner, E. R., Werner-Felmayer, G., Fuchs, D., Hausen, A., Reibnegger, G., Yim, J. J., and Wachter, H. (1991). Impact of tumour necrosis factor-alpha and interferon-gamma on tetrahydrobiopterin synthesis in murine fibroblasts and macrophages. *Biochem. J.* **280,** 709–714.

Werner, E. R., Pitters, E., Schmidt, K., Wachter, H., Werner-Felmayer, G., and Mayer, B. (1996). Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase. *Biochem. J.* **320,** 193–196.

Werner, E. R., Werner-Felmayer, G., and Mayer, B. (1998). Tetrahydrobiopterin, cytokines, and nitric oxide synthesis. *Proc. Soc. Exp. Biol. Med.* **219,** 171–182.

Werner-Felmayer, G., and Gross, S. S. (1996). Analysis of tetrahydrobiopterin and its role in nitric oxide synthesis. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. S. Stamler, eds.), pp. 271–299. Wiley, Sussex, England.

Werner-Felmayer, G., Golderer, G., Werner, E. R., Grobner, P., and Wachter, H. (1994). Pteridine biosynthesis and nitric oxide synthase in *Physarum polycephalum. Biochem. J.* **304,** 105–111.

Wever, R. M. F., van Dam., T., van Rijn, H. J. M., de Groot, P. G., and Rabelink, T. J. (1997). Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthesis. *Biochem. Biophys. Res. Commun.* **237,** 340–344.

Xia, Y., Tsai, A. L., Berka, V., and Zweier, J. L. (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J. Biol. Chem.* **273,** 25804–25808.

Xie, L., Smith, J. A., and Gross, S. S. (1998). GTP cyclohydrolase I inhibition by the prototypic inhibitor 2,4-diamino-6-hydroxypyrimidine. Mechanisms and unanticipated role of GTP cyclohydrolase I feedback regulatory protein. *J. Biol. Chem.* **273,** 21091–21098.

Yan, X., Day, P., Hollis, T., Monzingo, A. F., Schelp, E., Robertus, J. D., Milne, G. W., and Wong, S. (1998). Recognition and interaction of small rings with the ricin A-chain binding site. *Proteins* **31,** 33–41.

Yoneyama, T., and Hatakeyama, K. (1998). Decameric GTP cyclohydrolase I forms complexes with two pentameric GTP cyclohydrolase I feedback regulatory proteins in the presence of phenylalanine or of a combination of tetrahydrobiopterin and GTP. *J. Biol. Chem.* **273,** 20102–20108.

Zhuo, S., Fan, S., and Kaufman, S. (1996). Effects of depletion of intracellular tetrahydrobiopterin in murine erythroleukemia cells. *Exp. Cell Res.* **222,** 163–170.

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CHAPTER 11

Regulation of Arginine Availability and Its Impact on NO Synthesis

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BECAUSE ARGININE IS THE ONLY PHYSIOLOGICAL SOURCE OF THE NITROGEN ATOM IN NITRIC OXIDE (NO), ENZYMATIC REACTIONS AND OTHER PROCESSES THAT REGULATE THE AVAILABILITY OF THIS AMINO ACID PLAY CRUCIAL ROLES IN REGULATING RATES OF NO SYNTHESIS IN HEALTH AND DISEASE. THE PROCESSES OF ARGININE BIOSYNTHESIS, CATABOLISM, AND TRANSPORT ARE ESPECIALLY IMPORTANT IN MODULATING BOTH INTRACELLULAR AND PLASMA LEVELS OF ARGININE. THE KEY PLAYERS IN THESE PROCESSES ARE ARGININOSUCCINATE SYNTHASE, THE ARGINASES, AND THE CATIONIC AMINO ACID TRANSPORTERS (CATS), RESPECTIVELY. THEIR EXPRESSION NOT ONLY VARIES AMONG DIFFERENT CELL TYPES BUT IS DYNAMICALLY MODULATED BY BACTERIAL LIPOPOLYSACCHARIDE, CYTOKINES, AND OTHER AGENTS. IN PARTICULAR, ARGININOSUCCINATE SYNTHASE AND THE CATS ARE GENERALLY COINDUCED WITH INFLAMMATORY NITRIC OXIDE SYNTHASE (INOS), APPARENTLY TO MAXIMIZE CELLULAR CAPACITY FOR NO SYNTHESIS. THE METABOLIC ROLES AND EXPRESSION OF THE ARGINASES, WHICH COMPETE WITH THE NOS ENZYMES FOR A COMMON SUBSTRATE, ARE MORE COMPLEX AND INCOMPLETELY UNDERSTOOD. THE ARGININE METABOLITE AGMATINE, RECENTLY DISCOVERED TO BE SYNTHESIZED BY MAMMALS, HAS POTENTIAL FOR INHIBITING NO SYNTHESIS, BUT ITS FUNCTION *IN VIVO* REMAINS TO BE ESTABLISHED. AS VIRTUALLY ALL THE ENZYMES OF ARGININE METABOLISM, INCLUDING THE NOS ISOZYMES, CAN BE EXPRESSED WITHIN THE SAME CELL, THE COMPLEX INTERPLAY BETWEEN THE OFTEN CONFLICTING OR COMPETING REACTIONS REPRESENTS A CONSIDERABLE CHALLENGE TO OUR ABILITY TO UNDERSTAND THE FACTORS THAT CONTROL NO SYNTHESIS IN DIFFERENT CELL TYPES.

Introduction

The regulation of nitric oxide (NO) synthesis has proved to be remarkably complex. As described elsewhere in this volume, the synthesis of the nitric oxide synthase (NOS) isoforms and their cofactors, as well as NOS activity, is highly regulated, and our understanding of these processes is far from complete. The ever-burgeoning studies of NO synthesis during the 1990s have spurred a growing appreciation of and interest in the complex metabolism of arginine. Prior to 1987, this amino acid was most commonly recognized as a metabolite of the urea cycle and as a substrate in creatine biosynthesis. Following the discovery that arginine is the only physiologically significant source of the nitrogen atom in NO, interest in other aspects of arginine metabolism intensified.

Availability of arginine for NO synthesis is determined by five factors: exogenous supply (dietary intake by intact animals or concentration in the medium used to culture cells), endogenous release of arginine via protein degradation,

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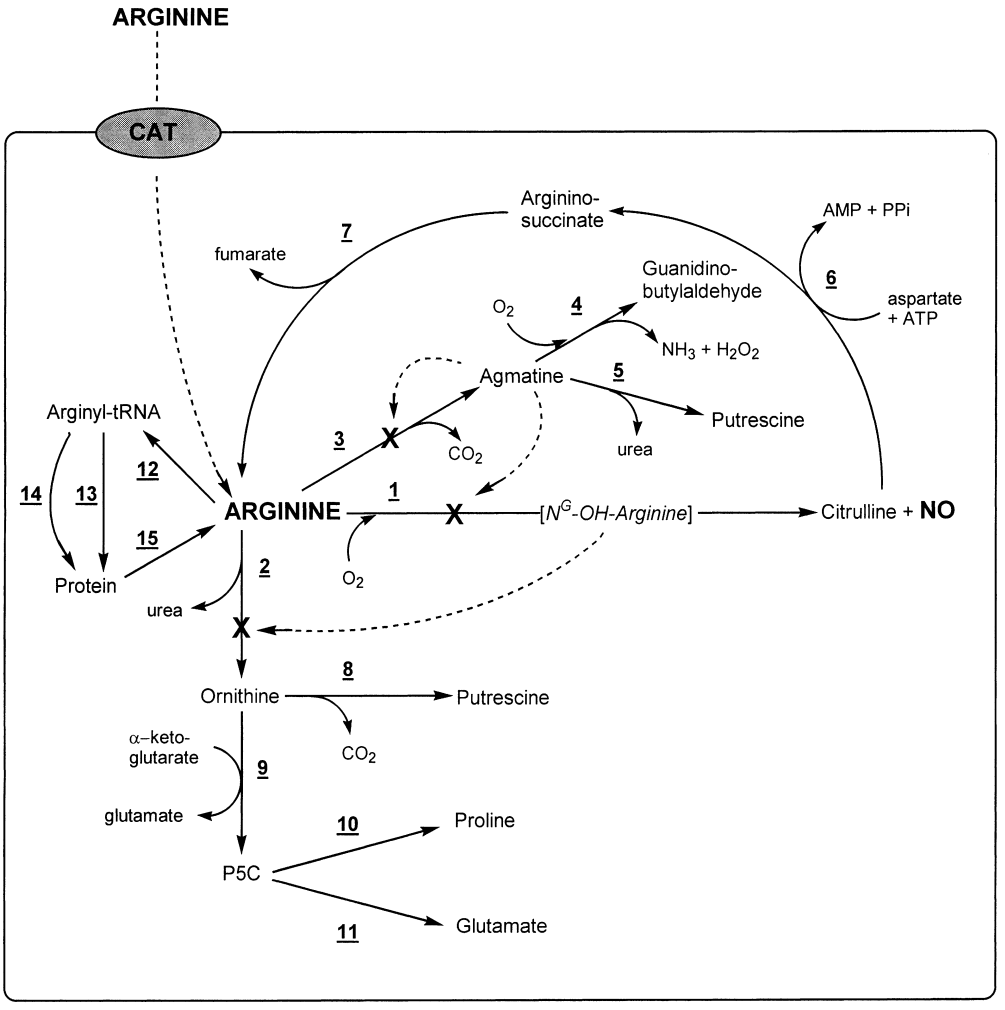


Figure 1 Overview of arginine metabolism in nonhepatic cells. For the sake of simplicity, subcellular compartments, multiple enzyme isoforms, and enzyme cofactors are not depicted. Structures of key metabolites are shown in Fig. 2. Key to enzymes: 1, NOS; 2, arginase; 3, arginine decarboxylase; 4, diamine oxidase; 5, agmatinase; 6, argininosuccinate synthase; 7, argininosuccinate lyase; 8, ornithine decarboxylase; 9, ornithine aminotransferase; 10, P5C reductase; 11, P5C dehydrogenase; 12, arginyl-tRNA synthetase; 13, protein synthesis; 14, arginyl-tRNA–protein transferase; 15, proteases. Abbreviations and symbols: P5C, Δ^1-pyrroline-5-carboxylate; CAT, cationic amino acid transporter.

endogenous arginine synthesis, arginine catabolism, and arginine transport. Enzymatic reactions involved in each of these processes are depicted in Fig. 1. The last four processes regulate availability of arginine at two levels: by controlling plasma arginine levels in the intact animal and by regulating the intracellular supply of arginine for NO synthesis in individual cell types. Complexity arises from the fact that relative contributions of each of these processes to overall arginine availability are not the same in every cell type, and they can vary considerably during development and in response to various stimuli, including those that regulate NO synthesis. In addition, the various enzymatic reactions are not always independent, that is, the product or intermediate of one enzyme may inhibit the activity of a different enzyme, the most notable example being inhibition of arginase by N^G-hydroxyarginine, an intermediate in NO synthesis (Fig. 1).

The structural similarity between arginine and several of its metabolites (Fig. 2) might suggest that several of these compounds could serve as substrates for NO synthesis, but the NOS enzymes are quite selective in abstracting one of the two chemically equivalent nitrogen atoms in the guanidino group of arginine. Agmatine, guanidinobutylaldehyde, and guanidinoacetate retain the guanidino group found in arginine itself, but they have very little or no activity as substrates for NO synthesis. N^G-Hydroxyarginine, a relatively stable intermediate in NO synthesis, is a substrate for NOS when provided exogenously to cells, but, unlike arginine, it is a potent inhibitor of arginase (Fig. 1). Only the L-enantiomers of arginine, its metabolites, or analogs are recognized by the NOS enzymes as substrates or inhibitors.

This chapter reviews the concept that competing enzymatic reactions and other processes which regulate arginine

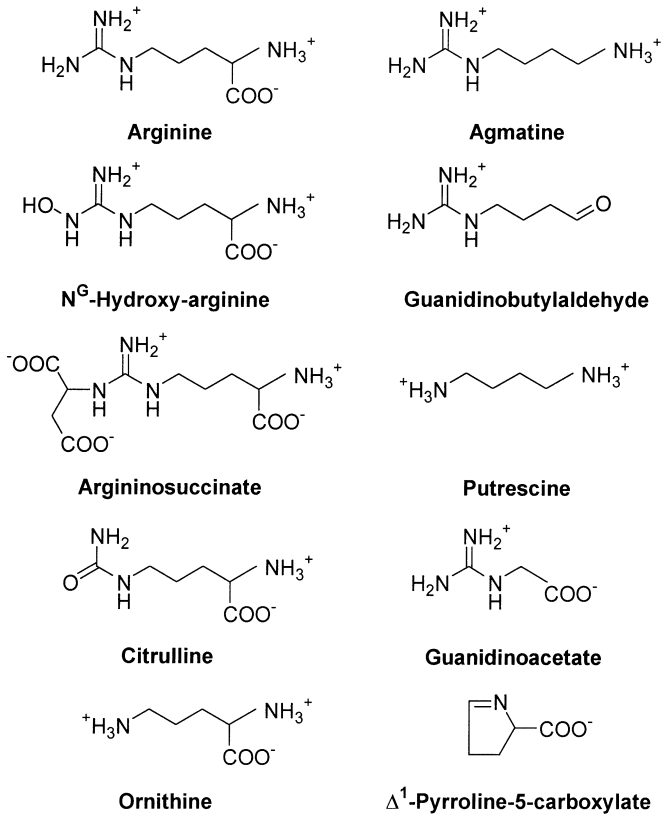


Figure 2 Structures of arginine and arginine-derived metabolites. Guanidinoacetate, derived from arginine and glycine, is the first intermediate in creatine biosynthesis.

availability can play important roles in determining rates of NO synthesis. The primary focus will be on arginine synthesis, with brief mention of the roles of arginine transport and the arginases because these topics will be considered in depth in subsequent chapters. Possible roles of agmatine, an arginine metabolite synthesized in mammalian cells, also will be noted. Some aspects of arginine metabolism that are represented in Fig. 1 will not be discussed because of inadequate information specifically relating these processes to NO synthesis. These reactions include ubiquitous processes involved in protein synthesis (reactions 12 and 13), posttranslational modification (reaction 14), and degradation (reaction 15). Although high-level production of NO in cells induced to express inflammatory NOS (iNOS) can result in inhibition of protein synthesis, no studies have evaluated the roles of protein synthesis and degradation in regulating availability of arginine for NO synthesis within specific cell types. This chapter also will not consider potential roles of arginine:glycine amidinotransferase, an arginine-requiring enzyme that catalyzes the first step in creatine biosynthesis (not illustrated), because it is expressed only in a limited number of specialized cell types and has not been shown to regulate arginine availability for NO synthesis.

Exogenous Arginine and Arginine Homeostasis

Owing to endogenous synthesis, arginine is classified as a conditionally essential dietary amino acid for many adult mammals, including humans and rodents. Consequently, feeding these species an arginine-free diet has relatively little impact on plasma arginine levels. However, in immature, growing animals and in cases where functions of the major organs involved in arginine synthesis (i.e., the small intestine and kidneys) have been compromised by injury, infection, or surgical resection, endogenous synthesis is insufficient to meet the needs of the organism, and arginine therefore is required in the diet.

Arginine is an essential dietary amino acid for some species, including chickens and carnivores such as cats, ferrets, and dogs. Owing to the dietary requirement, it is possible to modulate plasma arginine levels in these creatures over a wider range than in humans and rodents by varying dietary arginine intake. Interestingly, this biological property has not been exploited to evaluate the impact of varying plasma arginine levels on NO synthesis in these animals.

Plasma arginine levels for healthy adult mice and humans are approximately 100 μM. In contrast, concentrations of arginine in standard tissue culture media are considerably higher, for example, 400 μM for DMEM medium and 1150 μM for RPMI 1640 medium. Consequently, in cultured cells with active arginine transport systems, rates of NO synthesis may be much higher than *in vivo,* possibly giving rise to effects which are not physiological. Moreover, cultured cells are not continously perfused with fresh medium and substrates, so that availability of arginine declines with time in culture. As a result, total NO production in iNOS-expressing cells during, for example, an overnight incubation may be more a reflection of the ratio of the volume of culture medium to total cell number than of the capacity of the cells to produce NO during the incubation period. These points should be taken into consideration when extrapolating results obtained with cultured cells to *in vivo* conditions.

Plasma arginine levels can decline by as much as 50–60% during sepsis, but the basis for this is not known. Although it is tempting to speculate that this is the consequence of iNOS induction, it has become clear that the arginases are also induced by inflammatory stimuli such as bacterial lipopolysaccharide (LPS), so these enzymes may contribute significantly to the reduction in plasma arginine levels during sepsis. Expression of plasma arginine transporters also is increased by inflammatory stimuli, thus enhancing capacity for arginine clearance. In short, it is likely that changes in multiple components of arginine metabolism contribute to the decline in plasma arginine levels in septic individuals.

Arginine homeostasis is maintained by a balance between dietary arginine intake, arginine production (synthesis plus release from degraded protein), and arginine degradation. Although the high capacity for endogenous arginine synthesis limits the impact of an arginine-free diet on plasma arginine levels in most mammals, elevated dietary intake can exceed the capacity for arginine degradation. Thus, it is

possible to double or triple plasma arginine levels by providing supplemental arginine in the diet or drinking water.

Regulation of arginine homeostasis is poorly understood. On the basis of metabolic labeling studies, it has been proposed that arginine degradation and/or dietary arginine intake, rather than arginine synthesis, are the primary regulators of arginine homeostasis. Consistent with this hypothesis, preliminary studies have shown that plasma arginine levels in mice homozygous for a ''knockout'' of the type II arginase gene are severalfold higher than in wild-type mice. This result indicates not only that type II arginase plays a major role in arginine homeostasis, but also that compensatory changes in arginine biosynthesis or type I arginase expression, if any, are not sufficient to restore normal plasma arginine values.

Arginine Synthesis

Whole Body

Net arginine synthesis *in vivo* is accomplished primarily by a collaboration between intestinal epithelial cells, which utilize glutamate and glutamine to synthesize citrulline, and renal proximal tubular cells, which take up citrulline and convert it to arginine. In fact, the renal tubular cells are responsible for approximately 60% of net arginine synthesis in adult mammals. Rates of renal arginine synthesis in adults are limited by the supply of citrulline rather than by renal capacity for converting citrulline to arginine. Plasma levels of citrulline, produced primarily by the small intestine, are normally 30–40 μM. High rates of arginine synthesis occur also within the urea cycle in liver, but there is little or no net production of arginine because newly synthesized arginine is immediately hydrolyzed to ornithine and urea. It is important to recognize that net arginine synthesis accounts for only 5–15% of total plasma arginine flux in healthy adults, indicating that most of the arginine entering the plasma pool is derived from protein degradation and diet. Endogenous arginine synthesis can contribute up to 30% of the total plasma arginine flux in immature growing animals.

The Citrulline–NO Cycle

Although the highest rates of net arginine synthesis occur in renal tubules, the capability for synthesizing arginine from citrulline, one of the two products of the NOS-catalyzed reaction, exists to some degree in virtually all cells. This led eventually to appreciation of the possibility that synthesis of NO and arginine could be coupled in a pathway termed the arginine–citrulline cycle or the citrulline–NO cycle (Fig. 3), such that citrulline is recycled to arginine. This pathway is analogous to the classic urea cycle in liver, which also includes argininosuccinate synthase and argininosuccinate lyase (Fig. 3). The only route other than NOS for producing citrulline in vertebrates is via the sequential action of carbamyl phosphate synthetase I and ornithine carbamyltransferase (Fig. 3), which are expressed only in parenchymal hepatocytes, epithelial cells of the small intestine, or in certain hepatoma or intestinal epithelial cell lines.

The conversion of citrulline to arginine occurs via the sequential action of argininosuccinate synthase and argininosuccinate lyase (Fig. 3). Argininosuccinate synthase exists as a tetramer of four identical subunits with a subunit molecular weight of approximately 46 kDa. Argininosuccinate lyase also is comprised of four identical subunits, with a subunit molecular weight of approximately 51 kDa. No isozymes or posttranslationally modified forms of either enzyme are known to occur. The citrulline-to-arginine conversion requires aspartate as the nitrogen donor and energy in the form of ATP. Figure 3 indicates that the aspartate used in the citrulline–NO cycle is regenerated from fumarate as is commonly depicted for the urea cycle, but there is no direct evidence for this. The hydrolysis of ATP to AMP and inorganic phosphate represents an energy requirement of two high-energy phosphate bonds for each nitrogen atom which is replenished in the citrulline–NO cycle. A practical consequence of this is the fact that the presence of argininosuccinate synthase should not interfere with *in vitro* assays of NOS activity which measure conversion of arginine to citrulline unless appreciable amounts of both ATP and aspartate also are present in the assay.

The presence of a citrulline–NO cycle is usually inferred if the substitution of citrulline for arginine in culture media supports cellular NO synthesis, but this does not necessarily indicate that a major fraction of NOS-produced citrulline is recycled into arginine when cells are incubated in the presence of arginine. In healthy adult humans, for example, about half of the arginine used for NO synthesis is derived from the plasma arginine pool, the remainder coming from intracellular protein degradation and endogenous arginine synthesis. Although limited, the available data indicate that efficiency of the citrulline–NO cycle varies from one cell type to another, but in no case has the efficiency been shown to approach that of the urea cycle. This is clearly evident from the fact that citrulline accumulates in the medium of NO-producing cells cultured in the presence of arginine. The low efficiency may reflect the K_m of argininosuccinate synthase for citrulline, which is approximately 40–70 μM. Consequently, if the rate of cellular NO synthesis is modest and if citrulline is transported across the plasma membrane at an appreciable rate, recycling of citrulline to arginine would be expected to be inefficient if the reactions in the citrulline–NO cycle are diffusion-controlled. However, cycle efficiency can be enhanced if the enzymes in the pathway are in close apposition within the cell. Both argininosuccinate synthase and argininosuccinate lyase are found primarily in the cytosolic fraction of cell homogenates, but there is evidence that they are associated with mitochondria in hepatocytes and smooth muscle cells. The mitochondrial association in hepatocytes undoubtedly contributes to the high efficiency of metabolite ''channeling'' within the urea cycle. There is one report that iNOS, argininosuccinate synthase, and argininosuccinate lyase are colocalized on or near mitochondria in

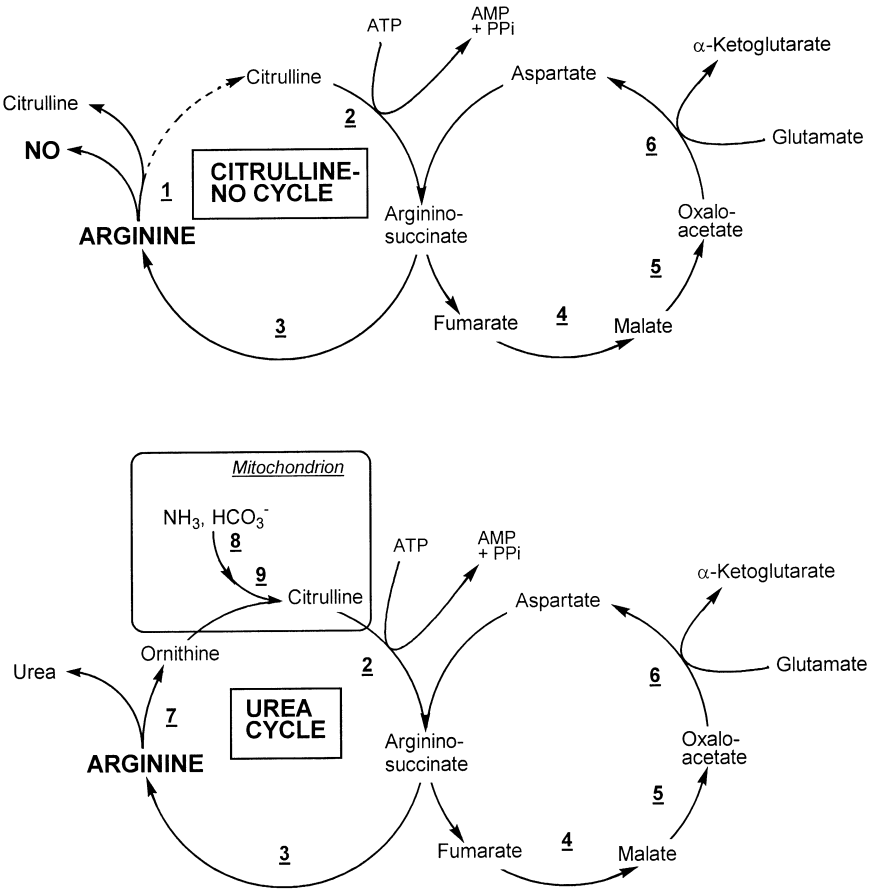


Figure 3 The citrulline–NO cycle and the urea cycle. The dashed line indicates that only a fraction of citrulline produced by NOS is recycled via the citrulline–NO cycle (adapted from Fig. 5 of Nussler *et al.,* 1994). Key to enzymes: 1, NOS; 2, argininosuccinate synthase; 3, argininosuccinate lyase; 4, fumarase; 5, malate dehydrogenase; 6, aspartate aminotransferase; 7, arginase (type I); 8, carbamyl phosphate synthetase I; 9, ornithine carbamyltransferase. Two ATP are required for reaction 8 in the urea cycle.

vascular smooth muscle cells, possibly to maximize efficiency of the citrulline–NO cycle. Whether such colocalization is a common feature of NO-producing cells remains to be determined.

Argininosuccinate synthase—but rarely argininosuccinate lyase—is coinduced with iNOS in nearly all mammalian cell lines and primary cultured cells examined to date (Table I). Argininosuccinate synthase and iNOS are induced in primary cultures of mixed neural cell types but are not coexpressed within the same cells, suggesting that an intercellular citrulline–NO cycle may exist in some cell populations. Virtually any stimulus that has been shown to induce iNOS also induces argininosuccinate synthase (Table I; Fig. 4). As basal expression of argininosuccinate synthase varies considerably from one cell type to another, the magnitude of argininosuccinate synthase induction following stimulation with LPS or cytokines varies enormously. In cells with high basal expression of argininosuccinate synthase, for example, expression may be induced only two- or threefold, whereas expression may be induced by as much as 50-fold in cells with low basal expression. Responses in vascular smooth muscle cells illustrate the remarkable induction of argininosuccinate synthase expression possible in cells with low basal expression (Fig. 4). One characteristic of iNOS expression is the dramatic synergism in its induction by combinations of specific agents. This feature is shared also by argininosuccinate synthase (e.g., compare the response to LPS + TNFα versus responses to LPS or TNFα alone in Fig. 4). As in cultured cells, argininosuccinate synthase and iNOS are co-induced in heart, lung, kidney, and spleen of LPS-treated rats. The response is unlike that of cultured cells, however, in that argininosuccinate lyase also is induced in these organs. The basis for the induction of argininosuccinate lyase *in vivo* but not in cultured cells is unknown.

The induction of argininosuccinate synthase rather than of argininosuccinate lyase in stimulated cells is consistent with the assumption that the former enzyme catalyzes the ''rate-limiting'' step in the conversion of citrulline to arginine. This assumption is based primarily on measurements of enzyme activities in cell homogenates, but it has not been established that this is indeed the case in intact cells. Without knowing the activity of argininosuccinate lyase and the

Table I Cultured Cells in Which Argininosuccinate Synthase Is Coinduced with iNOS

Cell type	Species	Stimulus[a]
Cell lines		
RAW 264.7 macrophage	Mouse	LPS, IFNγ
RIN insulinoma	Rat	IL-1β, IFNγ + TNFα
C6-BU-1 glioma	Rat	LPS, IFNγ
DLD-1 adenocarcinoma	Human	LPS + IFNγ + TNFα + IL-1β
AKN-1 hepatobiliary	Human	LPS + IFNγ + TNFα + IL-1β
Primary culture		
Arterial smooth muscle	Rat	IL-1β, TNFα, LPS
Aortic smooth muscle	Rat	LPS + IFNγ
Macrophage	Mouse	LPS + IFNγ
Pancreatic islets	Rat	IL-1β
Pancreatic islets	Human	IL-1β + IFNγ + TNFα
Cardiac myocyte	Rat	IL-1β + IFNγ
Endothelial cells	Pig	LPS

[a] Abbreviations used: LPS, lipopolysaccharide; IFNγ, interferon-γ; IL-1β, interleukin-1β; TNFα, tumor necrosis factor α.

availability of aspartate and ATP (Fig. 3) for the cells under investigation, it cannot be assumed that increases in expression of argininosuccinate synthase necessarily represent equivalent increases in cellular rates of citrulline recycling to arginine. Argininosuccinate synthase is subject to inhibition by arginine, thus raising the possibility that the citrulline–NO cycle may function more efficiently at low rather than high arginine concentrations. However, the inhibition constant for arginine is in the millimolar range, so appreciable inhibition will not occur unless intracellular arginine concentrations are in this range.

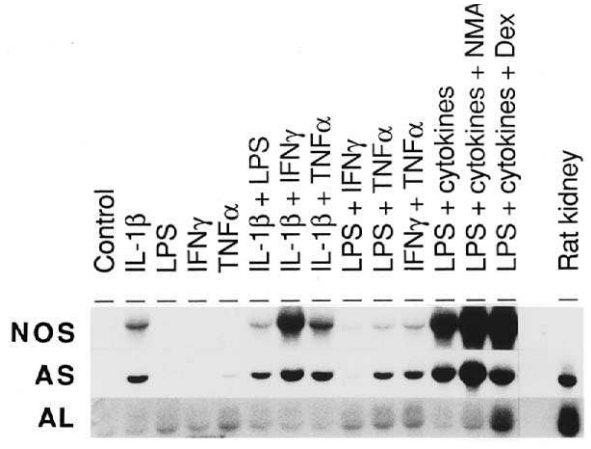


Figure 4 Expression of iNOS, argininosuccinate synthase (AS), and argininosuccinate lyase (AL) in rat pulmonary artery smooth muscle cells. Northern blots of total RNA are shown for cells exposed to the indicated agents for 24 hours. Total RNA from rat kidney was included as a positive control for argininosuccinate synthase and argininosuccinate lyase mRNAs. Key to abbreviations: IL-1β, interleukin-1β; LPS, lipopolysaccharide; IFNγ, interferon-γ; TNFα, tumor necrosis factor α; cytokines = IL-1β + IFNγ + TNFα; NMA, N^G-monomethyl-L-arginine; Dex, dexamethasone. (Results described in Morris *et al.,* 1994).

A special case of arginine metabolism is represented by hepatocytes, which not only express the urea cycle but also can be induced to express iNOS. As argininosuccinate synthase and argininosuccinate lyase normally are highly expressed in liver, their expression does not increase further when iNOS is induced. High rates of arginine synthesis occur within the urea cycle, but the enzymes in this pathway constitute a metabolon whereby the product of each reaction is efficiently channeled to the next enzyme in the pathway. As a consequence of this tight channeling, little or no arginine produced within the urea cycle can be utilized by iNOS. Most of the arginine for hepatic NO synthesis is probably generated by intracellular protein degradation.

Inhibition of arginine synthesis by nonhormonal stimuli has been observed in some NO-producing cells. For example, glutamine inhibits arginine synthesis in endothelial cells, cerebral perivascular neural tissue, and rat peritoneal macrophages but not in a murine macrophage line. This inhibition occurs via competitive inhibition of citrulline uptake and by decreasing activity of argininosuccinate synthase by an unknown mechanism. Hypoxia also decreases activity of argininosuccinate synthase in endothelial cells and inhibits its induction by LPS.

The contribution of endogenous arginine synthesis to NO synthesis has been difficult to evaluate because of the lack of NO-producing cells which do not express these enzymes or of potent, highly specific inhibitors of argininosuccinate

synthase or argininosuccinate lyase. α-Methyl-D,L-aspartate, an inhibitor of argininosuccinate synthase in the test tube, blocks NO synthesis in stimulated cells when citrulline is substituted for arginine. However, it also inhibits NO synthesis in stimulated cells even when ample arginine is provided, thus calling into question the specificity of its inhibitory effects in intact cells. Similar difficulties in interpretation of results arise from use of the general transaminase inhibitor (aminooxy)acetic acid, which inhibits NO synthesis when stimulated cells are cultured in the presence of either arginine or citrulline. The generation of homozygous genetic knock outs of argininosuccinate synthase or argininosuccinate lyase expression has not proved to be useful because these enzymes are essential components of the urea cycle (Fig. 3), and such knockout animals therefore die from hyperammonemia in the perinatal period. An alternative strategy is to enhance cellular arginine biosynthetic capacity and determine its effect on NO production. Using this approach, vascular smooth muscle cells that had been transfected to overexpress argininosuccinate synthase were found to produce more NO following cytokine stimulation than did control cells, thus providing further evidence for the notion that the citrulline–NO cycle can indeed play an important role in regulation of NO synthesis rates.

Arginase

As the interrelationships between arginase expression and NO synthesis are discussed in more detail in the next chapter, by Mori and Gotoh, this topic will be considered only briefly here. First, it is essential to recognize that two isozymes of arginase exist. Type I arginase is cytosolic and is best known as a component of the urea cycle within hepatocytes. Type II arginase, the product of a separate arginase gene, is localized within the mitochondrial matrix and is expressed by a wide variety of cell types. The different subcellular locations of the arginases may represent a mechanism for directing ornithine to different end products, as indicated in Fig. 5.

With regard to NO synthesis, interest in the arginases has grown because of the facts that both NOS and the arginases utilize arginine as substrate and that both enzyme activities are often expressed within the same cells. This initially led to the hypothesis that, owing to competition for a common substrate, simultaneous expression of arginase(s) and NOS—particularly iNOS—may result in reduced production of NO (and possibly of ornithine and urea as well).

Consideration of the properties of these two sets of enzymes lends credence to this notion: For the NOS enzymes, the K_m for arginine is in the 2–20 μ*M* range, but the V_{max} is approximately 1 μmol/min/mg; in the case of the arginases, the K_m is much higher—approximately 2–20 m*M*—but the estimated V_{max} is about 1400 μmol/min/mg. Thus, rates of arginine consumption by equivalent levels of NOS and arginase should be similar at low arginine concentrations but relative consumption of arginine by the arginases would be expected to be predominant at higher arginine concentrations, such as the concentrations found in tissue culture media. Indeed, early studies of arginine metabolism by activated murine macrophages found that the majority of arginine consumed was for the production of urea rather than of NO, and subsequent studies found that inhibition of arginase resulted in increased NO production by activated macrophages. Recent work has shown, however, that interactions between the NOS and arginase enzymes are more complicated than simple competition for substrate. For example, appreciable amounts of N^G-hydroxyarginine, an intermediate in NO synthesis, are released from iNOS in intact cells. As this compound is one of the most potent arginase inhibitors yet discovered, cells with high NO synthesis rates also produce N^G-hydroxyarginine in quantities sufficient to inhibit arginase activity, as indicated in Figs. 1 and 5. Such inhibition may reduce downstream production of key products such as polyamines, proline, or glutamate, an hypothesis which has yet to be tested. It should be borne in mind that these tantalizing findings were obtained in studies of cells cultured in supraphysiological concentrations of arginine. Thus, the nature and consequences of the balance between

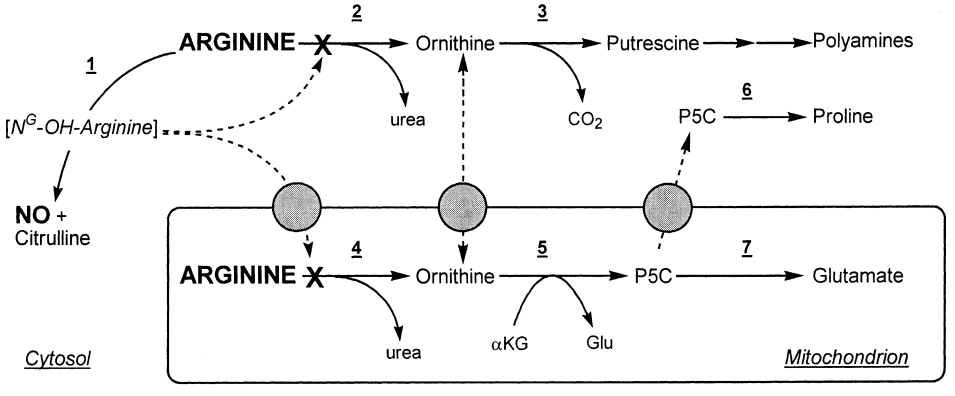


Figure 5 Potential relationships between NOS and arginases in cytosol and mitochondria. Shaded circles represent mitochondrial transmembrane transporters. Key to enzymes: 1, NOS; 2, type I arginase; 3, ornithine decarboxylase; 4; type II arginase; 5, ornithine aminotransferase; 6, P5C reductase; 7, P5C dehydrogenase. Abbreviations: P5C, Δ^1-pyrroline-5-carboxylate; αKG, α-ketoglutarate; Glu, glutamate. (Modified figure reprinted from Morris, 1999, p. 66, by courtesy of Marcel Dekker, Inc.)

NOS and arginase activities in specific cell types *in vivo* remain to be elucidated.

The challenge in comprehending the metabolic consequences of the changing balance in iNOS and arginase expression can be appreciated from the complex stimulus–response profile of NO synthesis and arginase expression in just one cell type—the RAW 264.7 murine macrophage cell line (Table II). Untreated RAW 264.7 cells express a low level of type II arginase but no type I arginase or iNOS. Arginase expression is induced under some but not all conditions in which iNOS also is induced, but the arginase isoforms are not coordinately regulated. As suggested above, these differences in expression of the arginase isozymes are likely significant because of the distinct subcellular localization of the isozymes. For example, the mitochondrial type II arginase may be less susceptible to inhibition in NO-producing cells than is the cytosolic type I arginase, depending on how readily N^G-hydroxyarginine traverses the mitochondrial membrane. In any case, it is clear from Table II that the arginases are more promiscuously induced in this macrophage cell line than is iNOS, raising the intriguing possibility of similarly complex stimulus–response profiles in other NO-expressing cell types. The physiological consequences of these complex patterns of expression are just beginning to be explored. The development of highly potent arginase inhibitors (though none are isoform-specific) and our development of a viable knockout mouse model lacking type II arginase expression should greatly aid in these studies.

Table II Stimulus–Response Profile for RAW 264.7 Macrophage Cells Treated for 18–24 Hours with the Indicated Stimuli[a]

Stimulus	NO	Arginase I	Arginase II
cAMP	—	▲	▲
Dex	—	—	—
cAMP + Dex	—	▽	▲▲
LPS	▲	—	▲
LPS + cAMP	▲	▲▲	▲▲
LPS + Dex	▲	—	▲▲
LPS + IL-4	▲	▲	▲
LPS + IL-10	▲	▲	▽
LPS + TGFβ	▲	▲▲	▽
IFNγ	▲	—	—
IFNγ + cAMP	▽	—	▲
IFNγ + Dex	▲	—	—
IFNγ + IL-4	▽	▲▲	▲
IFNγ + IL-10	▲	—	▲
IFNγ + TGFβ	▲	▲	▲
IL-4	—	▲	—
IL-4 + cAMP	—	▲▲	▲
IL-10	—	—	—
IL-10 + cAMP	—	▲▲	▲
IL-10 + IL-4	—	▲▲	—
TGFβ	—	▲	—
TGFβ + cAMP	—	▲▲	▲▲

[a] Key to abbreviations and symbols: cAMP, 8-bromo-cAMP; Dex, dexamethasone; LPS, lipopolysaccharide; IL-4, interleukin-4; IL-10, interleukin-10; IFNγ, interferon-γ; TGFβ, transforming growth factor-β; —, no effect; ▲, induction (no additive or synergistic response when multiple stimuli are used); ▲▲, synergistic or additive induction; ▽, antagonism.

Arginine Transport

This section will only highlight selected features of arginine transport because this topic is developed more fully in Chapter 14 by Mann and Closs. A large fraction of cellular arginine requirements are met by uptake of extracellular arginine via one or more specific transporters, including systems y^+, $b^{o,+}$, $B^{o,+}$, and y^+L. As noted above, about half of the arginine used for whole-body NO synthesis in healthy adult humans is derived from the plasma. Thus, arginine transport systems represent a major control point for regulating availability of arginine for NO synthesis. This notion is further supported by the fact that the apparent K_m of NO-producing cells for extracellular arginine is about the same as the K_m of the arginine transport systems. A mitochondrial arginine transport system exists, indicated schematically in Fig. 5, but has not been well characterized.

Although the expression of the different transport systems varies among different cell types, the most important system for arginine uptake in many NO-producing cells is system y^+, which transports lysine and ornithine in addition to arginine. The K_m of system y^+ for arginine is near the normal plasma concentration of this amino acid. Thus, this transport system is probably not saturated *in vivo* but may become saturated at the high arginine concentrations commonly found in tissue culture media.

Uptake of arginine can be competitively inhibited by lysine and ornithine, as well as by some positively charged NOS inhibitors such as N^G-monomethyl-L-arginine and N^G-iminoethyl-L-ornithine. As a result, these NOS inhibitors will not only reduce cellular NO synthesis by directly inhibiting NOS but also can reduce arginine availability by limiting its uptake. On the other hand, NOS inhibitors such as aminoguanidine, N^G-nitro-L-arginine, and N^G-nitro-L-arginine methyl ester do not compete with arginine for uptake by system y+.

System y^+ is commonly induced by the same inflammatory stimuli which induce iNOS. In fact, induced NO synthesis in some cell types has a strict requirement for coinduction of system y^+. Thus, induction of both the arginine transport system and argininosuccinate synthase serves to maximize availability of arginine for NO synthesis in activated cells. The need to maximize arginine availability is somewhat surprising because the K_m values of the NOS isozymes for arginine are in the 2–20 μ*M*, suggesting that these enzymes

would almost always be saturated with substrate in intact cells. In fact, rates of cellular NO synthesis can be varied according to extracellular arginine concentration, a phenomenon known as the arginine paradox. This suggests that measurements of intracellular arginine concentrations are highly inaccurate, that the K_m of the NOS isozymes inside cells is quite different than the K_m value determined with cell extracts or purified enzymes, or that multiple intracellular pools of arginine exist. Current evidence suggests that the last possibility may be most likely. For example, eNOS and the arginine transporter appear to be colocalized in plasma membrane caveolae, suggesting that, as arginine is taken up, it does not rapidly mix with the total intracellular arginine pool but is preferentially delivered to eNOS. This is analogous to the proposition that arginine may be preferentially delivered or channeled to iNOS in the citrulline–NO cycle.

Agmatine

Agmatine [4-(aminobutyl)guanidine], the decarboxylation product of arginine (Fig. 1), was discovered to be produced by mammalian cells only as recently as 1994. Although it can bind to α_2-adrenergic and imidazoline receptors, suggesting that it may represent a novel neurotransmitter, it also has potential for regulating NO production. Owing to its guanidino group (Fig. 2), it is a weak competitive inhibitor of NOS (Fig. 1), yet paradoxically it can stimulate cNOS activity in intact cells. The latter effect occurs at physiological levels of agmatine, probably via stimulation of intracellular calcium flux, whereas direct inhibition of NOS occurs at higher concentrations (K_i of 0.2–7.5 m*M,* depending on the NOS isoform). It is unlikely that endogenously produced agmatine acts as a NOS inhibitor *in vivo* because the concentrations required for inhibition are much higher than the low concentrations (approximately 1–10 μM) found in tissue. Moreover, agmatine acts as a feedback inhibitor of arginine decarboxylase, thus limiting its accumulation at the site of synthesis. Its accumulation can be further limited by the action of agmatinase and diamine oxidase (Fig. 1). Net production and accumulation of agmatine by specific cells will, of course, depend on the balance between activities of the synthetic and degradative enzymes, but this information is not yet available for most cell types. Nevertheless, relatively high local concentrations of agmatine can be achieved via its accumulation and release from storage vesicles in neural cells. Even so, it is not clear whether local concentrations become high enough to inhibit NOS activity.

Neither arginine decarboxylase nor agmatinase has been well characterized, though analysis of cell homogenate fractions indicates that both enzymes are localized within mitochondria. Limited information is available concerning the tissue distribution and regulation of arginine decarboxylase and agmatinase. Arginine decarboxylase activity and/or mRNA has been detected in brain, astrocytes, kidney, liver, adrenal gland, and gut. Agmatinase is expressed in brain, liver, and kidney. Both enzymatic activities are present in the RAW 264.7 murine macrophage line in the presence and absence of stimuli which induce iNOS. As no specific inhibitors for arginine decarboxylase or agmatinase have been identified, however, the impact of these activities on NO synthesis by these or any other cells has not been determined.

A partial cDNA sequence for a putative rat arginine decarboxylase was described in 1995, but no subsequent reports of the complete cDNA sequence or functional characterization of the expressed protein have appeared; its subunit size is unknown. Agmatinase is initially synthesized as a protein of 352 residues, and a short N-terminal fragment is probably removed as part of its transport into mitochondria. Inasmuch as both arginase and agmatinase catalyze the hydrolytic cleavage of a guanidino group from their respective substrates, there is a possibility that N^G-hydroxyarginine may inhibit agmatinase as it does the arginases.

Agmatine levels also may be regulated by diamine oxidase, a copper-containing enzyme which is a member of a family of amine oxidases. Diamine oxidase acts on agmatine to produce guanidinobutylaldehyde, hydrogen peroxide, and ammonia (Fig. 1). The structure of guanidinobutylaldehyde suggests that this compound may be an inhibitor of NOS activity. Mammalian diamine oxidase has been cloned, and two isoforms have been identified. It has a relatively broad substrate specificity, with the ability to catabolize various diamines and histamine as well as agmatine. Its contribution to agmatine catabolism is unknown.

Summary

1. Endogenous protein degradation and diet are major contributors to the free arginine pool *in vivo.* The arginine transport systems and the arginases play key roles in regulating the concentration and delivery of this pool of arginine to the NOS enzymes.

2. Argininosuccinate synthase, the arginases, and the arginine transporters are critical regulators of intracellular arginine availability for NO synthesis. However, little quantitative information currently exists regarding their precise contributions to the control of NO synthesis rates in specific cell types. Their expression can vary dramatically from one cell type to another and also can be regulated over a wide dynamic range in many cells by the same stimuli that regulate changes in NO synthesis and iNOS expression. Overall, these properties indicate that these proteins represent potential targets for development of drugs or gene therapy agents to modulate NO synthesis.

3. Endogenously synthesized agmatine is a potential regulator of NO synthesis *in vivo,* but insufficient information is available to evaluate this possibility.

Acknowledgments

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References

Beaumier, L., Castillo, L., Yu, Y.-M., Ajami, A. M., and Young, V. R. (1996). Arginine: New and exciting developments for an ''old'' amino acid. *Biomed. Environ. Sci.* **9,** 296–315.

Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkinson, C. P., Cederbaum, S. D., and Ignarro, L. J. (1996). Arginase activity in endothelial cells: Inhibition by N^G-hydroxyarginine during high-output nitric oxide production. *Am. J. Physiol.* **271,** H1988–H1998.

Castillo, L., Chapman, T. E., Sanchez, M., Yu, Y.-M., Burke, J. F., Ajami, A. M., Vogt, J., and Young, V. R. (1993). Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7749–7753.

Castillo, L., Sanchez, M., Vogt, J., Chapman, T. E., DeRojas-Walker, T. C., Tannenbaum, S. R., Ajami, A. M., and Young, V. R. (1995). Plasma arginine, citrulline, and ornithine kinetics in adults, with observations on nitric oxide synthesis. *Am. J. Physiol.* **268,** E360–E367.

Castillo, L., Beaumier, L., Ajami, A. M., and Young, V. R. (1996). Whole body nitric oxide synthesis in healthy men determined from [^{15}N]arginine-to-[^{15}N]citrulline labeling. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11460–11465.

Chang, C.-I., Liao, J. C., and Kuo, L. (1998). Arginase modulates nitric oxide production in activated macrophages. *Am. J. Physiol.* **43,** H342–H348.

Closs, E. I. (1996). CATs, a family of three distinct mammalian cationic amino acid transporters. *Amino Acids* **11,** 193–208.

Cohen, N. S., and Kuda, A. (1996). Argininosuccinate synthetase and argininosuccinate lyase are localized around mitochondria: An immunochemical study. *J. Cell. Biochem.* **60,** 334–340.

Deves, R., and Boyd, C. A. R. (1998). Transporters for cationic amino acids in animal cells: Discovery, structure, and function. *Physiol. Rev.* **78,** 487–545.

Flodstrom, M., Nieman, A., Bedoya, F. J., Morris, S. M., Jr., and Eizirik, D. L. (1995). Expression of the citrulline-nitric oxide cycle in rodent and human pancreatic β-cells. Induction of argininosuccinate synthetase by cytokines. *Endocrinology* **136,** 3200–3206.

Flodstrom, M., Morris, S. M., Jr., and Eizirik, D. L. (1996). Role of the citrulline-nitric oxide cycle in the functional response of adult human and rat pancreatic islets to cytokines. *Cytokine* **8,** 642–650.

Galea, E., Regunathan, S., Eliopoulos, V., Feinstein, D. L., and Reis, D. J. (1996). Inhibition of mammalian nitric oxide synthases by agmatine, an endogenous polyamine formed by decarboxylation of arginine. *Biochem. J.* **316,** 247–249.

Granger, D. L., Hibbs, J. B., Jr., Perfect, J. R., and Durack, D. T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Invest.* **85,** 264–273.

Grant, S. K., Green, B. G., Stiffey-Wilusz, J., Durette, P. L., Shah, S. K., and Kozarich, J. W. (1998). Structural requirements for human inducible nitric oxide synthase substrates and substrate analogue inhibitors. *Biochemistry* **37,** 4174–4180.

Hattori, Y., Campbell, E. B., and Gross, S. S. (1994). Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. *J. Biol. Chem.* **269,** 9405–9408.

Hattori, Y., Shimoda, S. I., and Gross, S. S. (1995). Effect of lipopolysaccharide treatment *in vivo* on tissue expression of argininosuccinate synthetase and argininosuccinate lyase mRNAs: Relationship to nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **215,** 148–153.

Hey, C., Boucher, J.-L., Vadon-Le Goff, S., Ketterer, G., Wessler, I., and Racke, K. (1997). Inhibition of arginase in rat and rabbit alveolar macrophages by N^{ω}-hydroxy-D,L-indospicine, effects on L-arginine utilization by nitric oxide synthase. *Br. J. Pharmacol.* **121,** 395–400.

Holt, A., and Baker, G. B. (1995). Metabolism of agmatine (clonidine-displacing substance) by diamine oxidase and the possible implications for studies of imidazoline receptors. *Prog. Brain Res.* **106,** 187–197.

Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D. (1996). Comparative properties of arginases. *Comp. Biochem. Physiol.* **114B,** 107–132.

Kilberg, M. S., Stevens, B. R., and Novak, D. A. (1993). Recent advances in mammalian amino acid transport. *Annu. Rev. Nutr.* **13,** 137–165.

Li, G., Regunathant, S., Barrow, J., Eshraghi, J., Cooper, R., and Reis, D. J. (1994). Agmatine: An endogenous clonidine-displacing substance in the brain. *Science* **263,** 966–969.

Lortie, M. J., Novotny, W. F., Peterson, O. W., Vallon, V., Malvey, K., Mendonca, M., Satriano, J., Insel, P., Thomson, S. C., and Blantz, R. C. (1996). Agmatine, a bioactive metabolite of arginine. Production, degradation, and functional effects in the kidney of the rat. *J. Clin. Invest.* **97,** 413–420.

Louis, C. A., Mody, V., Henry, W. L., Jr., Reichner, J. S., and Albina, J. E. (1999). Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. *Am. J. Physiol.* **276,** R237–R242.

McDonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the ''arginine paradox.'' *J. Biol. Chem.* **272,** 31213–31216.

MacLeod, C. L., and Kakuda, D. K. (1996). Regulation of CAT—Cationic amino acid transporter gene expression. *Amino Acids* **11,** 171–191.

Morris, S. M., Jr. (1992). Regulation of enzymes of urea and arginine synthesis. *Annu. Rev. Nutr.* **12,** 81–101.

Morris, S. M., Jr., and Billiar, T. R. (1994). New insights into the regulation of induced nitric oxide synthesis. *Am. J. Physiol.* **266,** E829–E839.

Morris, S. M., Jr., Nakayama, D. K., Nussler, A. K., Liu, Z. Z., Davies, P., Pitt, B. R., Simmons, R. L., and Billiar, T. R. (1994). Coinduction of nitric oxide synthase and argininosuccinate synthetase gene expression: Implications for regulation of nitric oxide synthesis. *In* ''The Biology of Nitric Oxide. Volume 4. Enzymology, Biochemistry, and Immunology'' (S. Moncada, M. Freelisch, R. Busse, and E. A. Higgs, eds.), pp. 301–303. Portland Press, London.

Morris, S. M., Jr., Kepka-Lenhart, D., and Chen, L. C. (1998). Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. *Am. J. Physiol.* **275,** E740–E747.

Morris, S. M., Jr. (1999). Arginine synthesis, metabolism, and transport: Regulators of nitric oxide synthesis. *In* ''Cellular and Molecular Biology of Nitric Oxide'' (J. D. Laskin and D. L. Laskin, eds.), pp. 57–86. Dekker, New York.

Morrissey, J., McCracken, R., Ishidoya, S., and Klahr, S. (1995). Partial cloning and characterization of an arginine decarboxylase in the kidney. *Kidney Int.* **47,** 1458–1461.

Nagasaki, A., Gotoh, T., Takeya, M., Yu, Y., Takiguchi, M., Matsuzaki, H., Takasuki, K., and Mori, M. (1996). Coinduction of nitric oxide synthase, argininosuccinate synthetase, and argininosuccinate lyase in lipopolysaccharide-treated rats. RNA blot, immunoblot, and immunohistochemical analyses. *J. Biol. Chem.* **271,** 2658–2662.

Norris, K. A., Schrimpf, J. E., Flynn, J. L., and Morris, S. M., Jr. (1995). Enhancement of macrophage microbicidal activity: Supplemental arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of *Trypanosoma cruzi*. *Infect. Immun.* **63,** 2793–2796.

Nussler, A. K., Billiar, T. R., Liu, Z. Z., and Morris, S. M., Jr. (1994). Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line. Implications for regulation of nitric oxide production. *J. Biol. Chem.* **269,** 1257–1261.

Nussler, A. K., Liu, Z. Z., Hatakeyama, K., Geller, D. A., Billiar, T. R., and Morris, S. M., Jr. (1996). A cohort of supporting metabolic enzymes is coinduced with nitric oxide synthase in human tumor cell lines. *Cancer Lett.* **103,** 79–84.

Pastor, C. M., Morris, S. M., Jr., and Billiar, T. R. (1995). Sources of arginine for induced nitric oxide synthesis in the isolated perfused liver. *Am. J. Physiol.* **269,** G861–G866.

Regunathan, S., Feinstein, D. L., Raasch, W., and Reis, D. J. (1995). Agmatine (decarboxylated arginine) is synthesized and stored in astrocytes. *Neuroreport* **6,** 1897–1900.

Reis, D. J., and Regunathan, S. (1998). Agmatine: An endogenous ligand at imidazoline receptors may be a novel neurotransmitter in brain. *J. Auton. Nerv. Syst.* **72,** 80–85.

Sastre, M., Regunathan, S., Galea, E., and Reis, D. J. (1996). Agmatinase activity in rat brain: A metabolic pathway for the degradation of agmatine. *J. Neurochem.* **67,** 1761–1765.

Sastre, M., Galea, E., Feinstein, D., Reis, D. J., and Regunathan, S. (1998). Metabolism of agmatine in macrophages: Modulation by lipopolysaccharide and inhibitory cytokines. *Biochem. J.* **330,** 1405–1409.

Schmidlin, A., and Wiesinger, H. (1998). Argininosuccinate synthetase: Localization in astrocytes and role in the production of glial nitric oxide. *Glia* **24,** 428–436.

Schmidlin, A., Kalbacher, H., and Wiesinger, H. (1997). Presence of argininosuccinate synthetase in glial cells as revealed by peptide-specific antisera. *Biol. Chem.* **378,** 47–50.

Schwartz, D., Peterson, O. W., Mendonca, M., Satriano, J., Lortie, M., and Blantz, R. C. (1997). Agmatine affects glomerular filtration via a nitric oxide synthase-dependent mechanism. *Am. J. Physiol.* **272,** F597–F601.

Simmons, W. W., Closs, E. I., Cunningham, J. M., Smith, T. W., and Kelly, R. A. (1996). Cytokines and insulin induce cationic transporter (CAT) expression in cardiac myocytes. *J. Biol. Chem.* **271,** 11694–11702.

Solimeo, H. T., Kepka-Lenhart, D., and Morris, S. M., Jr. (1998). Independent regulation of two arginase genes in RAW 264.7 macrophage cells by TGF-β and Th2 cytokines. *Nitric Oxide Biol. Chem.* **2,** 87.

Sonoki, T., Nagasaki, A., Gotoh, T., Takiguchi, M., Takeya, M., Matsuzakis, H., and Mori, M. (1997). Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues *in vivo* by lipopolysaccharide. *J. Biol. Chem.* **272,** 3689–3693.

Su, Y., and Block, E. R. (1997). Hypoxia inhibits the induction of argininosuccinate synthetase by endotoxin in lung endothelial cells. *Am. J. Physiol.* **272,** L934–L938.

Wang, W. W., Jenkinson, C. P., Griscavage, J. M., Kern, R. M., Arabolos, N. S., Byrns, R. E., Cederbaum, S. D., and Ignarro, L. J. (1995). Coinduction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. *Biochem. Biophys. Res. Commun.* **210,** 1009–1016.

Wu, G., and Brosnan, J. T. (1992). Macrophages can convert citrulline into arginine. *Biochem. J.* **281,** 45–48.

Wu, G., and Meininger, C. J. (1993). Regulation of L-arginine synthesis from L-citrulline by L-glutamine in endothelial cells. *Am. J. Physiol.* **265,** H1965–H1971.

Wu, G., and Morris, S. M., Jr. (1998). Arginine metabolism: Nitric oxide and beyond. *Biochem. J.* **336,** 1–17.

Xie, L., and Gross, S. S. (1997). Argininosuccinate synthetase overexpression in vascular smooth muscle cells potentiates immunostimulant-induced NO production. *J. Biol. Chem.* **272,** 16624– 16630.

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CHAPTER 12

Relationship between Arginase Activity and Nitric Oxide Production

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NITRIC OXIDE (NO) IS SYNTHESIZED FROM ARGININE BY NO SYNTHASE (NOS), AND THE AVAILABILITY OF ARGININE IS ONE OF THE RATE-LIMITING FACTORS IN CELLULAR NO PRODUCTION. ARGINASE CATALYZES THE HYDROLYSIS OF ARGININE TO UREA AND ORNITHINE. THUS, BOTH NOS AND ARGINASE USE ARGININE AS A COMMON SUBSTRATE. ARGINASE EXISTS IN TWO KNOWN ISOFORMS, HEPATIC CYTOSOLIC TYPE (ARGINASE I) AND NONHEPATIC MITOCHONDRIAL TYPE (ARGINASE II). SEVERAL LINES OF EVIDENCE HAVE INDICATED THAT ARGINASE COMPETES WITH NOS FOR THE SUBSTRATE AND DOWNREGULATES NO PRODUCTION. INDUCIBLE NOS (iNOS) AND ARGINASE ACTIVITIES ARE REGULATED RECIPROCALLY IN MACROPHAGES BY CYTOKINES, AND THIS MAY GUARANTEE THE EFFICIENT PRODUCTION OF NO. WHEN RAT PERITONEAL MACROPHAGES ARE STIMULATED WITH BACTERIAL LIPOPOLYSACCHARIDE (LPS), iNOS IS INDUCED FIRST, FOLLOWED BY ARGINASE I, SUGGESTING THAT ARGINASE I PREVENTS SUSTAINED OVERPRODUCTION OF NO. DIFFERENT TIME COURSES OF iNOS AND ARGINASE I INDUCTION ARE ALSO OBSERVED *IN VIVO* IN RAT TISSUES. ON THE OTHER HAND, ONLY ARGINASE II IS INDUCED IN ACTIVATED MOUSE MACROPHAGE-LIKE RAW 264.7 CELLS, AND BOTH ARGINASE FORMS ARE INDUCED IN PRIMARY CULTURES OF MOUSE MACROPHAGES. NO PRODUCTION IN ACTIVATED MACROPHAGES IS ENHANCED BY THE ARGINASE INHIBITORS N^{ω}-HYDROXY-D,L-INDOSPICINE AND L-NORVALINE. IN LPS-ACTIVATED MACROPHAGES AND ENDOTHELIAL CELLS, N^{G}-HYDROXY-L-ARGININE (NOHA), AN INTERMEDIATE OF THE NOS REACTION, ACCUMULATES AND INHIBITS ARGINASE ACTIVITY, WHICH MAY BE A MECHANISM TO ENSURE SUFFICIENT ARGININE AVAILABILITY FOR HIGH-OUTPUT PRODUCTION OF NO. IN IMMUNOSTIMULATED RAW 264.7 CELLS, NO PRODUCTION IS ELEVATED, AND NO-MEDIATED APOPTOSIS FOLLOWS. WHEN ARGINASE II IS INDUCED, NO PRODUCTION IS MUCH DECREASED, AND THE CELLS ARE RESCUED FROM APOPTOSIS. NO-MEDIATED APOPTOSIS CAN ALSO BE PREVENTED BY TRANSFECTION WITH EITHER AN ARGINASE I OR AN ARGINASE II EXPRESSION PLASMID. ALL THESE RESULTS INDICATE THAT NO PRODUCTION IS MODULATED BY ARGINASE ISOFORMS AT THE LEVEL OF BOTH ACTIVITY AND ENZYME AMOUNT IN VARIOUS CELL TYPES.

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Metabolism of Arginine and NO

Arginine is a precursor for synthesis of urea, polyamines, creatine phosphate, agmatine, nitric oxide (NO), and proteins (Fig. 1). Arginine is synthesized from citrulline by the successive actions of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL), the third and fourth enzymes of the urea cycle (ornithine cycle). The major site of arginine synthesis in ureotelic animals is the liver, where arginine generated in the urea cycle is rapidly converted to urea and ornithine by arginase with no net synthesis of arginine occurring. Another major site is the kidney, where arginine is synthesized from citrulline and is released into the blood circulation. However, many other tissues and cell types also contain low levels of AS and AL. In adult animals, citrulline is produced primarily in the small intestine from NH_3, CO_2, and ornithine by carbamylphosphate synthetase I (CPS I) and ornithine transcarbamylase (OTC), the first two enzymes of the urea cycle, and is supplied to the kidney and probably to other tissues for synthesis of arginine. Citrulline is also formed from arginine as a coproduct of the NOS reaction, forming a cycle which is called the citrulline–NO cycle. On the other hand, arginine is hydrolyzed to urea and ornithine by arginase. Thus, arginase and NOS use arginine as a common substrate. Evidence is accumulating to show that arginase competes with NOS for arginine and downregulates NO production by decreasing intracellular arginine. Arginine metabolism was reviewed by Wu and Morris (1998).

Arginase I

Arginase exists in two isoforms, liver-type arginase I and nonhepatic-type arginase II. Arginase I has been purified from a number of sources, including rat (Schimke, 1964; Hirsch-Kolb and Greenberg, 1968; Tarrab *et al.,* 1974), mouse (Spolarics and Bond, 1988), and human (Beruter *et al.,* 1978; Brusdeilins *et al.,* 1985), and the crystal structure of the trimeric rat enzyme was revealed (Kanyo *et al.,* 1996). cDNAs for the rat (Kawamoto *et al.,* 1987) and human enzyme (Haraguchi *et al.,* 1987) were isolated, with the predicted polypeptides being composed of 323 and 322 amino acid residues, respectively. Human arginase I was expressed in *Escherichia coli* and was purified (Ikemoto *et al.,* 1990).

The Arginase I Gene

The human arginase I gene was mapped to chromosone 6q23 (Sparkes *et al.,* 1986). The genes from rat (Ohtake *et al.,* 1988) and human (Takiguchi *et al.,* 1988) are about 12 and 11.5 kb long, respectively, and consist of 8 exons (Fig. 2). The 5' region of the rat gene from −193 to +286 bp exihibited a moderately liver-selective promoter activity in an *in vitro* transcription analysis using nuclear extracts of rat liver and brain (Takiguchi and Mori, 1991). Deletion analysis of the rat arginase I promoter region in hepatoma cell lines showed multiple negative regulatory elements between positions −1500 and −299 bp, and multiple positive elements between −299 and −52 bp (Gotoh *et al.,* 1994). There are two binding sites for CCAAT/enhancer binding protein (C/EBP), one at a position around −90 bp and the other around −55 bp. Binding of C/EBP family members to the region around −55 bp stimulates promoter activity (Chowdhury et al., 1996). Hepatocyte nuclear factor-4 (HNF-4) represses the promoter activity without directly binding the promoter region, and the region overlapping with the C/EBP-binding site at around −55 bp is responsible for the HNF-4 repression (Chowdhury *et al.,* 1996).

Transcription of the rat arginase I gene is induced by glucocorticoids in a delayed secondary manner. A glucocorticoid-responsive and hepatoma cell-selective enhancer is

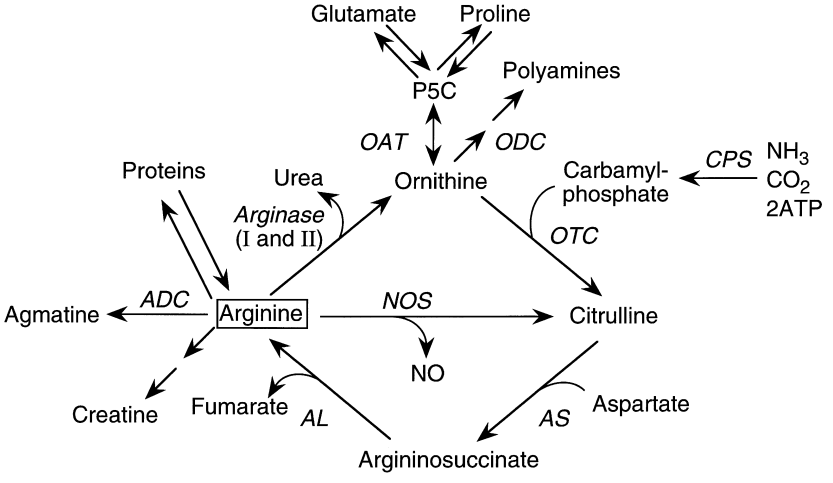


Figure 1 Arginine metabolism. The urea cycle is composed of carbamylphosphate synthetase I (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS), argininosuccinate lyase (AL), and arginase I. The citrulline–NO cycle is composed of nitric oxide synthase (NOS), AS, and AL. OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; P5C, pyrroline-5-carboxylate.

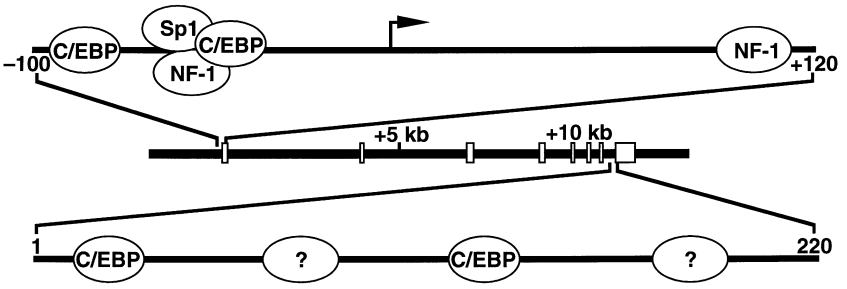


Figure 2 Schematic representation of the rat arginase I gene and binding factors to the promoter and enhancer regions. The transcription start site is indicated by the hooked arrow. The enhancer is located 11 kb downstream from the start site of the gene.

located 11 kb downstream of the promoter and around intron 7 (Gotoh *et al.,* 1994). In this enhancer region, there are four protein binding sites; two of the sites are recognized by C/EBP family members, and the other two bind with unknown factors. Induction of arginase I mRNA by glucocorticoid was preceded by augmentation of the C/EBPβ mRNA (Matsuno *et al.,* 1996; Gotoh *et al.,* 1997a). These results are consistent with the notion that the glucocorticoid response of the arginase I gene is mediated by C/EBPβ. In C/EBPα-deficient mice, mRNA and protein levels for arginase I and other urea cycle enzymes decrease markedly, and hyperammonemia is evident (Kimura *et al.,* 1998). Regulation of the arginase I gene along with other urea cycle enzyme genes was reviewed (Takiguchi and Mori, 1995).

Arginase II

Besides liver-type arginase I, an isozyme (arginase II) is present in some nonhepatic tissues. Distribution of arginase II mRNA along with arginase I mRNA in normal rat tissues is shown in Fig. 3 (Ozaki *et al.,* 1999). Arginase II mRNA is most abundant in the gut and kidney, and it is present at very low levels in some other tissues. In the gut, the mRNA was most abundant in the jejunum, followed by the ileum, duodenum, and colon, but was barely detectable in the stomach. The tissue distribution of arginase II activity (Herzfeld and Raper, 1976) and protein (Ozaki *et al.,* 1999) is similar to that of mRNA. In the small intestine, arginase II is colocalized with ornithine aminotransferase in absorptive epithelial cells (Ozaki *et al.,* 1999). In the kidney, the enzyme is located in the outer stripes of the outer medulla, presumably in the proximal straight tubules (Miyanaka *et al.,* 1998). The localization of arginase II in the kidney differs from those of AS and AL, the last two enzymes of arginine biosynthesis, which are localized in the proximal tubules in the cortex. Arginase II in the small intestine and kidney may supply ornithine for the synthesis of polyamines and proline. This enzyme is also abundant in lactating mammary gland and is thought to be involved in proline synthesis (Yip and Knox, 1972).

cDNAs for human (Gotoh *et al.,* 1996; Vockley *et al.,* 1996; Morris *et al.,* 1997), rat (Iyer *et al.,* 1998), and mouse enzyme (Iyer *et al.,* 1998) have been isolated. The predicted sequence of the human arginase II precursor is shown along with that of human arginase I in Fig. 4. A polypeptide of 354 amino acid residues, including the putative NH_2-terminal presequence for mitochondrial targeting and import, is predicted. Arginase I is located in the cytosol, whereas arginase II is located in the mitochondrial matrix (Spector *et al.,* 1994). It is 59% identical with arginase I. The arginase II precursor synthesized *in vitro* can be imported into isolated mitochondria and proteolytically processed (Gotoh *et al.,* 1996). Comparative properties of arginases have been summarized (Jenkinson *et al.,* 1996).

The human arginase II gene was mapped to chromosome 14q24.1–24.3 (Gotoh *et al.,* 1997b). The mouse gene was isolated (Shi et al., 1998). It contains 8 exons like the arginase I gene, and the identity of the exon–intron boundaries among the two isoform genes is consistent with a gene duplication. The sequence of the promoter region of the mouse gene was presented.

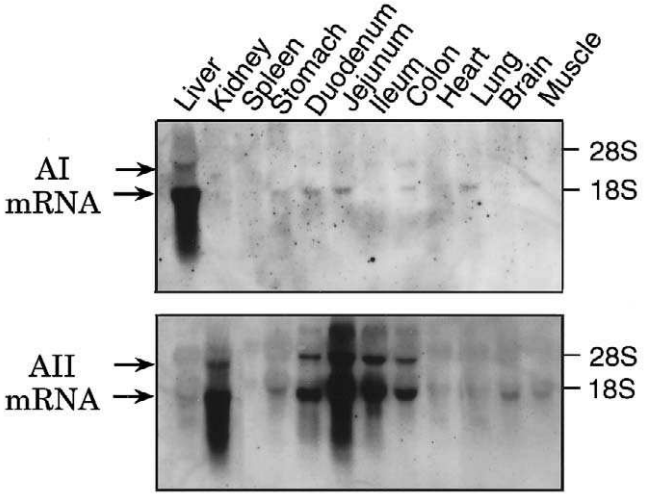


Figure 3 Distribution of arginase I and II mRNAs in normal rat tissues. Total RNAs (10 μg) from rat tissues were electrophoresed in formaldehyde-containing 1% agarose gels and transferred to nylon membranes. The filters were hybridized using digoxigenin-labeled antisense RNAs for arginase (AI) or arginase II (AII) as probes. From Ozaki *et al.* (1999).

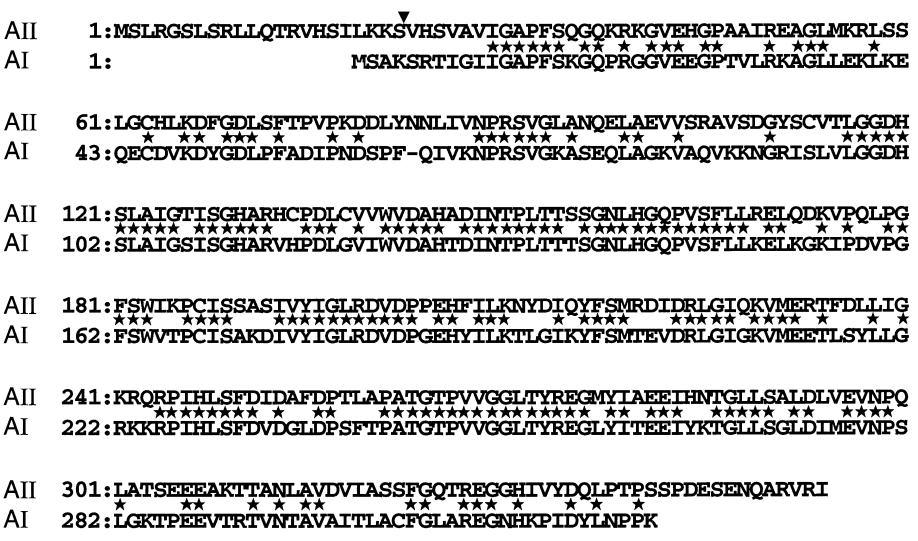


Figure 4 Predicted amino acid sequence of human arginase II precursor (AII) (Gotoh *et al.,* 1996) and comparison with that of human arginase I (AI) (Haraguchi *et al.,* 1987). Identical amino acids are shown by stars, and the triangle shows the putative cleavage site.

Regulation of the Genes for iNOS and Arginase Isoforms in Macrophages

The macrophage is a phagocytic cell of the myeloid lineage found in all organs and connective tissues, and it plays an important part in nonspecific immune reactions. When macrophages are immunostimulated, iNOS is induced and a large amount of NO is produced. Arginine was found to be completely depleted from cell-free supernatants of mixed leukocyte cultures suppressed by the addition of excess mouse peritoneal macrophages (Kung *et al.,* 1977). A marked increase in arginase activity was found in macrophages that had been cultured *in vitro* for 24 hours and in peritoneal cells activated by prior injection of thioglycollate. In contrast, Currie (1978) reported that thioglycollate-induced mouse peritoneal macrophages do not contain detectable arginase activity unless exposed to lipopolysacchanite (LPS) or incubated for 3 days. He showed that activation of macrophages by zymosan or LPS induces the production and release of arginase and kills tumor cells as a consequence of arginine deprivation. However, release of arginase from activated macrophages has not been confirmed.

Albina *et al.* (1988) investigated arginine metabolism in wounds in the rat and found decreased arginine and elevated ornithine contents and high arginase activity. Wound and peritoneal macrophages were shown to convert arginine not only to ornithine (arginase activity) but also to citrulline (arginine deiminase activity was first suggested, but represents NOS activity). Granger *et al.* (1990) analyzed the metabolic fate of arginine in LPS-activated mouse peritoneal macrophages and showed that one-third of arginine is metabolized to NO and citrulline by iNOS and two-thirds is metabolized to urea and ornithine by arginase. Albina *et al.* (1990) examined temporal expression of the two pathways in an experimental wound model and showed that the NO pathway predominates during the early period, whereas the arginase pathway predominates in a later stage. Modolell *et al.* (1995) tested the induction of arginase in mouse bone marrow-derived macrophages and found that NOS and arginase activities are regulated reciprocally by Th1 and Th2 cytokines. Shearer *et al.* (1997) also observed reciprocal changes in the activities of NOS and arginase when adherent mouse peritoneal exudate cells were treated with mediators of leukocyte activation and inhibition, namely, LPS, γ-interferon (IFN-γ), transforming growth factor β (TGF-β), and corticosterone. In rat alveolar macrophages, Hey *et al.* (1995) reported that 3.8 and 4.6% of added [^{3}H]arginine (about 20 n*M*) were utilized by NOS ([^{3}H]citrulline formation) and arginase ([^{3}H]ornithine formation), respectively. When the cells were activated with LPS, the formation of [^{3}H]citrulline was increased about 30-fold, and this was accompanied by a reduction in [^{3}H]ornithine formation of about 50%.

Wang *et al.* (1995) investigated coinduction of iNOS and arginase using LPS-activated mouse macrophage-like RAW 264.7 cells and immunoprecipitation experiments, and they showed that arginase II is induced. They also showed that the inhibitor of NF-κB activation, pyrrolidine dithiocarbamate, inhibits the induction of iNOS but not arginase II, and that IFN-γ causes iNOS induction and abolishes arginase II induction. We isolated arginase II cDNA and showed that arginase II mRNA is coinduced with iNOS mRNA in RAW 264.7 cells by LPS, and that this induction of arginase II mRNA is enhanced by dexamethasone and dibutyryl-cAMP, and is prevented by IFN-γ (Gotoh *et al.,* 1996). Surprisingly, however, we found that arginase I, not arginase II, is coinduced with iNOS in rat peritoneal macrophages (Sonoki *et al.,* 1997) (Fig. 5). Coinduction of arginase I and iNOS by LPS was also observed *in vivo* in rat lung (Fig. 6) and spleen. It is of note that, both *in vitro* and *in vivo,* iNOS is induced

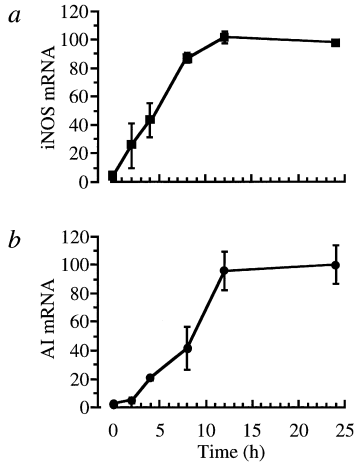


Figure 5 Time course of induction of (a) iNOS and (b) arginase I (AI) mRNAs in LPS-treated rat peritoneal macrophages. The cells were treated with 10 μg/ml of LPS for the indicated times, and total RNAs (0.5 μg) were subjected to blot analysis. The results were quantified and are represented by means ± SD. Maximal values are set at 100. Modified from Sonoki *et al.* (1997).

rapidly after LPS treatment, whereas arginase I is induced much later. Induction of arginase I is mediated at least partly by the preceding induction of C/EBPβ, a transactivator of the arginase I gene (Sonoki *et al.,* 1997; Gotoh *et al.,* 1997a).

Louis *et al.* (1998) performed experiments to identify arginase isoforms expressed in primary and transformed rodent macrophages. They showed that arginase I is induced in primary cultures of rat and mouse peritoneal macrophages by LPS and that arginase II is induced in mouse, but not rat, peritoneal macrophages in RAW 264.7 cells after LPS treatment; neither J774A.1 nor P388D1 cells contain arginase mRNA. They also found that arginase I is increased in rat wound-derived and mouse peritoneal macrophages by hypoxic or anoxic culture, whereas arginase II is suppressed by O_2 deprivation. They further studied the effect of interleukin-4 (IL-4), a Th2 cytokine which had been shown to increase arginase activity in concert with serum; IL-4 increases arginase I, but not arginase II, mRNA in mouse peritoneal macrophages.

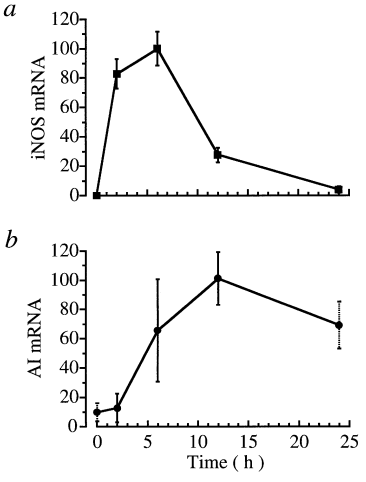


Figure 6 Time course of induction of (a) iNOS and (b) arginase I (AI) mRNAs in the lung after LPS treatment. Total RNA was isolated from the rat lung at the indicated times after LPS injection. Two rats at 0 and 24 hours and three rats at 2, 6, and 12 hours were used. RNAs (1.0 μg) were subjected to blot analysis. The results were quantified and are represented by means ± SD (solid bar; $n = 3$) or means ± range (dotted bar; $n = 2$). Maximal values are set at 100. From Sonoki *et al.* (1997).

Morris *et al.* (1998) analyzed expression of iNOS and arginase isoforms in RAW 264.7 cells in detail and showed that one or both arginase isoforms may be induced and that arginase expression is regulated independently of iNOS expression. For example, 8-bromo-cAMP strongly induced both arginase I and II mRNAs but not iNOS. Whereas IFN-γ induced iNOS but not arginase, 8-bromo-cAMP and IFN-γ mutually antagonized induction of iNOS and arginase I mRNAs. Dexamethasone, which did not induce either arginase or iNOS, almost completely abolished induction of arginase I mRNA by 8-bromo-cAMP but enhanced induction of arginase II mRNA. LPS induced arginase II mRNA, but 8-bromo-cAMP plus LPS resulted in synergistic induction of both arginase I and II mRNAs. All these results show that the genes for iNOS, arginase I, and arginase II are regulated differentially by LPS and cytokines, and that the arginase isoforms may play distinct, although partially overlaping, functional roles in macrophage arginine metabolism.

We have performed a detailed kinetic analysis of the induction of iNOS, arginase isoforms, and related enzymes in mouse tissues and peritoneal macrophages after LPS treatment (Salimuddin *et al.,* 1999). Both arginase I and II as well as iNOS were induced in peritoneal macrophages; arginase II was induced early, whereas arginase I was induced much later. Similar results were obtained in the lung of LPS-treated mice (Fig. 7). NOS activity was rapidly induced after LPS treatment, reached a maximum at 12 hours, and decreased thereafter. Arginase II activity was present prior to LPS treatment, increased early on and up to 24 hours, and then decreased. In contrast, arginase I activity was not detectable for up to 12 hours and increased at 24 to 36 hours. Arginase II activity was still higher than arginase I activity at 36 hours but arginase I activity may dominate after a while. On the basis of these kinetic studies *in vivo,* we speculate the following events in the endotoxemia. iNOS and arginase II are induced early in activated macrophages. NO produced by the iNOS reaction is cytotoxic to the bacteria. On the other hand, arginase I is induced later than arginase II. Overproduction of NO is toxic to macrophages and neighboring cells. Therefore, there must be a mechanism to prevent sustained overproduction of NO. iNOS activity decreases after early induction *in vivo,* suggesting that NO production decreases in the late stage of endotoxemia. Furthermore, arginase I is induced later and may function in downregulating NO production. The ornithine formed by arginase I and II in endotoxemia may be utilized for synthesis of polyamines and proline (and thus collagen), which are required for cell growth and tissue repair.

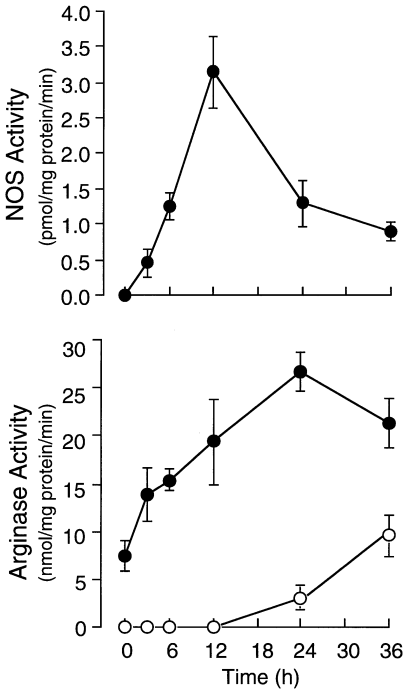


Figure 7 Time course of induction of iNOS, arginase I (O), and arginase II (●) activities in the mouse lung after LPS treatment. Mice were injected intraperitoneally with LPS (10 mg/kg body weight). iNOS activity was assayed by measuring conversion of [^{3}H]arginine to [^{3}H]citrulline. Arginase I and arginase II activities were calculated by measuring with and without immunodepletion with an antiarginase I antibody. From Salimuddin *et al.* (1999).

To be noted here is that the maxmal activity of arginase II (27 nmol/min/mg protein) was about 10,000 times higher than that of iNOS (3.2 pmol/min/mg protein). Assuming that the K_m value of iNOS for arginine is 1–20 μ*M* (Knowles and Moncada, 1994), this enzyme is expected to be operating at a velocity close to V_{max} at intracellular concentrations of arginine (50–200 μ*M*). However, when the arginine concentration decreases to lower levels, it becomes one of the rate-limiting factors of iNOS activity. On the other hand, assuming the K_m value of arginase for arginine to be 10 m*M* (Jenkinson *et al.,* 1996), arginase activity at 50–200 μ*M* arginine is calculated to be 14–54 pmol/min/mg protein on the basis of simple Michaelis–Menten kinetics. Arginase activity is roughly proportional to physiological concentrations of arginine, and when the intracellular concentration of arginine further decreases, arginase and iNOS activities will become of the same order.

Regulation of NO Production by Arginase in Macrophages

N^G-Hydroxy-L-arginine (NOHA), an intermediate in the biosynthesis of NO, was found to be a uniquely potent competitive inhibitor of arginase I (Daghigh *et al.,* 1994; Boucher *et al.,* 1994). The K_i value (40–150 μ*M*) for arginase I is much lower than the K_m value (1–10 m*M*) for its natural substrate arginine. Since substantial amounts of this intermediate appear to be liberated from the active site of NOS (Klatt *et al.,* 1993; Chenais *et al.,* 1993), it is conceivable that NOHA may act as an endogenous arginase inhibiter in NO-producing cells. In fact, Hecker *et al.* (1995) showed that exogenously applied NOHA strongly inhibited the arginase activity in rabbit or rat alveolar macrophages, and that up to 37 μ*M* NOHA is released in the medium of rat alveolar macrophages exposed to LPS for 18 hours. Thus, the inhibition of arginase by NOHA during marked iNOS induction may be a mechanism to ensure sufficient availability of arginine for high-output production of NO in activated macrophages.

However, NOHA cannot be used as a pharmacological tool to elucidate interactions between the arginase and NOS pathways, because NOHA itself is a substrate of NOS. Hey *et al.* (1997) addressed this problem by using N^{ω}-hydroxy-D,L-indospicine, another potent inhibitor of arginase, to show that inhibition of arginase by this agent in rabbit alveolar macrophages can cause a shift of arginine metabolism to the NOS pathway. Futhermore, Chang *et al.* (1998) showed that another arginase inhibitor, L-norvaline, enhances NO production from LPS-activated J774A.1 mouse macrophages.

We addressed the same problem using another approach (Gotoh and Mori, 1999). When RAW 264.7 cells were exposed to LPS and IFN-γ, iNOS was induced and NO production was elevated. When dexamethasone and dibutyryl-cAMP were added, both iNOS and arginase II were induced and NO production was much decreased (Fig. 8). This suggests that the induced arginase II downregulates NO production by depleting intracellular arginine. Downregulation of NO production by arginase was demonstrated using NO-mediated apoptosis. When RAW 264.7 cells were treated with LPS and IFN-γ, the cells underwent NO-dependent apoptosis. This apoptosis was prevented when arginase II was induced by further addition of dexamethasone and dibutyryl-cAMP (Fig. 9). Furthermore, when the cells were transfected with arginase II or arginase I expression plasmid, they were rescued from apoptosis. These results indicate that both cytosolic arginase I and mitochondrial arginase II are effective in downregulating NO production and in preventing NO-mediated apoptosis in activated macrophages.

Regulation of NO Production by Arginase in Other Cells and Tissues

Vascular Endothelial Cells

Enthothelial cells express high iNOS activity and produce a large amount of NO on stimulation with LPS and cytokines. Buga *et al.* (1996) found that rat aortic endothelial cells contain both arginase isoforms. LPS induces both iNOS and arginase II, but not arginase I. Induction of large

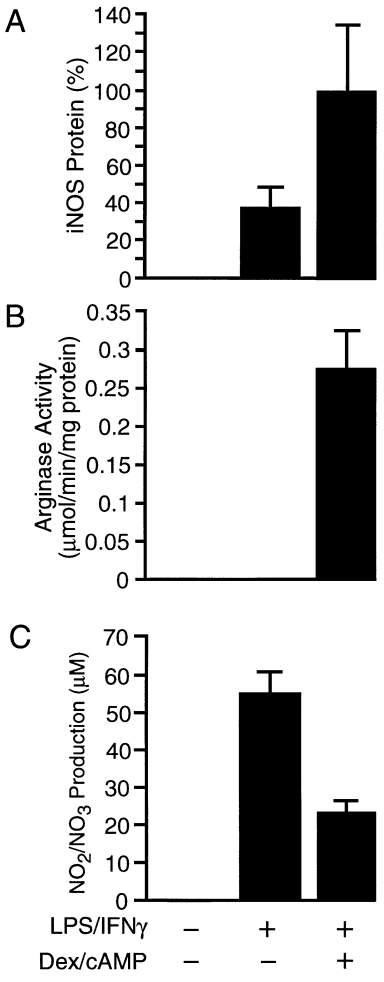


Figure 8 Effect of various reagents on iNOS protein (A), arginase activity (B), and NO_2^-/NO_3^--production (C) in RAW 264.7 cells. The cells were treated with or without 1 μ*M* dexamethasone (Dex) and 1 m*M* dibutyryl-cAMP for 24 hours and then with LPS (150 μg/ml) and IFN-γ (100 U/ml) as indicated at the bottom for 12 hours (A and B) or 18 hours (C). (A) Cell extracts (30 μg protein) were subjected to immunoblot analysis for iNOS protein, and the immunoblots were quantitated and are shown as means ± SE ($n = 3$). The maximal value is set at 100%. (B) The arginase activity of the cell extracts was measured, and the results are shown as means ± SE ($n = 3$). (C) NO_2^- plus NO_3^- in the medium was measured and the results are shown as means ± SE ($n = 3$). From Gotoh and Mori (1999).

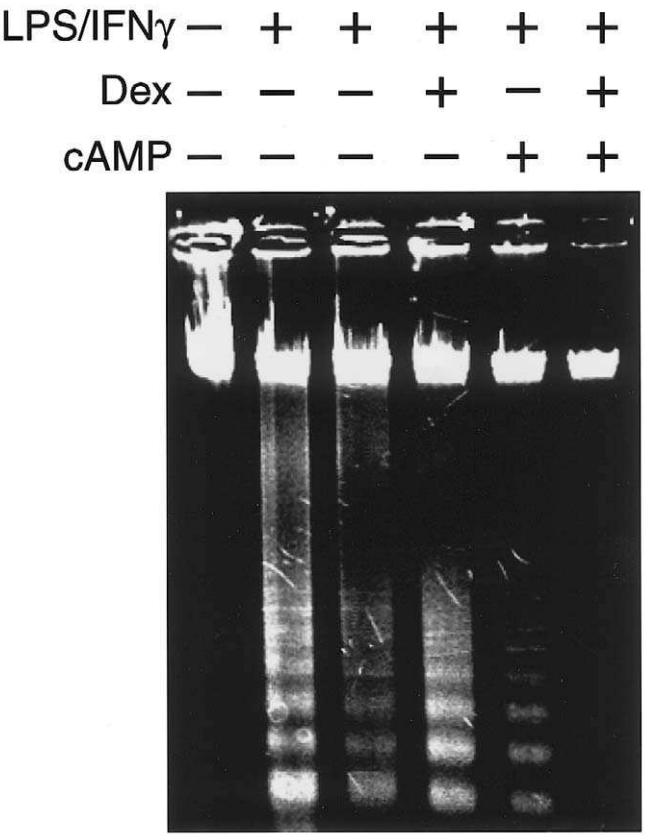


Figure 9 Effect of arginase II induction on apoptosis of RAW 264.7 cells. The cells were treated with 1 μ*M* dexamethasone (Dex) and/or 1 m*M* dibutyryl cAMP for 24 hours, and then treated with LPS (150 μg/ml) and IFN-γ (100 U/ml) for 6 hours. DNAs were isolated, resolved on an agarose gel, stained with SYBR Green I, and visualized for DNA fragmentation by UV transillumination. From Gotoh and Mori (1999).

amounts of iNOS by LPS and cytokines results in large quantities of NO, citrulline, and NOHA, but urea production is markedly diminished. Decreased urea production is attributed to increased formation of NOHA. Thus, arginase inhibition by NOHA may also ensure an arginine supply for NO production in endothelial cells.

Lung

In the lung, NO plays an important role in pulmonary capillary transport function and associates with inflammation. Among the arginase isoforms, arginase II is the major form present in normal rat and mouse lung (Carraway *et al.,* 1998; Salimuddin *et al.,* 1999). After LPS administration, iNOS and arginase I are markedly induced in the rat lung (Sonoki *et al.,* 1997), whereas iNOS and both arginase isoforms are induced in the mouse lung (Salimuddin *et al.,* 1999). On the other hand, Carraway *et al.* (1998) reported that iNOS is induced in the lung in sepsis from cecal ligation and puncture in the rat, whereas arginase II becomes almost undetectable. Que *et al.* (1998) examined expression of arginase isoforms in hyperoxic lung injury. They found that both arginase I and II are upregulated after exposure to 100% O_2 for 60 hours. Immunostaining showed that increases in arginase I and II occur mainly in the peribronchial and perivascular connective tissues. iNOS was not induced under these conditions. Therefore, the role of arginase in hyperoxic lung may be linked to connective tissue elements rather than regulation of NO production. Schapira *et al.* (1998) studied metabolism of arginine in lung macrophages and neutrophils following intratracheal instillation of silica into rat lungs *in vivo.* The results show that lung inflammatory cells increase arginine uptake and metabolism by both NOS and arginase following *in vivo* silica exposure. Therefore, NOS and arginase appear to compete for arginine in these cells.

Kidney

Arginase II is highly expressed in the proximal straight tubules in the outer medulla and in a subpopulation of the proximal tubules in the cortex (Miyanaka *et al.,* 1998), but its role in the regulation of NO production is not known. NO is thought to be important in regulating blood flow in glomeruli and in glomerulonephritis. Both NOS and arginase activities were found to be present in glomeruli, and arginase activity was increased by more than five-fold in nephritic glomeruli (Jansen *et al.,* 1992). By using an acute glomerulonephritis model in rats, Cook *et al.* (1994) found the highest NOS activity in glomeruli on day 1 and the highest arginase activity on day 4; both declined by day 7. Macrophages were shown to be a major source of glomerular NOS, whereas glomerular mesangial cells appeared to be a major source of arginase. Ketteler *et al.* (1996) also reported that, in antithymocyte serum-induced glomerulonephritis in rats, iNOS gene expression and NO production are increased very early, whereas arginase activity was increased later and until 5 days of disease. In glomerular acute immune complex inflammation, arginase I was induced in nephritic glomeruli, and in mesangial cells stimulated with IL-4 and cAMP, whereas arginase II was constitutively expressed (Waddington *et al.,* 1998a). We found that arginase II is elevated in rat kidney in response to LPS, dibutyryl-cAMP, and dexamethasone, but the cell type with the induced enzyme was not identified (Ozaki *et al.,* 1999). To understand regulation of arginase and its relationship to NO production, Waddington *et al.* (1998b) studied effects of NOHA and IL-4 on urea and NO synthesis by glomeruli and mesangial cells during rat immune glomerulonephritis. The results show that arginase is inhibited during high-output NO production and is stimulated with NO suppression. Taking these results together, one can see that the complicated regulation of NOS and arginase isoform genes is important in controlling the balance of inflammatory and repair mechanisms during the course of glomerulonephritis.

Pancreatic β-Cell

A constitutive NOS pathway is thought to negatively control arginine-stimulated insulin release by pancreatic β-cells. Gross *et al.* (1997) investigated the effect of glucose on this mechanism and whether it could be accounted for by NO production. They found that a NOS inhibitor L-NAME strongly potentiates arginine-induced insulin secretion at 5 m*M* glucose. It was concluded that the potentiation of arginine-induced insulin secretion resulting from the blockade of NOS activity is not due to deceased NO production but is probably accounted for by decreased levels of NOHA or citrulline, resulting in the attenuation of an inhibitory effect on arginase activity.

Induction of NOS and generation of NO in β-cells may mediate cytokine-induced dysfunction, leading to insulin-dependent diabetes mellitus. Cunningham *et al.* (1997) investigated the effect of IL-1β, NOHA, and NO donors on arginase activity in the rat insulinoma-derived RINm5F cells. When the cells were treated with IL-1β, NOS activity was increased and arginase activity was decreased. Arginase was significantly inhibited by NOHA and NO donors. On the basis of these results, they concluded that during cytokine-directed β-cell assault, NOS-catalyzed production of NOHA and NO may inhibit arginase, thereby increasing the availability of arginine for NO production.

Central Nervous System

NO is involved in many physiological and pathological processes in the brain. Possible regulation of NO production by arginase in the brain is of great interest and potentially important. However, little is known about arginase in this tissue. Nakamura *et al.* (1990) reported that arginase I-like immunoreactivity is present rather diffusely throughout the rat brain. On the other hand, RNA blot analysis showed that arginase II is expressed at low levels in various portions of human brain, whereas arginase I expression was barely detectable (Gotoh *et al.,* 1997b). Braissant *et al.* (1999) performed an *in situ* hybridization study of mRNAs for the enzymes of arginine metabolism in rat brain; they showed that neuronal NOS (nNOS) mRNA is restricted to a subpopulation of neurons, whereas arginase II mRNA is present ubiquitously in glial cells and neurons, including the nNOS-positive neurons. Therefore, in the neurons expressing both nNOS and arginase II, arginase II may regulate nNOS-dependent NO production. Further studies on the relationship between the NOS and arginase isoforms under physiological and pathological conditions are awaited.

References

Albina, J. E., Mills, C. D., Barbul, A., Thirkill, C. E., Henry, W. L., Jr., Mastrofrancesco, B., and Caldwell, M. D. (1988) Arginine metabolism in wounds. *Am. J. Physiol.* **254,** E459–E467.

Albina, J. E., Mills, C. D., Henry, W. L., Jr., and Caldwell, M. D. (1990). Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.* **144,** 3877–3880.

Beruter, J., Colombo, J. P., and Bachmann, C. (1978). Purification and properties of arginase from human liver and erythrocytes. *Biochem. J.* **175,** 449–454.

Boucher, J. L., Custot, J., Vadon, S., Delaforge, M., Lepoivre, M., Tenu, J. P., Yapo, A., and Mansuy, D. (1994). N^G-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. *Biochem. Biophys. Res. Commun.* **203,** 1614–1621.

Braissant, O., Gotoh, T., Loup, M., Mori, M., and Bachmann, C. (1999). L-Arginine uptake, the citrulline-NO cycle and arginase II in the rat brain: An in situ hybridization study. *Mol. Brain Res.* **70,** 231–241.

Brusdeilins, M., Kuhner, R., and Schumacher, K. (1985). Purification, affinity to anti-human arginase immunoglobulin-Sepharose 4B and subunit molecular weights of mammalian arginases. *Biochim. Biophys. Acta.* **840,** 79–90.

Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkinson, C. P., Cederbaum, S. D., and Ignarro, L. J. (1996). Arginase activity in endothelial cells: Inhibition by N^G-hydroxy-L-arginine during high-output NO production. *Am. J. Physiol.* **271,** H1988–H1998.

Carraway, M. S., Piantadosi, C. A., Jenkinson, C. P., and Huang, Y. C. (1998). Differential expression of arginase and iNOS in the lung in sepsis. *Exp. Lung Res.* **24,** 253–268.

Chang, C. I., Liao, J. C., and Kuo, L. (1998). Arginase modulates nitric oxide production in activated macrophages. *Am. J. Physiol.* **274,** H342–H348.

Chenais, B., Yapo, A., Lepoivre, M., and Tenu, J. P. (1993). N^{ω}-hydroxy-L-arginine, a reactional intermediate in nitric oxide biosynthesis, induces cytostasis in human and murine tumor cells. *Biochem. Biophys. Res. Commun.* **196,** 1558–1565.

Chowdhury, S., Gotoh, T., Mori, M., and Takiguchi, M. (1996). CCAAT/enhancer-binding protein-β (C/EBPβ) binds and activates while hepatocyte nuclear factor-4 (HNF-4) does not bind but represses the liver-type arginase promoter. *Eur. J. Biochem.* **236,** 500–509.

Cook, H. T., Jansen, A., Lewis, S., Largen, P., O'Donnell, M., Reaveley, D., and Cattell, V. (1994). Arginine metabolism in experimental glomerulonephritis: interaction between nitric oxide synthase and arginase. *Am. J. Physiol.* **267,** F646–F653.

Cunningham, J. M., Mabley, J. G., and Green, I. C. (1997). Interleukin 1β-mediated inhibition of arginase in RINm5F cells. *Cytokine* **9,** 570–576.

Currie, G. A. (1978). Activated macrophages kill tumour cells by releasing arginase. *Nature* **273,** 758–759.

Daghigh, F., Fukuto, J. M., and Ash, D. E. (1994). Inhibition of rat liver arginase by an intermediate in NO biosynthesis, N^{G}-hydroxy-L-arginine: Implications for the regulation of nitric oxide biosynthesis by arginase. *Biochem. Biophys. Res. Commun.* **202,** 174–180.

Gotoh, T., and Mori, M. (1999). Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J. Cell. Biol.* **144,** 427–434.

Gotoh, T., Araki, M., and Mori, M. (1997b). Chromosomal localization of the human arginase II gene and tissue distribution of its mRNA. *Biochem. Biophys. Res. Commun.* **233,** 487–491.

Gotoh, T., Chowdhury, S., Takiguchi, M., and Mori, M. (1997a) The glucocorticoid-responsive gene cascade. Activation of the rat arginase gene through induction of C/EBPβ. *J. Biol. Chem.* **272,** 3694–3698.

Gotoh, T., Haraguchi, Y., Takiguchi, M., and Mori, M. (1994). The delayed glucocorticoid-responsive and hepatoma cell-selective enhancer of the rat arginase gene is located around intron 7. *J. Biochem. (Tokyo)* **115,** 778–788.

Gotoh, T., Sonoki, T., Nagasaki, A., Terada, K., Takiguchi, M., and Mori, M. (1996). Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. *FEBS Lett.* **395,** 119–122.

Granger, D. L., Hibbs, J. B., Jr., Perfect, J. R., and Durack, D. T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Invest.* **85,** 264–273.

Gross, R., Roye, M., Manteghetti, M., Broca, C., Hillaire-Buys, D., Masiello, P., and Ribes, G. (1997). Mechanisms involved in the effect of nitric oxide synthase inhibition on L-arginine-induced insulin secretion. *Br. J. Pharmacol.* **120,** 495–501.

Haraguchi, Y., Takiguchi, M., Amaya, Y., Kawamoto, S., Matsuda, I., and Mori, M. (1987). Molecular cloning and nucleotide sequence of cDNA for human liver arginase. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 412–415.

Hecker, M., Nematollahi, H., Hey, C., Busse, R., and Racke, K. (1995). Inhibition of arginase by N^{G}-hydroxy-L-arginine in alveolar macrophages: implications for the utilization of L-arginine for nitric oxide synthesis. *FEBS Lett.* **359,** 251–254.

Herzfeld, A., and Raper, S. M. (1976). The heterogeneity of arginases in rat tissues. *Biochem. J.* **153,** 469–478.

Hey, C., Wessler, I., and Racke, K. (1995). Nitric oxide synthase activity is inducible in rat, but not rabbit alveolar macrophages, with a concomitant reduction in arginase activity. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **351,** 651–659.

Hey, C., Boucher, J. L., Vadon-Le Goff, S., Ketterer, G., Wessler, I., and Racke, K. (1997). Inhibition of arginase in rat and rabbit alveolar macrophages by N^{ω}-hydroxy-D,L-indospicine, effects on L-arginine utilization by nitric oxide synthase. *Br. J. Pharmacol.* **121,** 395–400.

Hirsch-Kolb, H., and Greenberg, D. M. (1968). Molecular characteristics of rat liver arginase. *J. Biol. Chem.* **243,** 6123–6129.

Ikemoto, M., Tabata, M., Miyake, T., Kono, T., Mori, M., Totani, M., and Murachi, T. (1990). Expression of human liver arginase in *Escherichia coli.* Purification and properties of the product. *Biochem. J.* **270,** 697–703.

Iyer, R. K., Bando, J. M., Jenkinson, C. P., Vockley, J. G., Kim, P. S., Kern, R. M., Cederbaum, S. D., and Grody, W. W. (1998). Cloning and characterization of the mouse and rat type II arginase genes. *Mol. Genet. Metab.* **63,** 168–175.

Jansen, A., Lewis, S., Cattell, V., and Cook, H. T. (1992). Arginase is a major pathway of L-arginine metabolism in nephritic glomeruli. *Kidney Int.* **42,** 1107–1112.

Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D. (1996). Comparative properties of arginases. *Comp. Biochem. Phys. B* **114,** 107–132.

Kanyo, Z. F., Scolnick, L. R., Ash, D. E., and Christianson, D. W. (1996). Structure of a unique binuclear manganese cluster in arginase. *Nature* **383,** 554–557.

Kawamoto, S., Amaya, Y., Murakami, K., Tokunaga, F., Iwanaga, S., Kobayashi, K., Saheki, T., Kimura, S., and Mori, M. (1987). Complete nucleotide sequence of cDNA and deduced amino acid sequence of rat liver arginase. *J. Biol. Chem.* **262,** 6280–6283.

Ketteler, M., Ikegaya, N., Brees, D. K., Border, W. A., and Noble, N. A. (1996). L-arginine metabolism in immune-mediated glomerulonephritis in the rat. *Am. J. Kidney Dis.* **28,** 878–887.

Kimura, T., Christoffels, V. M., Chowdhury, S., Iwase, K., Matsuzaki, H., Mori, M., Lamers, W. H., Darlington, G. J., and Takiguchi, M. (1998). Hypoglycemia-associated hyperammonemia caused by impaired expression of ornithine cycle enzyme genes in C/EBPα knockout mice. *J. Biol. Chem.* **273,** 27505–27510.

Klatt, P., Schmidt, K., Uray, G., and Mayer, B. (1993). Multiple catalytic functions of brain nitric oxide synthase. Biochemical characterization, cofactor-requirement, and the role of N^{G}-hydroxy-L-arginine as an intermediate. *J. Biol. Chem.* **268,** 14781–14787.

Knowles, R. G., and Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.* **298,** 249–258.

Kung, J. T., Brooks, S. B., Jakway, J. P., Leonard, L. L., and Talmage, D. W. (1977). Suppression of in vitro cytotoxic response by macrophages due to induced arginase. *J. Exp. Med.* **146,** 665–672.

Louis, C. A., Reichner, J. S., Henry, W. L., Jr., Mastrofrancesco, B., Gotoh, T., Mori, M., and Albina, J. E. (1998). Distinct arginase isoforms expressed in primary and transformed macrophages: Regulation by oxygen tension. *Am. J. Physiol.* **274,** R775–R782.

Matsuno, F., Chowdhury, S., Gotoh, T., Iwase, K., Matsuzaki, H., Takatsuki, K., Mori, M., and Takiguchi, M. (1996). Induction of the C/EBPβ gene by dexamethasone and glucagon in primary-cultured rat hepatocytes. *J. Biochem. (Tokyo)* **119,** 524–532.

Miyanaka, K., Gotoh, T., Nagasaki, A., Takeya, M., Ozaki, M., Iwase, K., Takiguchi, M., Iyama, K. I., Tomita, K., and Mori, M. (1998). Immunohistochemical localization of arginase II and other enzymes of arginine metabolism in rat kidney and liver. *Histochem. J.* **30,** 741–751.

Modolell, M., Corraliza, I. M., Link, F., Soler, G., and Eichmann, K. (1995). Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur. J. Immunol.* **25,** 1101–1104.

Morris, S. M., Jr., Bhamidipati, D., and Kepka-Lenhart, D. (1997). Human type II arginase: Sequence analysis and tissue specific expression. *Gene* **193,** 157–161.

Morris, S. M., Jr., Kepka-Lenhart, D., and Chen, L. C. (1998). Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. *Am. J. Physiol.* **275,** E740–E747.

Nakamura, H., Saheki, T., and Nakagawa, S. (1990). Differential cellular localization of enzymes of L-arginine metabolism in the rat brain. *Brain Res.* **530,** 108–112.

Ohtake, A., Takiguchi, M., Shigeto, Y., Amaya, Y., Kawamoto, S., and Mori, M. (1988). Structural organization of the gene for rat liver-type arginase. *J. Biol. Chem.* **263,** 2245–2249.

Ozaki, M., Gotoh, T., Nagasaki, A., Miyanaka, K., Takeya, M., Fujiyama, S., Tomita, K., and Mori, M. (1999). Expression of arginase II and related enzymes in the rat small intestine and kidney. *J. Biochem. (Tokyo)* **125,** 586–593.

Que, L. G., Kantrow, S. P., Jenkinson, C. P., Piantadosi, C. A., and Huang, Y. C. (1998). Induction of arginase isoforms in the lung during hyperoxia. *Am. J. Physiol.* **275,** L96–L102.

Salimuddin, N. A., Gotoh, T., Isobe, H., and Mori, M. (1999). Differential regulation of the genes for arginase isoforms and related enzymes in mouse tissues and peritoneal macrophages by lipopolysaccharide. *Am. J. Physiol.* **277,** E110–E117.

Schapira, R. M., Wiessner, J. H., Morrisey, J. F., Almagro, U. A., and Nelin, L. D. (1998). L-arginine uptake and metabolism by lung macrophages and neutrophils following intratracheal instillation of silica in vivo. *Am. J. Respir. Cell. Mol. Biol.* **19,** 308–315.

Schimke, R. T. (1964). The importance of both synthesis and degradation in the control of arginase levels in rat liver. *J. Biol. Chem.* **239,** 3808–3817.

Shearer, J. D., Richards, J. R., Mills, C. D., and Caldwell, M. D. (1997). Differential regulation of macrophage arginine metabolism: A proposed role in wound healing. *Am. J. Physiol.* **272,** E181–E190.

Shi, O., Kepka-Lenhart, D., Morris, S. M., Jr., and O'Brien, W. E. (1998). Structure of the murine arginase II gene. *Mamm. Genome* **9,** 822–824.

Sonoki, T., Nagasaki, A., Gotoh, T., Takiguchi, M., Takeya, M., Matsuzaki, H., and Mori, M. (1997). Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues *in vivo* by lipopolysaccharide. *J. Biol. Chem.* **272,** 3689–3693.

Sparkes, R. S., Dizikes, G. J., Klisak, I., Grody, W. W., Mohandas, T., Heinzmann, C., Zollman, S., Lusis, A. J., and Cederbaum, S. D. (1986). The gene for human liver arginase (ARG1) is assigned to chromosome band 6q23. *Am. J. Hum. Genet.* **39,** 186–193.

Spector, E. B., Jenkinson, C. P., Grigor, M. R., Kern, R. M., and Cederbaum, S. D. (1994). Subcellular location and differential antibody specificity of arginase in tissue culture and whole animals. *Int. J. Dev. Neurosci.* **12,** 337–342.

Spolarics, Z., and Bond, J. S. (1988). Multiple molecular forms of mouse liver arginase. *Arch. Biochem. Biophys.* **260,** 469–479.

Takiguchi, M., and Mori, M. (1991). In vitro analysis of the rat liver-type arginase promoter. *J. Biol. Chem.* **266,** 9186–9193.

Takiguchi, M., and Mori, M. (1995). Transcriptional regulation of genes for ornithine cycle enzymes. *Biochem. J.* **312,** 649–659.

Takiguchi, M., Haraguchi, Y., and Mori, M. (1988). Human liver-type arginase gene: Structure of the gene and analysis of the promoter region. *Nucleic Acids Res.* **16,** 8789–8802.

Tarrab, R., Rodriguez, J., Huitron, C., Palacios, R., and Soberon, G. (1974). Molecular forms of rat-liver arginase. Isolation and characterization. *Eur. J. Biochem.* **49,** 457–468.

Vockley, J. G., Jenkinson, C. P., Shukla, H., Kern, R. M., Grody, W. W., and Cederbaum, S. D. (1996). Cloning and characterization of the human type II arginase gene. *Genomics* **38,** 118–123.

Waddington, S. N., Mosley, K., Cook, H. T., Tam, F. W., and Cattell, V. (1998a). Arginase AI is upregulated in acute immune complex-induced inflammation. *Biochem. Biophys. Res. Commun.* **247,** 84–87.

Waddington, S. N., Tam, F. W., Cook, H. T., and Cattell, V. (1998b). Arginase activity is modulated by IL-4 and HOArg in nephritic glomeruli and mesangial cells. *Am. J. Physiol.* **274,** F473–F480.

Wang, W. W., Jenkinson, C. P., Griscavage, J. M., Kern, R. M., Arabolos, N. S., Byrns, R. E., Cederbaum, S. D., and Ignarro, L. J. (1995). Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. *Biochem. Biophys. Res. Commun.* **210,** 1009–1016.

Wu, G., and Morris, S. M., Jr. (1998). Arginine metabolism: Nitric oxide and beyond. *Biochem. J.* **336,** 1–17.

Yip, M. C., and Knox, W. E. (1972). Function of arginase in lactating mammary gland. *Biochem. J.* **127,** 893–899

CHAPTER 13

The Physical Properties of Nitric Oxide

Determinants of the Dynamics of NO in Tissue

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ALTHOUGH MUCH IS KNOWN REGARDING THE BIOLOGICALLY IMPORTANT CHEMICAL REACTIVITY OF NITRIC OXIDE (NITROGEN MONOXIDE, NO) AND ITS DERIVATIVES, MUCH LESS IS KNOWN REGARDING ITS DYNAMICS *IN VIVO.* SINCE NO IS HIGHLY MOBILE, ITS ACTIONS WILL BE WIDESPREAD, AND THIS MOVEMENT WILL BE CRITICALLY IMPORTANT IN DETERMINING ITS EXTENT OF ACTION. THE SPATIAL MOVEMENT OF NO WILL BE DETERMINED BY ITS PHYSICAL PROPERTIES, SPECIFICALLY, ITS VOLATILITY/SOLUBILITY AND ITS DIFFUSIBILITY. THESE PROPERTIES ARE DISCUSSED HERE AND APPLIED TO THE BIOLOGICAL SITUATION WHERE THERE ARE SPECIFIC SPATIAL ARRANGEMENTS OF SOURCES, SINKS, AND TARGETS FOR NO. THE INCREASED SOLUBILITY OF NO IN HYDROPHOBIC PHASES PREDICTS THAT MEMBRANES ARE IMPORTANT SITES FOR NO REACTIVITY. THE RAPID AND WIDESPREAD DIFFUSIBILITY OF NO PREDICTS THAT IT IS NOT A LOCALLY ACTING MEDIATOR, AND ONE NO-PRODUCING CELL AFFECTS MANY HUNDREDS OF ITS NEIGHBORS. THEORETICAL AND EXPERIMENTAL EVIDENCE SUGGESTS THAT ANY GIVEN NO MOLECULE WHICH REACTS INTRACELLULARLY ENTERS FROM OUTSIDE THE CELL, EVEN IF THE CELL ITSELF SYNTHESIZES NO. THE OVERALL USEFULNESS OF THE CONCEPT OF A ''STORAGE FORM'' OF NO TO PREVENT ITS RAPID DISAPPEARANCE IS DISCUSSED AND SHOWN TO BE EFFECTIVE ONLY UNDER CERTAIN RESTRICTIVE CONDITIONS. THE EFFECTS OF REACTIONS OF NO WITH HEMOGLOBIN ARE DISCUSSED, INCLUDING HEMOGLOBIN AS AN NO CARRIER. IN LIGHT OF RECENT FINDINGS THAT SHOW THAT ERYTHROCYTE CONSUMPTION OF NO IN THE BLOOD VESSEL IS SUBSTANTIALLY SLOWER THAN EQUIVALENT AMOUNTS OF FREE HEMOGLOBIN, IT IS SUGGESTED THAT THERE IS NO NECESSITY TO POSTULATE AN NO CARRIER TO PRESERVE ITS ACTION AS THE ENDOTHELIUM-DERIVED RELAXING FACTOR.

Introduction

The properties of substances are their characteristic qualities, and these are of two types[1] (Pauling, 1964). Chemical properties are those properties of a substance that relate to its participation in chemical reactions, where the chemical identity of the substance changes. Physical properties are those properties of a substance that can be observed without changing the substance into other substances. Here I will address the physical properties of nitric oxide (nitrogen monoxide). In addition to paramagnetism [which is not dealt with here but is covered elsewhere (Henry *et al.,* 1993; Wilcox and Smith, 1995; Singel and Lancaster, 1996; Henry and

[1] Ligand–receptor binding can be considered a type of chemical reaction, although no new covalent bonds are formed.

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Singel, 1996; Kalyanaraman, 1996; Henry *et al.,* 1997)], the two most important physical properties of nitric oxide (NO) in biology are its volatility/solubility and its diffusibility. As we will see, an understanding of these properties of NO are as important to understanding its biological actions as an understanding of its chemical properties.

How Reactions Occur

In order for two molecules to react, they must collide. However, of the billions of chemical reactions, only a handful are rapid enough that the reaction will occur after only a few collisions. Thus, virtually all reactions occur after the two molecules collide in ''just the right way.'' In the gaseous phase under high vacuum, the distance between molecules is great enough to ensure that molecules will separate rapidly after a collision. In the condensed phase, however (in aqueous solution or tissue), individual molecules are surrounded by a ''cage'' of solvent (H_2O) molecules. Thus, parts of the ''walls'' of these two cages must move away from the two solutes (reactants) in order for the two cages to coalesce and collisions to occur.

Once this happens, however, the molecular pair is surrounded by the solvent cage, which means that during the lifetime of the cage (which is 10^{-10}–10^{-8} s), the reactant molecules are colliding repeatedly. This is termed an ''encounter'' (Ridd, 1978). If the probability of the reaction is high enough that the reaction will occur most every time there is an encounter, then the rate of the reaction will depend only on how fast the two molecules will form an encounter. This is called the ''diffusion limit.'' No reaction can occur more rapidly than this limit. This limit is in the range of 10^9–10^{10} M^{-1} s^{-1}. In this case, the reaction is essentially ''zero order'' with respect to concentrations of the reactants; all that is needed is that the amounts of reactions approximate their relative reaction stoichiometry in order for complete reaction of the reactants to occur. If the reaction is slower than the diffusion limit, the rate of the reaction will be dependent on concentration because the rate of formation of encounters is determined by the concentrations of the reactants.

The concentration of NO at any spatial location is determined by its physical properties (the phase where the reaction takes place and the position of the location relative to NO sources and sinks). These properties are described below.

Volatility/Solubility of NO

Volatility of NO

If an aqueous solution is equilibrated with a headspace gas composed of pure NO at 1 atm pressure and 25°C the concentration of NO in solution will be approximately 1.9 m*M* (Shaw and Vosper, 1977). It is a simple matter to calculate that under these conditions greater than 20 times more NO molecules will exist per unit volume in the gas than in solution, and so NO is regarded as relatively insoluble in aqueous solution (although its solubility is slightly greater than the solubility of O_2). This means that if a solution containing dissolved NO is exposed to a gas interface (headspace) which is not pure NO, the NO in solution will readily volatilize to exist mostly in the headspace, depending on the volume of the headspace compared to the solution volume. This phenomenon has important consequences (which are not always appreciated) for certain experiments where solutions (e.g., containing cells) are injected with small volumes of NO-saturated solutions. In the absence of a gas–liquid interface (such as in *in vivo* compartments except for the lung), unless the concentration approaches 1–2 m*M* (a highly unlikely condition), NO will exist completely as a dissolved nonelectrolyte. Thus, in virtually all its biological actions NO is not a gas.

Solubility of NO

On a molar basis NO is approximately nine times more soluble in organic solvents than water (Shaw and Vosper, 1977). This means NO will concentrate in hydrophobic phases in tissue (membranes, lipid inclusions, chylomicrons, and lipoprotein complexes). This effect could have several important consequences biologically. First, the hydrophobic nature of NO will mean that the lipid bilayer of membranes will pose no barrier to its movement in tissue (Collander and Barna, 1933; Subczynski *et al.,* 1996). This is one of the most important properties of NO for its biological actions, since this means there is no need for ''packaging'' of the molecule in order for NO to exert its effects on additional cells, other than the cell that makes it. In addition, the concentration of NO in hydrophobic compartments (i.e., on a per volume basis) will be higher than in the aqueous phase, as has been observed experimentally (Malinski *et al.,* 1993). This could mean that hydrophobic compartments may serve as substantial ''reservoirs'' for NO. However, in most parenchymal tissue the total volume of the membrane is much less than the total cellular aqueous volume, so the total amount of NO (i.e., total number of moles in each compartment at any one time) will still be much higher in the aqueous than membrane compartment. In addition, the extremely rapid diffusivity of NO [both in the aqueous phase as described below and in the membrane phase (Vanderkooi *et al.,* 1994; Denicola *et al.,* 1996)] means that both depletion of NO (from reaction) and also production in either compartment will occur essentially simultaneously in both compartments.

The increased concentration of NO (and also O_2) within the hydrophobic compartment could increase reaction rates there, meaning that the membrane could become an important site for the reactions of NO [including reactions with lipids (Rubbo *et al.,* 1994)] and for the generation of its products. Another potentially important effect is that because chemical reactions are greatly influenced by the solvent in which the reaction takes place, these and other possible reactions may occur with mechanisms that are dif-

ferent from those of reaction in water. Unfortunately, little is known about the reactions of NO in hydrophobic phases, although the general phenomenon of acceleration of reactions by the presence of hydrophobic phases is well documented (Cordes and Dunlap, 1969; Fendler and Fendler, 1975); this phenomenon has also been considered on a theoretical basis for both small radical molecules in general (Babbs and Steiner, 1990) and NO (Gordin and Nedospasov, 1998). We have studied the reactions of NO with dioxygen in hydrophobic phases (Liu *et al.,* 1998a), as described below.

The reaction of NO with O_2 in aqueous solution (Ford *et al.,* 1993) results in the formation of nitrous acid:

$$4\,NO + O_2 + 2H_2O \rightarrow 4\,H^+ + 4\,NO_2^- \qquad (1)$$

The rate of this reaction is second order in NO concentration and first order in oxygen concentration:

$$\frac{-d[NO]}{dt} = 4k[NO]_2[O_2] \qquad (2)$$

This is because although the overall stoichiometry involves 4 moles of NO per mole of O_2, the rate-limiting step is the initial reaction of two NO with one O_2 to produce two NO_2:

$$2\,NO + O_2 \rightarrow 2\,NO_2 \qquad (3)$$

Consider the reaction of NO and O_2 taking place in a heterogeneous system (i.e., two different immiscible phases). Assuming the concentration of O_2 is approximately threefold higher in the hydrophobic phase than in the aqueous phase (Subczynski and Hyde, 1983) and that NO is nine times more concentrated in the membrane (Shaw and Vosper, 1977), as depicted in Fig. 1, it can be shown that the rate of autoxidation in the membrane will be nearly 250 times faster than in the aqeuous phase, solely because of the concentration effects of the reactants. It is important to appreciate that this does not mean that the overall rate of NO autoxidation in the heterogeneous solution will be 250 times faster, because this figure refers to the rate occurring within each of the respective phases, that is, on a per volume basis. Much less of the volume of a cell is the membrane compared to the aqueous compartment [approximately 3% in tissue (Anonymous, 1961)].

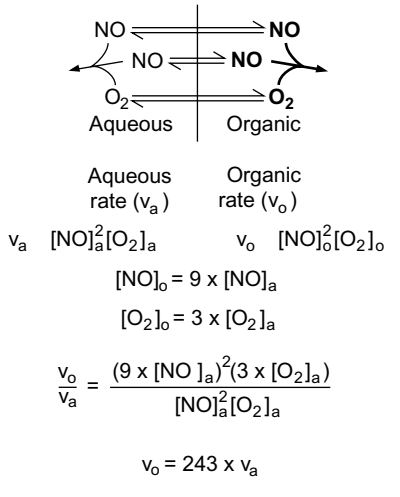


Figure 1 Effects of a hydrophobic phase on NO autoxidation.

Does the greater rate of NO autoxidation in membranes prevail at the whole-cell level? Even though the total volume of the membrane phase in tissue is only 3% of the total volume (the other 97% is aqueous), this factor of 250 would mean that the overall reaction would occur $1 + 250 \times 0.03 = 8.5$ times faster when membranes are present than when they are not. Thus, 7.5/8.5, or 88%, of the total reaction in tissue occurs within membranes (and other hydrophobic phases). To test this we measured NO disappearance using an NO-selective electrode and tested whether addition of phospholipid vesicles accelerates NO disappearance. Figure 2A shows that, indeed, NO disappears much faster when liposomes (40 mg/ml) are present.

According to simple principles of chemical kinetics, the change in NO concentration with time (under conditions where the concentration of O_2 does not change appreciably, which is true here because the concentration of O_2 in

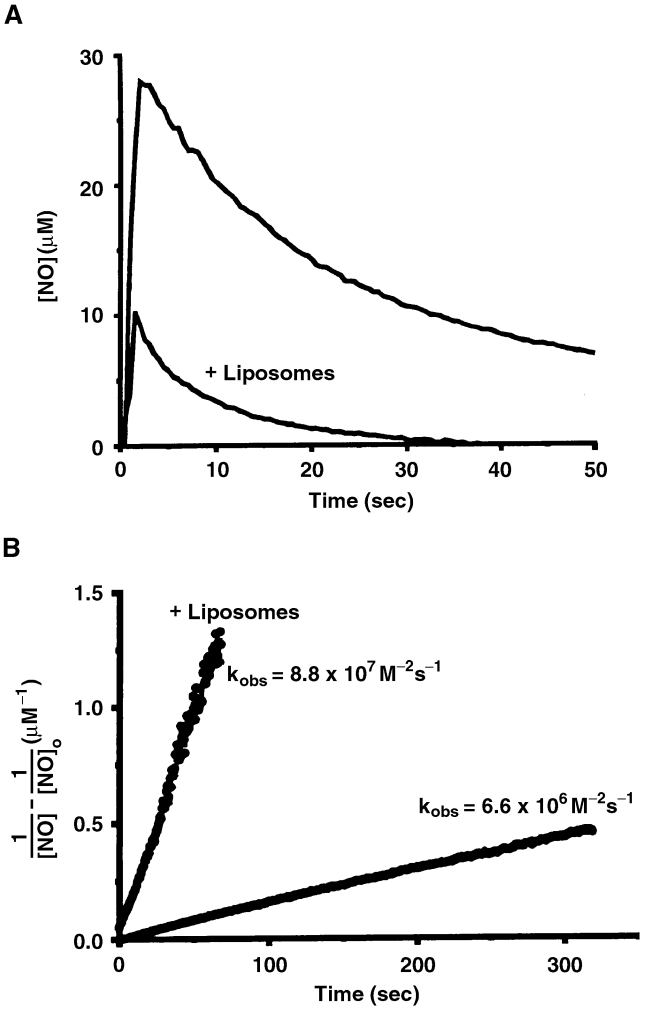


Figure 2 Increased rate of NO autoxidation in the presence of phospholipid vesicles (liposomes).

air-equilibrated buffer is 0.25 m*M,* much greater than that of NO) is given by the following equation:

$$\frac{1}{[NO]} - \frac{1}{[NO]_0} = k[O_2]t \quad (4)$$

where [NO] is the NO concentration at time *t,* $[NO]_0$ is the NO concentration at zero time, and *k* is the rate constant. A plot of $1/[NO] - 1/[NO]_0$ will thus yield a straight line with slope equal to the rate constant *k.* From Fig. 2B two things are clear: (1) the rate of the reaction is 88/6.6 = 13.3 times faster when lipid is present compared to when it is not, and (2) the reaction is still second order with respect to NO concentration (because only a second-order reaction will yield a straight line). The second result strongly suggests that we are still studying NO autoxidation, since this is the only known reaction of NO which is second order in NO concentration (reaction with metals or lipid radicals would be first order).

The real proof that this acceleration of reaction is due to the ''sopping up'' and thus concentrating of NO (and also of O_2) by membranes came from studies with detergents. These amphiphilic molecules exist as isolated monomers at low concentrations, but at a certain concentration (called the critical micelle concentration, or CMC) micelles form (Fig. 3). Above this concentration, the added detergent molecules incorporate into the micelles instead of dispersing as monomers free in solution, and a distinct hydrophobic phase forms, which increases in volume with increasing detergent added.

Figure 4 shows what happens to the rate of NO reaction with increasing concentrations of three detergents with different CMCs (the CMCs are designated by the arrows). The rate of NO reaction does not change with increasing detergent until the CMC is reached, and above that concentration the rate increases linearly with detergent concentration. Perhaps most importantly, the extent to which each of these detergents increases NO reaction (i.e., the slope of the line as detergent concentration is increased above the CMC) is the same for these detergents. This is true for a variety of detergents and also for isolated cell membranes. This demonstrates that this effect is due solely to the presence of the hydrophobic phase, even for biological membranes.

A complete theoretical analysis reveals that on a per volume basis the rate of reaction of NO with O_2 occurs 300 times faster in the hydrophobic versus aqueous phase. In addition, these calculations reveal that, in tissue, greater than 90% of NO autoxidation occurs within membranes (even with only 3% total tissue volume being membrane volume).

It is important to point out that although the autoxidation reaction of NO will be very slow for physiologically relevant concentrations of NO (generally in the nanomolar range under noninflammatory conditions), there is no doubt that nitrosative reactions take place in tissue (e.g., nitrosothiol formation, as described below). This reaction is generally regarded as the primary mechanism whereby nitrosative chemistry of NO occurs (e.g., DNA base modification and mutagenesis, carcinogenesis), and so these results suggest that the membrane is an important location for these reactions. In addition, these results highlight the reactions of NO with lipid radical species produced during oxidative stress conditions (Rubbo *et al.,* 1996) and also highlight the antioxidant role for vitamin E, which is generally thought to exert its actions in the membrane milieu (Hogg *et al.,* 1996).

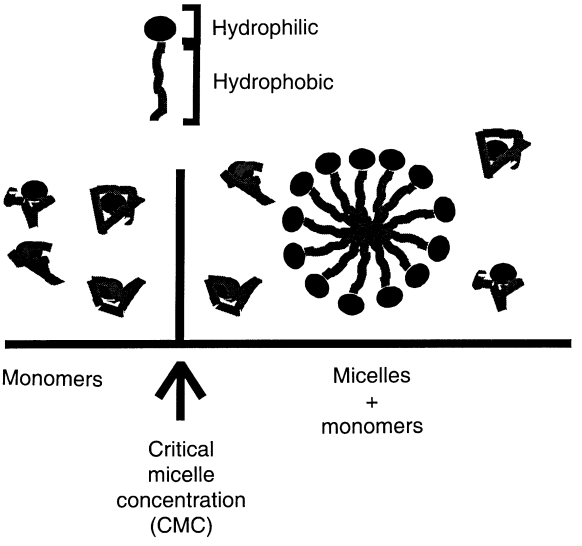


Figure 3 Formation of micelles from amphipathic molecules.

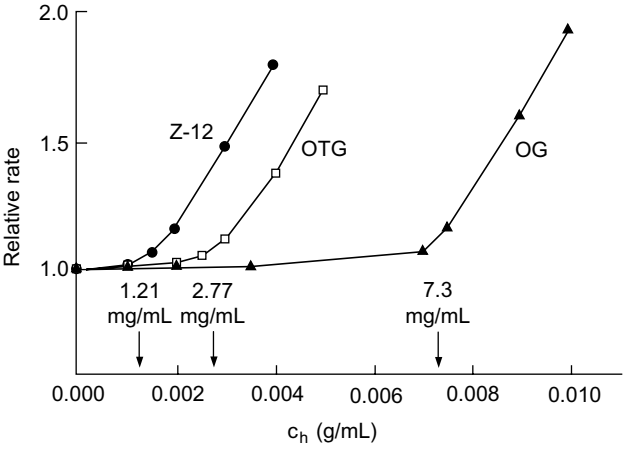


Figure 4 Effects of micelle formation on NO autoxidation.

Diffusibility of NO

The rate at which any reaction occurs at a specific location is determined by the concentrations of the reactants at that location. The concentration of NO is governed by the ratio of the rate at which it appears at that location to the rate at which it disappears. Although much effort has been devoted to delineating the rates of synthesis of NO and its reactions, the equally important[2] process of NO diffusion has

[2] Actually, NO diffusion is more important because NO synthase is not uniformly distributed at every location in tissue, and so diffusion into any specific location is the more common mechanism of NO appearance there.

received much less attention, and the subject is fraught with misconceptions in the literature.

It is common to see figures such as Fig. 5 when referring to the biological actions of NO. A single cell produces NO, which diffuses to act on a separate cell. However, there are two major inaccuracies in this simple figure: first, NO does not move in a straight line, and, second, NO acts on many more than two cells. As described below, we can be confident in making this assertion because the movement of NO, even in complex media such as tissue, is dictated by the laws of physics.

Movement by Random Diffusion

According to the kinetic theory of gases, the average energy of a gas molecule ($\overline{E}$) in a collection of molecules is given by

$$\overline{E} = \frac{1}{2} m\overline{v}^2 \tag{5}$$

where m is mass and $\overline{v}$ is the average velocity. The thermodynamic manifestation of the average kinetic energy of a collection of molecules is heat, which is given by

$$\overline{E} = \frac{3}{2} kT \tag{6}$$

where k is the Boltzmann constant and T is the absolute temperature. Combining Eqs. (5) and (6) yields the following relationship:

$$\overline{v} = \left(\frac{3kT}{m}\right)^{1/2} \tag{7}$$

This relationship means that the only quantities which determine the velocity of a molecule (how fast it moves) are the temperature and its mass. In the gas phase in a high vacuum (large distances between molecules), individual gas molecules move in a straight line. In the condensed phase, however, molecules collide much more frequently because of the much greater denisty. In fact, as described above, solvent (H_2O) molecules form a cage such that movement of solute is dependent on movement (rearrangement) of the solvent molecules in the ''wall'' so that the solute can ''escape.'' The result is that the direction in which an NO molecule moves out of the cage is completely random. NO is just as likely to move some arbitrarily defined ''forward'' direction as it is to move in exactly the opposite ''backward'' direction. These two probabilities, being of equal magnitude and opposite sign, cancel each other out, and so the average probability for movement is thus zero.

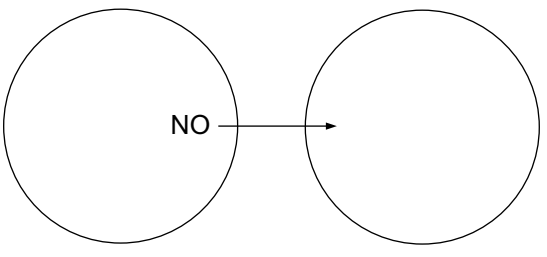


Figure 5 Depiction of NO diffusion between two cells.

We know, however, that molecules in solution do move. This is because, although the overall, net probability for a large number of molecules equally (isotropically) distributed throughout the solution[3] is zero, each individual molecule experiences thermal variations in the movement of individual molecules surrounding it. This means that a molecule will move in a random walk, each ''step'' occurring in a random direction depending on where an ''opening'' appears in the solvent cage surrounding the NO molecule (Lancaster, 1997).

Quantitation of Random Diffusion

The physical quantity which describes the diffusion of a molecule through a solvent is the diffusion constant, D, which is given by Stokes' law[4]:

$$D = \frac{kT}{4\pi\eta r} \tag{8}$$

where η is the viscosity of the medium and r is the radius of the diffusing molecule. With a value of $\eta = 0.695$ cp (water, 37°C) and $r = 1.4 \times 10^{-10}$ m (for NO), we calculate that $D = 3360$ $\mu m^2\ s^{-1}$, which compares remarkably well to the value measured in water [3300 $\mu m^2\ s^{-1}$ (Malinski *et al.,* 1993)] and also in brain [3810 $\mu m^2\ s^{-1}$ (Meulemans, 1994)]. The fidelity of these predictions to the results of experimental measurement in tissue (brain) assures us that the fundamental physical laws of diffusion of NO apply to its movement in tissue (as well it should!).

Although the probability for movement in any direction for random diffusion is zero, it is possible to consider the square of the probability (which will be nonzero). In 1905 Einstein proved that the average of the square of the displacement in the x direction $<(x)^2>$ is defined by the diffusion constant D and the time of movement by the following relationship (called the Einstein–Smolokowski equation):

$$<(x)^2> = 2Dt \tag{9}$$

For NO ($D = 3300$ $\mu m^2\ s^{-1}$), Fig. 6 shows the average distance, $<(x)^2>^{1/2}$, traveled as a function of time. From this it is clear that NO travels a very considerable distance in a short amount of time. In fact, on average it takes less than 2 ms for an NO molecule to escape from a 5-μm-diameter cell, which means (as described in more detail later) if the NO disappears on reaction within the cell with a rate slower than the 2-ms half-life (corresponding to a first-order rate constant of $3.46 \times 10^2\ s^{-1}$), then the NO is more likely to ''escape'' the cell than react. This would mean that the actions of NO would be mainly paracrine in nature (as opposed to autocrine). Also, as shown in Fig. 6, NO will diffuse enor-

[3] This will be important when we discuss the nonisotropic (heterogeneous) distribution of a collection of NO molecules.

[4] For diffusion of a molecule with a size similar to the solvent, which is a good approximation for NO in aqueous solution or in tissue/cells.

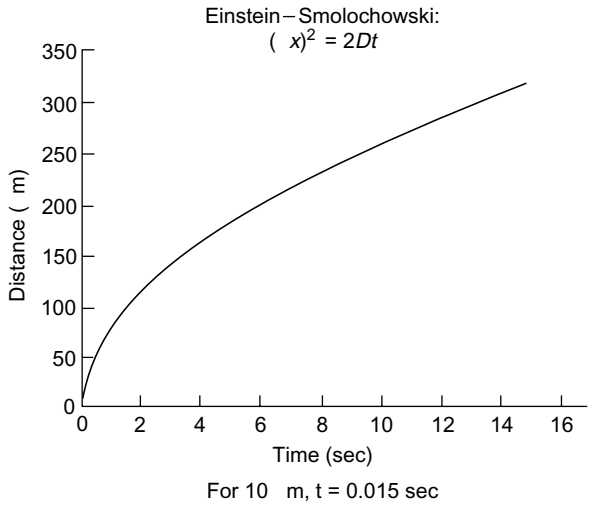


Figure 6 Root-mean-square distance of diffusion for nitric oxide with increasing times.

mous distances (0.2–0.25 mm) in 5–10 s, which is within the range generally considered to be the half-life of NO.

How many cells will be affected by a single NO-producing cell, with random diffusion away from the producer and different half-lives for the NO? Defining a ''sphere of influence'' around a single cell (5 μm in diameter), which corresponds to the distance traveled by NO in one half-life (Fig. 7A), Fig. 7B shows the number of 5-μm-diameter cells within this sphere as a function of half-life. For an NO half-life of 5–10 s, this number is 400,000 to 1,000,000. Even with a half-life of 20–200 ms the number of cells affected by a single NO producer is in the hundreds to thousands (Fig. 7B, inset). It seems inescapable, therefore, that for any reasonable NO half-life, NO is not a locally acting mediator but acts at large distances from a single cellular source and thus affects an enormous number of other cells surrounding this one source.

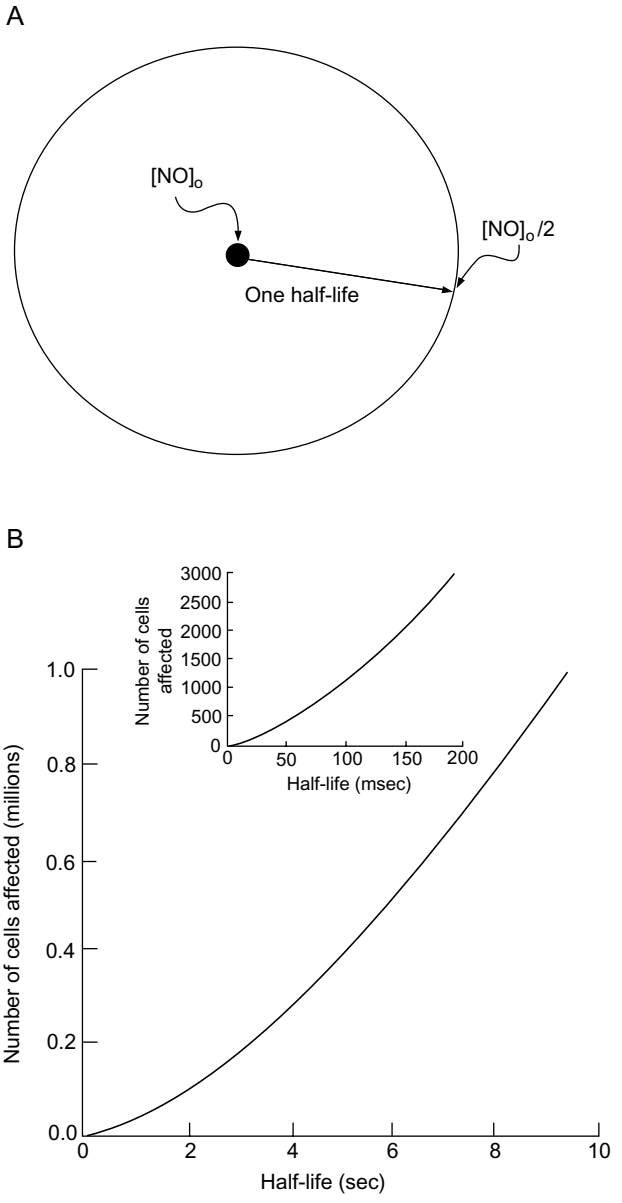


Figure 7 Root-mean-square distance of diffusion for NO with increasing half-life, in terms of numbers of 5-μm-diameter cells within the sphere of influence.

Net Movement of Collections of NO Molecules

Because diffusion is random, we can say that molecules have no ''memory''; at any moment in time, a molecule is just as likely to reverse its direction and go ''backward'' as it is to continue in the direction it has just moved. For example, an NO molecule that has moved halfway from an endothelial cell to a smooth muscle cell is just as likely to turn around and end up back at the endothelial cell as it is to move into the smooth muscle cell. In fact, it makes no difference where this NO molecule was synthesized; the probability of its movement away from that position is identical no matter where it originated. How then can net movement of molecules occur?

Consider two adjacent compartments (A and B), which each contain molecules that diffuse randomly (Fig. 8). The probability for a molecule to cross the boundary from A to B ($P_{A\rightarrow B}$) is determined solely by how many molecules are in compartment A: $P_{A\rightarrow B} = k[NO]_A$. The converse is true for movement from B to A: $P_{B\rightarrow A} = k[NO]_B$. If the concentrations of NO are the same in both A and B, there will be no net movement because the probabilities are the same; although there is movement of NO across this boundary, no net movement occurs (the molecules simply ''change places''). However, if the concentration of NO is less in one compartment than in another, the probability for NO to ''escape'' this compartment is less than the probability for NO to enter from the adjacent compartment, and so net movement will occur. Thus, although movement of individual molecules has no ''direction'' in space, net movement of a collection of molecules does occur (in fact, must occur), and the direction of this movement is always from a region of higher to lower concentration.

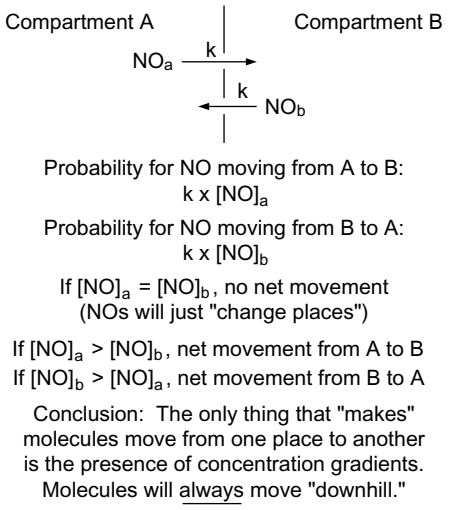


Figure 8 Probabilities of diffusion between two compartments.

DIFFUSION AWAY FROM A POINT SOURCE

There are two phenomena that will result in disappearance of NO in tissue. One is the existence of chemical reactions that consume NO (sinks), and the other can be characterized as ''diffusion into infinity.''

Let us consider the formation of NO from a point source, and we will first assume that NO is not degraded. Figure 9 plots the change in concentration of NO at this point source, as NO production is turned on and then off. There are two time regimes, corresponding to regions where the concentration is changing and where the concentration is unchanging. During the initial phase, the NO concentration increases as NO is synthesized. With time, however, the net rate of appearance of NO slows, even though the rate of NO production by the source may remain the same. This is because the rate of diffusion away from the source, at first slow because the concentration of NO at the source is small, becomes more and more significant because the NO concentration at the source is increasing (probability of moving outward increases). Eventually a steady state is reached where the rate of NO diffusion out of the volume element containing the source becomes equal to the rate of NO production.

If there were spatial boundaries beyond which the NO did not diffuse, the NO would continue to increase with time as long as it is produced (similar to filling a bucket with water). However, with no boundaries (which for all practical purposes applies to NO production *in vivo*) this steady-state constant NO concentration will be maintained as long as the rate of NO production is unchanged. This would be analogous to a fountain in the middle of an infinite parking lot: the height of the fountain is unchanged as long as the water flow is constant, and the water ''diffuses into infinity.''

At steady state, the concentration of NO will be a maximum at the source, and NO will spread out away from the source, approaching zero concentration at infinite distance. For a given production rate, the width of this ''diffusional spread'' (Wood and Garthwaite, 1994) will be determined solely by the magnitude of the diffusion constant (assuming no disappearance of NO by reaction). The greater the value for D, the ''broader'' will be this spread and the lower the concentration will be at the source.

The other factor that will contribute to diffusional spread is the loss of NO due to its disappearance from reaction, which will lower the NO concentration at the location(s) where the reaction takes place. For reaction with O_2, the simplest assumption would be that this loss is uniform throughout the tissue (due to uniform O_2 distribution, although this is not a completely valid assumption), and so it can be included by specifying a rate expression for NO reaction at all locations.

Figure 10 is a graphical depiction of the diffusional spread of NO away from a point source. At zero time, NO synthesis is initiated at the zero spatial coordinate, and the concentration is monitored at locations ± 200 μm from the source. NO concentration rises at all locations until approximately 20 s, when a steady state is reached throughout, with NO concentration declining as distance from the source increases. At $t = 40$ s, NO production at the source is terminated, and NO concentration declines and eventually falls to zero, also at every location.

This particular representation is based on a previously published computer simulation (Lancaster, 1994), which was developed to closely match experimental data[5] for which NO concentration was measured with a microsensor positioned either immediately adjacent to or 100 μm distant from a single endothelial cell (Malinski *et al.,* 1993). Thus, at the NO-producing cell the NO concentration increases and

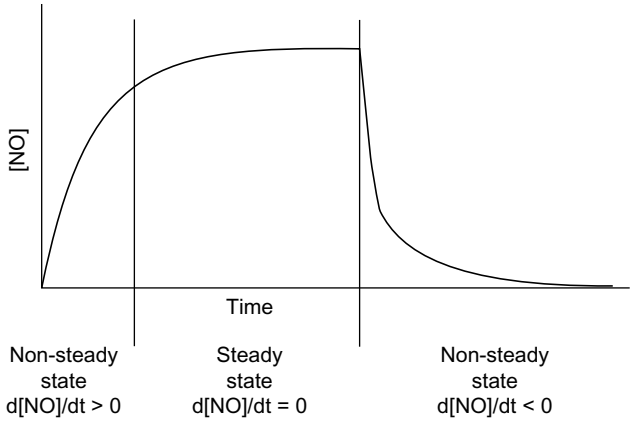


Figure 9 Initiation, steady state, and termination of NO synthesis at a source.

[5] The method (Lancaster, 1994) involves an empirical finite-difference approach wherein the spatial coordinate is subdivided into contiguous compartments, and the rate constants for NO intracompartment formation/reaction and intercompartment movement are defined. The values of these constants are adjusted until the resulting time and distance profile (on solution of the rate equations using numerical methods with selected initial conditions) match the experimental data published by Malinski *et al.* (1993). The results, which were specifically designed to mimic experimentally measured quantities, are comparable to predictions from analytical solution to Fick's equations.

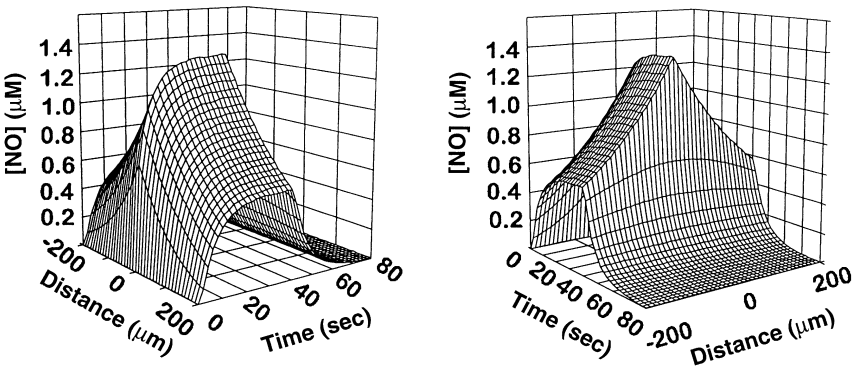


Figure 10 Representation of the diffusional spread of NO from a source, with initiation, steady state, and termination.

reaches a steady-state value of 1.3 μ*M* after 15 to 20 s, with a similar time course 100 μm away and a steady-state concentration of 0.83 μ*M*. The usefulness of this representation is that it allows us to ''fill in'' the values of NO concentration at all distances within our chosen volume element. From Fig. 10, it is clear that the diffusional spread of NO (as predicted by experimentally measured values) is very large, and it is in fact comparable to that predicted using the Einstein–Smolokowski equation.

Overlap of Multiple Sources of NO

If more than one cell is producing NO in a given tissue volume, the concentration of NO at any location will be the sum of the overlaps of the diffusional spread of NO away from each source. Thus, if the distance between NO-producing cells is large compared to the diffusional spread, the NO concentration will be confined to cells surrounding each of the sources, with no overlap. However, as shown in Figs. 7 and 10, the diffusional spread of NO is very broad, and in order for there to be no appreciable overlap between the profiles of multiple NO-producing cells, there should be less than one cell making NO in a total of more than 1 million. This means that there will always be substantial overlap of NO from multiple sources under virtually any realistic situation.

What would be the difference in NO concentration distribution for a collection of cells producing NO when they are ''spread out'' versus clustered together? In Fig. 11, five cells are either equally spread out along a quite large distance (0.4 mm) (Fig. 11B) or all clustered together in the center (Fig. 11C), as denoted by the small dots on the distance axis. The profiles reveal that the steady-state concentration is naturally more spread out when the cells are equally spaced as opposed to clustered together, but the difference in NO concentration at any location for the two configurations is actually not major. This is shown specifically in Fig. 11D, which is a plot of the ratio of the steady-state NO concentration as a function of distance for the two situations, which reveals that the difference is a maximum of less than 40%. Thus, for a tissue segment within which multiple NO sources are located, the concentration of NO at any location is relatively insensitive to exactly where the location is with relation to the sources, and it depends only on how many total NO-producing cells are ''in the general neighborhood.''

Reaction versus ''Escape'' of NO from an NO-Producing Cell

So far we have been discussing the actions of NO at some location as though they were determined solely by its steady-state concentration at that location. Although this is probably true for many (if not most) of the actions of NO in biology, what about the situation where a target for NO (such as guanylate cyclase) is located immediately adjacent to a source? Independent of the steady-state concentration (apparently), NO molecules released from NOS are more likely to collide with a target immediately next to the NOS than a target farther away.

We have examined this question both experimentally (Stadler *et al.,* 1993; Kim *et al.,* 1994) and theoretically (Lancaster, 1996). This question can be diagrammed as shown in Fig. 12. The question we ask is whether the reaction of NO with its intracellular target is rapid enough that it can occur faster than the NO can ''escape'' the cell. Using the Einstein–Smolokowski equation we can calculate that the ''escape time'' for NO from a 5-μm-diameter cell is less than 1 ms. In order to compete with this, the intracellular reaction should occur with a faster rate. Using this as a half-life we calculate that for a second-order reaction (reaction of NO with its target) and an intracellular concentration of the target of 0.1 m*M* or less, the rate constant must be $\geq 7.3 \times 10^6\ M^{-1}\ s^{-1}$. This is a quite rapid reaction.

Using isolated rat hepatocytes, we have studied this question experimentally. We have found that coculture of NO-producing hepatocytes with intact erythrocytes (which rapidly consume NO, as described below) results in virtually complete prevention of two intracellular reactions of NO within the hepatocyte, namely, cGMP formation (Kim *et al.,* 1994) and formation of dithioldinitrosyl complexes, as detected by EPR spectroscopy (Stadler *et al.,* 1993). Kinetic modeling reveals that the only way this result can be obtained is if the diffusion of NO out of the cell is more rapid than its reaction within the cell (Lancaster, 1996). In essence,

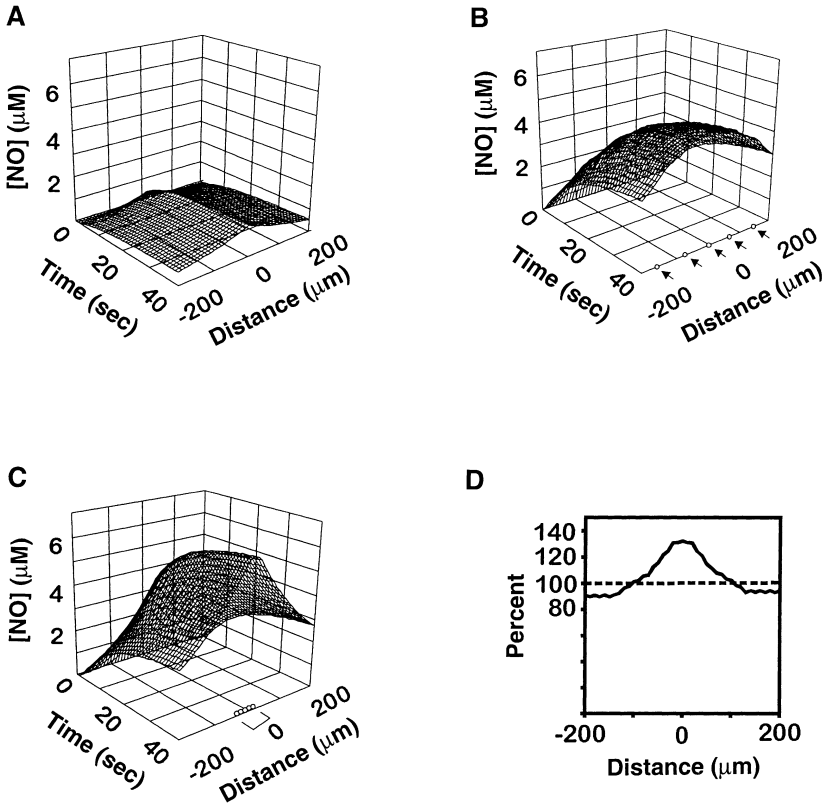


Figure 11 Differences in concentration of NO with five sources, either evenly distributed spatially (B) or clustered together (C).

this means that on average the NO molecule which reacts within a cell (even a cell producing NO) has entered the cell from the outside instead of having just been synthesized there. Thus, although it is most definitely true that a target will respond to NO when it is closer to a source than when it is farther away, this is only because the concentration of NO is higher closer to the source. This means that under these conditions NO is a paracrine effector.

SINKS FOR NO

As described in more detail below, the blood consumes NO rapidly, thus representing a potent sink. For any sink to have an appreciable effect on NO spatial distribution, the rate of reaction at the location of the sink must be comparable to or exceed the rate of exit by diffusion away from that location. This means that not only must the intrinsic rate constant(s) for the reaction be sufficiently rapid, but the reactant(s) which consume the NO must be present at a concentration that can ''keep pace'' with the rate of NO diffusion into the location. These reactants thus must either be present in great excess compared to NO or be constantly generated at a rate that is comparable to the NO diffusion. In the case of the erythrocyte, the reaction rate is very fast, and also the reactant (hemoglobin) is present at high concentrations.

The concept of the blood as a sink for NO (Lancaster, 1994), and thus a problem with respect to the postulate that the endothelium-derived relaxing factor (EDRF) is free NO, was not generally appreciated because it was thought that only ''half'' of the NO produced by the endothelium is scavenged, namely, the ''half'' that diffuses into the vessel lumen. The ''other half,'' the NO which moves into the smooth muscle cell, would not be scavenged. However, this is based on the implicit assumption that NO movement is linear and not random, as illustrated in Fig. 13. If movement were linear (Fig. 13, top) the presence of a sink would indeed consume only half the NO, because the NO that started moving a direction away from the sink would continue on in that direction and never be scavenged. However, with random movement (Fig. 13, bottom), at any instant a molecule is just

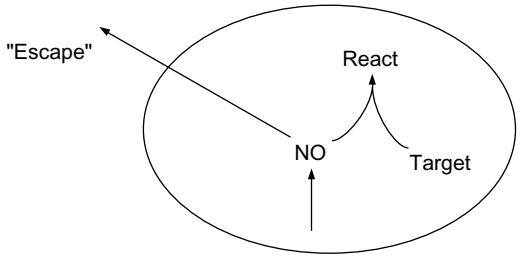


Figure 12 Reaction versus escape of NO from an NO-producing cell.

as likely to completely reverse its direction as it is to continue on in the same direction. In this case, if the sink mechanism is rapid enough, much more than 50% of the NO will be consumed, because any given NO molecule must move into the vessel lumen only once in order to be scavenged. Put another way, only those few molecules whose path never takes them into the vessel lumen escape being scavenged. This will be far less than 50%, owing to the closeness of the endothelium to the vessel lumen. A useful analogy is a large vat of water with a constant source at one end and a leak at the other end. Because the water molecules diffuse rapidly and isotropically, increasing the leak at one end will lower the water level throughout the vat, even immediately adjacent to the water inflow source.

The specific question of hemoglobin scavenging of endothelially produced NO is covered later, but next I discuss the general idea of stabilization of NO by formation of a ''bound'' form.

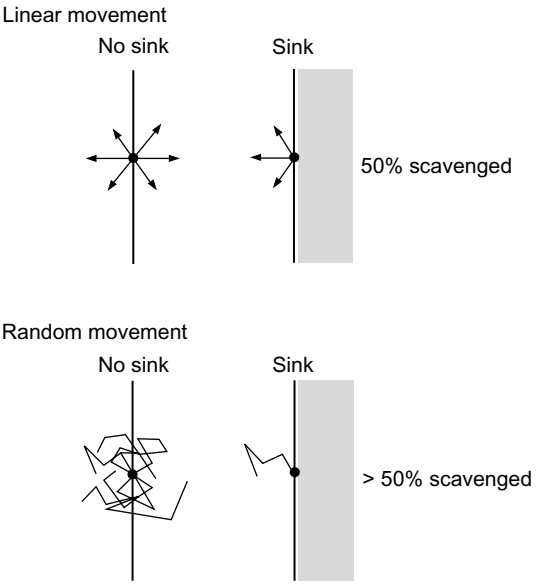


Figure 13 Effects of a sink with linear versus random movement.

"Stabilization" of NO by Binding

Although binding or reaction of NO to form a more stable molecule may seem an attractive mechanism for preventing its scavenging and thus loss of biological actions (Stamler *et al.,* 1992; Vanin, 1991), in fact this process removes NO just as surely as scavenging. In other words, making NO unavailable for reaction with a scavenger (e.g., hemoglobin) also makes it just as unavailable for reaction with its biological target(s) (e.g., guanylate cyclase). It is thus not a straightforward matter to predict the effects of binding or reaction on the biological effects of NO scavenging.

Binding and Free NO Concentration

Synthesis of NO, production of bound form, and disappearance by degradation can be represented as follows:

$$\mathrm{RNO} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{NO} \overset{k_1}{\rightarrow} \qquad \uparrow k_3 \tag{10}$$

for which the following expressions hold:

$$\frac{d[\mathrm{NO}]}{dt} = k_3 + k_{-2}[\mathrm{RNO}] - [\mathrm{NO}](k_1 + k_2) \tag{11}$$

$$\frac{d[\mathrm{RNO}]}{dt} = k_2[\mathrm{NO}] - k_{-2}[\mathrm{RNO}] \tag{12}$$

I will first consider the effects of bound NO formation on the steady-state free NO level. Under this condition the rate equations above equal zero, and solution for steady-state NO concentration ($[\mathrm{NO}]_{ss}$) yields

$$[\mathrm{NO}]_{ss} = k_3/k_1 \tag{13}$$

This relationship also holds when no NO binding takes place ($k_2 = 0$, $k_{-2} = 0$). Thus, the existence of a bound form of NO has no effect on the steady-state NO concentration with ongoing synthesis and disappearance. It can be shown that this will be true with any arrangement of multiple bound forms (not shown).

What are the effects of a bound form on changes in free NO under non-steady-state conditions, that is, when synthesis is initiated and then terminated (Fig. 9)? This can be accomplished by numerical integration of the above rate equations. At zero time, NO formation is initiated, with a rate constant that yields a steady-state concentration of 100 n*M*. After reaching this steady state, NO synthesis is terminated and NO disappearance is calculated.

Initial calculations revealed that there is no effect of binding on free NO appearance and disappearance under two general conditions, namely, (a) if binding (k_2) is slower than NO disappearance (k_1) or (b) if bound form breakdown (k_{-2}) is slower than formation (k_2). In the first case, NO never has a chance to bind, and in the second, binding takes place but the amount of bound form is small.

Figure 14 presents representative results for all other possible cases ($k_2 \geq k_1$ and $k_{-2} \leq k_2$), where $k_2 = 5k_1$ (Fig. 14A) and $k_2 = 20k_1$ (Fig. 14B). First of all, it is important to point out that, in all cases, at long enough times the steady-state concentration of free NO becomes identical to that when no binding occurs, as predicted above (not shown). The general effect of binding NO is to slow not only the rate of free NO appearance on initiation of synthesis, but also the rate of disappearance on cessation of synthesis. Under conditions where the rate of bound NO breakdown is slower than NO disappearance ($k_{-2} < k_1$, the lower two curves in both plots), the effect is clearly biphasic, with a rapid increase or decrease followed by a slower change.

To summarize, the presence of a bound form of NO will have no effect on the steady-state free NO concentration, only on the rates of change when this steady state is perturbed. Thus, bound form production will not protect free NO against scavenging by blood hemoglobin, a long-term effect.

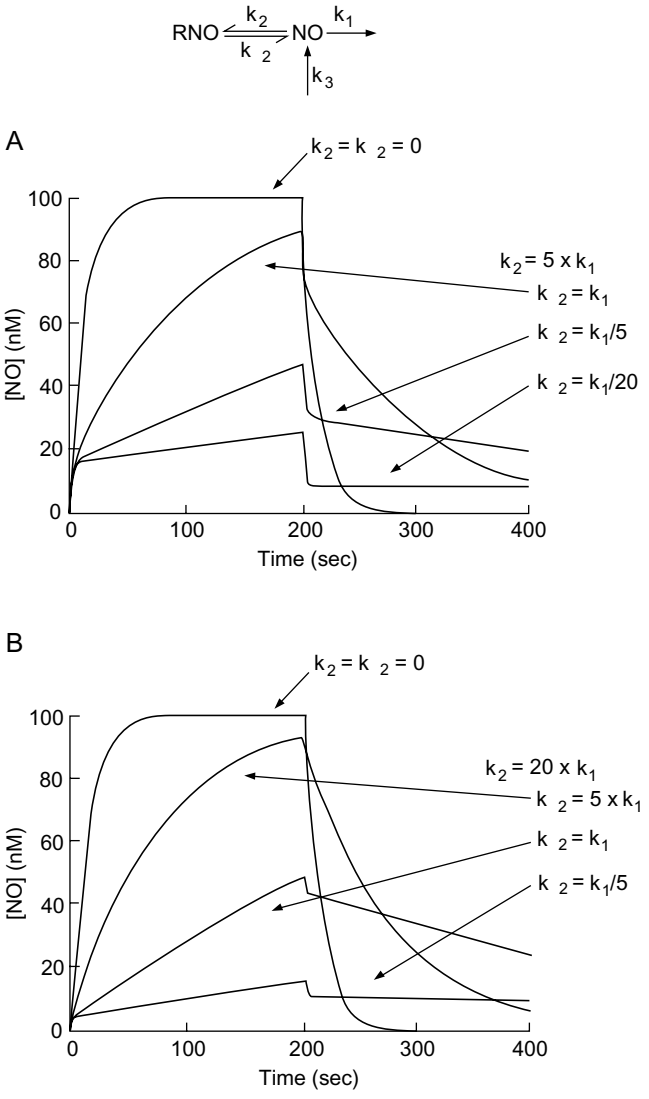
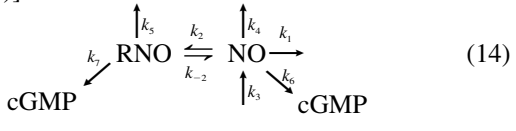


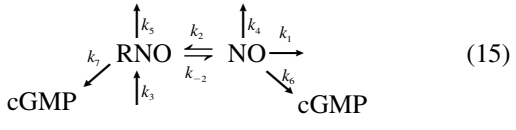
Figure 14 Effects of a ''bound'' or ''storage'' form of NO on free NO concentrations with initiation, steady state, and termination of NO production.

Bound NO: Synthesis by NO Synthase and Stimulation of Guanylate Cyclase

It has been suggested that at least one bound form of NO, nitrosothiol, may stimulate guanylate cyclase directly, thus potentially avoiding the inhibition of cGMP production by NO scavengers (Stamler, 1994). Two schemes describe this and other possibilities, specifically, cGMP synthesis stimulated by both bound and free NO, and NO synthase (NOS) production of either free NO [Eq. (14)] or bound NO [Eq. (15)]:



(14)



(15)

where k_4 is the rate constant for free NO scavenging (hemoglobin) and k_5 is a rate constant for breakdown of RNO not involving NO liberation (the importance of this becomes clear later). Describing cGMP production rate as directly proportional to NO or RNO concentration (either k_6[NO] or k_7[RNO]) and solution of the rate equations at steady state as previously yield the results presented in Table I.

Several important insights emerge from this analysis. First, if free NO is the product of NOS [Eq. (14)] or if free NO is responsible for guanylate cyclase stimulation [even if the product of NOS is RNO; Eq. (15), right-hand side], then the effect of hemoglobin scavenging (k_4) is the same. As shown in Table I, the terms which contain k_4 (the denominator) are identical for all three cases [denominator = $k_5(k_1 + k_2 + k_4) + k_{-2}(k_1 + k_4)$]. Under conditions where hemoglobin scavenging (k_4) is greater than NO autoxidation (k_1) and also the rate of RNO formation (k_2), the denominators in all three cases simplify to $k_4(k_5 + k_{-2})$. Thus, the rates of cGMP formation will be inversely proportional to the magnitude of k_4, and thus increasing k_4 will directly decrease cGMP formation.

Second, when the product of NOS is bound NO (RNO) and RNO directly stimulates guanylate cyclase [Eq. (15), left-hand side], then according to Table I (bottom right equation) there are two mathematically possible conditions where large values for k_4 will have no effect on RNO-driven cGMP formation (k_7[RNO]): if $k_5 > k_{-2}$ or if $k_1 > k_2$. *If* $k_5 > k_{-2}$, then free NO is never formed since RNO disappears via a mechanism not involving NO liberation. If $k_1 > k_2$, then free NO, although formed from RNO breakdown, will disappear before reforming RNO. Thus, although NO is formed, it is only a ''by-product,'' and so increasing its disappearance by scavenging will have no effect. Importantly, under these conditions, free NO will never have a vasodilatory effect, since it will disappear and never form the active species, RNO.

Thus, to summarize, the only two conditions under which bound NO formation will prevent hemoglobin ablation of NOS-induced cGMP formation are if bound (not free) NO is the immediate stimulant for guanylyl cyclase and if either (1) NO is never produced in the path from NOS to guanylate cyclase stimulation or (2) NO is a mere by-product of the path and incapable of vasodilatory actions. Thus, free NO would never be involved.

It is important to point out that for all the situations in Eqs. (14) and (15), reactions are occurring in the same compartment. There is indeed one situation where production of a ''bound'' form of NO will prevent scavenging, and that is when the bound form moves to an area farther away from a sink and then the NO is liberated. This concept is discussed next in the context of the proposal by Stamler that hemoglobin acts as an NO ''carrier.''

Hemoglobin as an NO Sink

Eich *et al.* (1996) have measured the rate of reaction of NO with oxyhemoglobin and oxymyoglobin, and they found that this is an exceptionally rapid reaction, with a rate

Table I Rate Equations for Equations (14) and (15)

NOS makes NO [Eq. (14)]	NOS makes RNO [Eq. (15)]
$k_6[\mathrm{NO}] = \dfrac{k_6k_3(k_5 + k_{-2})}{k_5(k_1 + k_2 + k_4) + k_{-2}(k_1 + k_4)}$	$k_6[\mathrm{NO}] = \dfrac{k_6k_3k_{-2}}{k_5(k_1 + k_2 + k_4) + k_{-2}(k_1 + k_4)}$
$k_7[\mathrm{RNO}] = \dfrac{k_7k_3k_2}{k_5(k_1 + k_2 + k_4) + k_{-2}(k_1 + k_4)}$	$k_7[\mathrm{RNO}] = \dfrac{k_7k_3}{k_5 + k_{-2}(k_1 + k_4)/(k_1 + k_2 + k_4)}$

constant of $3.4 \times 10^7\ M^{-1}\ s^{-1}$. Considering the very high concentration of NO in the blood, this would predict a half-life of NO in the vascular lumen of approximately 2 μs, and would make the blood an unacceptably potent sink for NO produced at its periphery (the endothelium). There are several possible solutions to this problem, beginning with the proposal by Stamler that in fact, rather than being a sink, hemoglobin may be a carrier of NO.

Hemoglobin Nitrosation

Hemoglobin is marvelously designed to transport oxygen to cells in tissue that need it for respiration. Binding of O_2 exhibits cooperativity, which means that binding of one O_2 increases the binding of the next O_2 (hemoglobin can bind a total of four dioxygens). This also means that the loss of an O_2 will increase the loss of the next O_2. What this means physiologically is that hemoglobin will very efficiently ''load up'' with oxygen in the lungs, where O_2 concentration is high, and also will ''dump'' its O_2 in the tissues, where O_2 is less abundant.

In the 1950s and 1960s it became clear that this cooperativity must involve major changes in the fundamental structure of the hemoglobin, when it has oxygen bound compared to when it does not. One of the most important pieces of evidence for this was the finding that a particular cysteine in the β chain changes its reactivity to chemical reagents in the oxy versus deoxy states. In particular, this thiol is more reactive when oxygen is bound to the heme than when it is not (Riggs, 1961; Benesch and Benesch, 1962).

Thiols are targets for modification in cells and tissue in the presence of nitric oxide. Through poorly defined mechanisms, NO can be oxidized and transferred to a nucleophilic acceptor such as a thiol to form nitrosothiols. Jonathan Stamler showed that the reactivity of this thiol in hemoglobin toward nitrosation, like that of other chemical reagents which react with thiols, also is different depending on whether hemoglobin is in the oxy versus deoxy state (Jia *et al.,* 1996; Stamler *et al.,* 1997). Using free hemoglobin in solution, he found evidence for a mechanism involving rapid NO binding to heme followed by nitrosative attack of the Cys-β93, resulting in nitrosothiol, more rapid than the irreversible consumption of NO through oxidation to produce nitrate (Gow *et al.,* 1999). Thus, rather than consuming NO by irreversible reaction with oxyheme [to form nitrate, as measured by Eich *et al.* (1996)], hemoglobin would become a nitrogen oxide carrier. In addition, the allosteric nature of NO and oxygen interactions suggest that such a mechanism could result in hemoglobin delivering both oxygen and a vasodilator (NO) simultaneously to tissue.

There are important mechanistic questions that may seem like chemical ''details'' but which must be answered, including the identities of the ''nitrogen oxides'' involved [since chemically speaking the ''nitrogen oxide'' in the hemoglobin nitrosothiol is not nitric oxide (NO) but its oxidation product nitrosonium (NO^+)]. In addition, it is unclear how the nitrosonium moiety [which does not exist as a distinct entity except under extremely acidic conditions (Williams, 1988)] transfers from the proximal heme position to the thiol, which is located on the back side of the heme group in the hemoglobin molecule (Stamler *et al.,* 1997). Finally, there is very little information on the mechanisms of transfer of nitrogen oxide from the nitrosohemoglobin through the entire path to the smooth muscle cell. Specifically, somehow the nitrosonium on hemoglobin must travel from within the red blood cell, across the red blood cell membrane, through the plasma, across the endothelial cell, enter the smooth muscle cell, and liberate nitric oxide, all within the ''dwell time'' for hemoglobin (a matter of seconds).

In addition, there is much data indicating that the blood is in fact a sink, rather than a reservoir, for NO. For example, acute hemorrhage increases exhaled NO (Carlin *et al.,* 1997), hemodilution causes NO-mediated vasodilation (Mellander *et al.,* 1997), vasodilation in anemic patients is due to increased NO and resistance to normal is restored on infusion of hemoglobin (Anand *et al.,* 1995), and increased hematocrit augments pulmonary hypoxic vasoconstriction and decreases expired NO in isolated lung (Deem *et al.,* 1998).

There are also studies with isolated hemoglobin that are not consistent with the overall concept. The transnitrosation kinetics of *S*-nitrosohemoglobin (i.e., transfer of the nitrosonium group between *S*-nitrosohemoglobin and glutathione) (Patel *et al.,* 1999) and the vasorelaxant properties of *S*-nitrosohemoglobin (when the effects of changes in oxygen tension alone on vascular tone are considered) (Wolzt *et al.,* 1999) are not particularly sensitive to the oxygenation state of hemoglobin. Also, transfer of the nitrosonium group to glutathione [which is the likely mechanism whereby the nitrosonium group would begin its movement from hemoglobin to the smooth muscle cell (Stamler *et al.,* 1997)] does not appear to be fast enough to accomplish nitrogen oxide trans-

fer to the smooth muscle cell during the transit time of an erythrocyte through the precapillary vasculature (Patel *et al.,* 1999; Wolzt *et al.,* 1999), and if such transfer occurs, it will most likely occur in regions of very low oxygen tension (Patel *et al.,* 1999). In addition, under conditions that stimulate those inside the erythrocyte (relatively high concentrations of reduced glutathione), the predominant pathway for *S*-nitrosohemoglobin breakdown appears to be nucleophilic attack by thiol, generating mixed disulfides and liberating nitric oxide and nitrous oxide (N_2O), with only minor amounts of *S*-nitrosohemoglutathione being formed (Wolzt *et al.,* 1999).

Perhaps most importantly, reactions with hemoglobin in the test tube may not be physiologically relevant, in light of the finding that the reactions of NO with hemoglobin occur nearly 1000 times slower when the hemoglobin is packaged inside the red blood cell than when it is evenly distributed in solution (Liu *et al.,* 1998b). This raises the possibility that reactions with hemoglobin inside the red blood cell may be fundamentally different than with hemoglobin free in solution. An additional important finding is that the presence of an erythrocyte-free zone in blood may also decrease the consumption of endothelial NO by intravascular erythrocytes (Vaughn *et al.,* 1998; Butler *et al.,* 1998; Liao *et al.,* 1999). These effects may well mean that scavenging of NO by oxyhemoglobin in the blood is not a major problem *in vivo.* In fact, this may be one reason Nature places hemoglobin inside a circulating cell rather than free in the blood vessel: to slow its reaction with NO. This possibility is dealt with in more detail in the next section.

Finally, the theory needs to be tested under physiologically relevant conditions, meaning, experiments with intact red blood cells or whole blood and measurements of the rate of formation and movement of nitrogen oxide from intracellular hemoglobin all the way to its target, guanylate cyclase in the smooth muscle cell.

Modeling Erythrocyte Consumption of NO

Exactly how potent is the consumption of NO by erythrocytes, and how might it affect the biological actions of free NO as an EDRF? In order to address this question, the model in Fig. 15 was utilized. This simplified kinetic scheme depicts three compartments containing NO, the vessel lumen (NO_L), the endothelial cell layer (NO_E), and the abluminal space (NO_A). NO is synthesized by the endothelium with rate constant k_3, diffuses between compartments via rate constants k_8, k_{-8} and k_9, k_{-9}, disappears with rate constant k_1 in all compartments (corresponding to the half-life of NO in parenchymal tissue), and disappears within the vessel lumen by reaction with intraerythrocytic hemoglobin with constant k_R. In Fig. 15, r_L is the vessel radius, d_E is the thickness of the endothelial layer, and d_A is the abluminal distance away from the endothelium containing the smooth muscle cell layer. Making the assumption that diffusion is faster than autoxidation (k_8, k_9, k_{-8}, $k_{-9} > k_1$), solution of the steady-state rate equations yields the following relation for the concentration of NO at the smooth muscle cell (i.e., in the abluminal compartment):

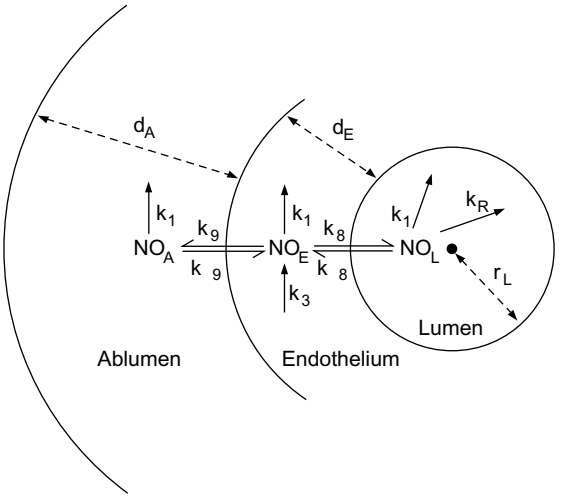


Figure 15 Model to predict NO concentration at the smooth muscle cell as a function of vessel radius with erythrocyte consumption of NO.

$$[NO]_A = \frac{k_3}{k_8}\frac{k_9}{k_{-9}}\frac{k_{-8} + k_R}{k_R} \tag{16}$$

In general, the steady-state concentration of an intermediate is equal to the ratio of the rate of formation to the rate of disappearance. Since the rate of formation of NO is k_3, then from Eq. (16) the rate of loss of NO from the abluminal compartment (k_L) is equal to the product $k_8k_{-9}k_R/k_9(k_{-8} + k_R)$. Assuming an effective permeability coefficient for NO across membranes that is similar to the value we have measured in a single erythrocyte ($P = 5.73 \times 10^{-2}$ cm s^{-1}),[6] and expressing k_L in terms of P using cylindrical geometries and the values for distance denoted in Fig. 15, yields

$$k_L + \frac{P}{d_A}\left(\frac{r_L}{r_L + d_E + d_A/2}\right)\left(\frac{SA_R}{SA_R + SA_V}\right) \tag{17}$$

where SA_R is the total erythrocyte surface area per luminal volume and SA_V is the vessel surface area per luminal volume.

The two terms in parentheses represent the two major effects of geometry on NO scavenging by erythrocytes. In this model, the most important sink for NO is the erythrocyte, which consumes NO at a rate determined solely by the rate at which it can enter the cell (Liu *et al.,* 1998b). In blood, this will be a function of only the permeability coefficient P and the total erythrocyte surface area per unit volume. However, plasma NO can ''escape'' by diffusing out of the vessel and into the endothelial compartment, and this process is

[6] Consumption of NO is limited by the rate at which NO can enter the erythrocyte (Liu *et al.,* 1998b), so this rate can be expressed in terms of an apparent permeability coefficient, *P.* Utilizing the rate constant for loss of NO of 7.74×10^{-8} s^{-1} for one erythrocyte per milliliter (Liu *et al.,* 1998b) and a surface area of 135 μm^2 per erythrocyte (Evans and Fung, 1972), the value for P becomes 5.73×10^{-2} cm s^{-1}.

also a transmembrane simple diffusion process and thus dictated by a permeability coefficient that should be similar in magnitude to the P for movement across the erythrocyte membrane. The rate of this escape will be determined by the vessel surface area per unit internal volume. Thus, the relative fraction of NO in the plasma that is consumed is given by the ratio $SA_R/(SA_R + SA_V)$. This number is the fraction of total membrane area available for NO diffusion that is erythrocyte membrane.

The other geometrical factor that is manifested in Eq. (17) and will determine the extent of NO scavenging (specifically, the concentration of NO in the abluminal compartment) is the size of the vessel with respect to the size of the surrounding endothelial and abluminal compartments. The larger the vessel, the more NO will be scavenged because the endothelial and abluminal compartments surround a large versus small pool of NO-consuming substance. This is expressed in the first term. Specifically, for larger vessels r_L is greater than d_E or d_A, and so this term approaches unity. As the vessel decreases in size, r_L becomes smaller and this term approaches zero; thus, k_L decreases as vessel size decreases.

As pointed out by Liao and co-workers (Vaughn *et al.,* 1998) and Butler *et al.* (1998), there is a third effect of vessel size on the extent of NO scavenging, which is the Fahraeus effect, whereby streaming of blood through small-diameter vessels results in decreased hematocrit. This phenomenon was incorporated into Eq. (17) using an empirical relationship derived from the data of Lipowski *et al.* (1980), which relates the microvessel hematocrit (H_{micro}) to the systemic hematocrit ($H_{systemic}$) in the cat mesentery using the equation $H_{micro} = \alpha H_{systemic}$ where $\alpha = 1.9 \times 10^{-2} \times r_L + 0.1336$. Assuming a surface area to volume ratio for the erythrocyte (β) of 1.436 μm^{-1} (Evans and Fung, 1972), the final equation is thus

$$k_L = \frac{P}{d_A}\left(\frac{r_L}{r_L + d_E + d_A/2}\right)\left(\frac{r_L}{r_L + 2/(\alpha\beta H_{systemic})}\right) \quad (18)$$

Using this model it is possible, given the systemic hematocrit ($H_{systemic}$), to predict the extent to which NO will be scavenged by the blood using solely geometrical parameters (d_A, d_E, r_L) and experimentally available data (P, α, and β). Using our experimentally determined value for the apparent permeability coefficient of NO ($P = 5.73 \times 10^{-2}$ cm s^{-1}, see earlier), a value of 2.5 μm for d_E (Evans and Fung, 1972), and a value of 50 μm for d_A (which seems a reasonable distance within which is located the smooth muscle cell target), Fig. 16 depicts the value of k_L as a function of vascular luminal diameter.

The rate of NO disappearance at the smooth muscle cell (k_L) is very sensitive to vessel radius. As described above, this is primarily because with larger vessels there is more erythrocyte surface area per volume of blood compared to vessel wall surface area; movement of NO across the former results in consumption, whereas movement across the latter results in vasodilator action. Using these values, the half-life

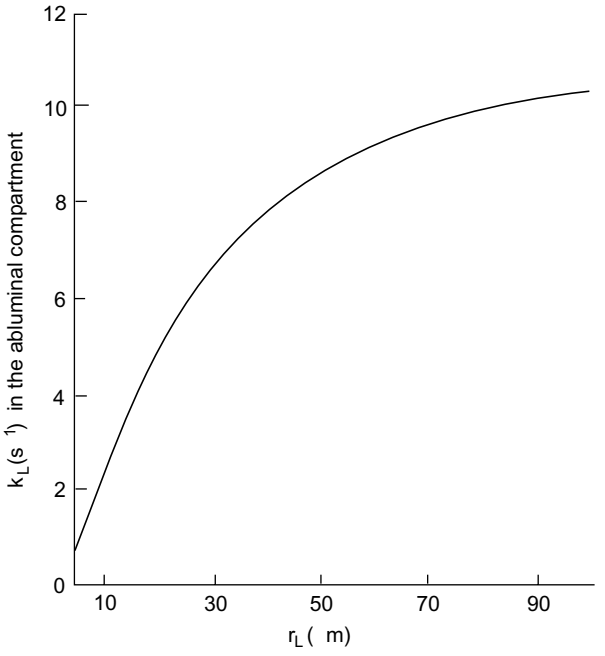


Figure 16 The effects of vessel radius on the rate of NO disappearance at the smooth muscle cell, using the model in Fig. 15.

of NO at the smooth muscle cell ($t_{1/2} = \ln 2/k_L$) varies from approximately 0.3 to 0.1 s over the size range 10–40 μm, which is the range of the arteriolar vascular bed. Although this modeling must be considered only an approximation, it would appear that the ''packaging'' of hemoglobin within the erythrocyte will indeed slow NO consumption to a level where there will be substantial amounts of endothelially produced NO that survive reaction with oxyhemoglobin. This suggests that EDRF is indeed free NO and that there is no necessity to postulate ''bound forms'' of NO for its biological actions, at least in the vasculature under normal (i.e., noninflammatory) conditions.

References

Anand, I. S., Chandrashekhar, Y., Wander, G. S., and Chawla, L. S. (1995). Endothelium-derived relaxing factor is important in mediating the high output state in chronic severe anemia. *J. Am. Coll. Cardiol.* **25,** 1402–1407.

Anonymous (1961). ''Biochemists' Handbook'' (C. Long, ed.), pp. 677. Van Nostrand, Princeton, New Jersey.

Babbs, C. F., and Steiner, M. G. (1990). Simulation of free radical reactions in biology and medicine: A new two-compartment kinetic model of intracellular lipid peroxidation. *Free Radicals Biol. Med.* **8,** 471–485.

Benesch, R. E., and Benesch, R. (1962). The influence of oxygenation on the reactivity of the -SH groups of hemoglobin. *Biochemistry* **1,** 735–738.

Butler, A. R., Megson, I. L., and Wright, P. G. (1998). Diffusion of nitric oxide and scavenging by blood in the vasculature. *Biochim. Biophys. Acta* **1425,** 168–176.

Carlin, R. E., McGraw, D. J., Camporesi, E. M., and Hakim, T. S. (1997). Increased nitric oxide in exhaled gas is an early marker of hypovolemic states. *J. Surg. Res.* **69,** 362–366.

Collander, R., and Barna, M. (1933). Permeabilitatsstudien in *Chara ceratophylla. Acta Bot. Fennica* **11,** 1–114.

Cordes, E. H., and Dunlap, R. B. (1969). Kinetics of organic reactions in micellar systems. *Acc. Chem. Res.* **2,** 329–337.

Deem, S., Swenson, E. R., Alberts, M. K., Hedges, R. G., and Bishop, M. J. (1998). Red-blood-cell augmentation of hypoxic pulmonary vasoconstriction: Hematocrit dependence and the importance of nitric oxide. *Am. J. Respir. Crit. Care Med.***157,** 1181–1186.

Denicola, A., Souza, J. M., Radi, R., and Lissi, E. (1996). Nitric oxide diffusion in membranes determined by fluorescence quenching. *Arch. Biochem. Biophys.* **328,** 208–212.

Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N. J., and Olson, J. S. (1996). Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* **35,** 6976–6983.

Evans, E., and Fung, Y. C. (1972). Improved measurements of the erythrocyte geometry. *Microvasc. Res.* **4,** 335–347.

Fendler, J. H., and Fendler, E. J. (1975). ''Catalysis in Micellar and Macromolecular Systems.'' Academic Press, New York.

Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993). Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* **326,** 1–3.

Gordin, V. A., and Nedospasov, A. A. (1998). NO catastrophes in vivo as a result of micellar catalysis. *FEBS Lett.* **424,** 239–242.

Gow, A. J., Luchsinger, B. P., Pawloski, J. R. Singel, D. J., and Stamler, J. S. (1999). The oxyhemoglobin reaction of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 9027–9032.

Henry, Y., and Singel, D. J. (1996). Metal–nitrosyl interactions in nitric oxide biology probed by electron paramagnetic resonance spectroscopy. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. S. Stamler, eds.), pp. 357–372. Wiley, New York.

Henry, Y., Lepoivre, M., Drapier, J. C., Ducrocq, C., Boucher, J. L., and Guissani, A. (1993). EPR characterization of molecular targets for NO in mammalian cells and organelles. *FASEB J.* **7,** 1124–1134.

Henry, Y., Guissani, A., and Ducastel, B. (1997). ''EPR Spectroscopy of Nitrosylated Compounds.'' Landes Bioscience, Austin, Texas.

Hogg, N., Singh, R. J., Goss, S. P., and Kalyanaraman, B. (1996). The reaction between nitric oxide and alpha-tocopherol: A reappraisal. *Biochem. Biophys. Res. Commun.* **224,** 696–702.

Jia, L. Bonaventura, J., and Stamler, J. S. (1996). *S*-Nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* **380,** 221–226.

Kalyanaraman, B. (1996). Detection of nitric oxide by electron spin resonance in chemical, photochemical, cellular, physiological, and pathophysiological systems. *Methods Enzymol.* **268,** 168–187.

Kim, Y.-M., Nussler, A., Sweetland, M., Billiar, T., Simmons, R. L., and Lancaster, J. R., Jr. (1994). Apparent differential sensitivity of nitric oxide synthase to feedback inhibition (by nitric oxide) in cells versus extracts. *In* ''The Biology of Nitric Oxide. 4. Enzymology, Biochemistry and Immunology'' (S. Moncada, M. Feelisch, R. Busse, and E. A. Higgs, eds.), pp. 77–80. Portland Press, London.

Lancaster, J. R., Jr. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8137–8141.

Lancaster, J. R., Jr. (1996). Diffusion of free nitric oxide. *Methods Enzymol.* **268,** 31–50.

Lancaster, J. R., Jr. (1997). A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* **1,** 18–30.

Liao, J. C., Hein, W., Vaughn, M. W., Huang, K. T., and Kuo, L. (1999). Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 8757–8761.

Lipowsky, H. H., Usami, S., and Chien, S. (1980). In vivo measurements of ''apparent viscosity'' and microvessel hematocrit in the mesentery of the cat. *Microvasc. Res.* **19,** 297–319.

Liu, X., Miller, M. S., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998a). Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2175–2179.

Liu, X., Miller, M. J., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster, J. R., Jr. (1998b). Diffusion-limited reaction of free nitric oxide with erythrocytes. *J. Biol. Chem.* **273,** 18709–18713.

Malinski, T., Taha, Z., Grunfeld, S., Patton, S., Kapturczak, M., and Tomboulian, P. (1993). Diffusion of nitric oxide in the aorta wall monitored in situ by porphyrinic microsensors. *Biochem. Biophys. Res. Commun.* **193,** 1076–1082.

Mellander, S., Bjornberg, J., Ekelund, U., and Alm, P. (1997). Cardiovascular regulation by endogenous nitric oxide is essential for survival after acute haemorrhage. *Acta Physiol. Scand.* **160,** 57–65.

Meulemans, A. (1994). Diffusion coefficients and half-lives of nitric oxide and *N*-nitroso-L-arginine in rat cortex. *Neurosci. Lett.* **171,** 89–93.

Patel, R. P., Hogg, N., Spencer, N. Y., Kalyanaraman, B., Matalon, S., Darley, U., and Darley-Usmar, V. (1999). Biochemical characterization of human *S*-nitrosohemoglobin. Effects on oxygen binding and transnitrosation. *J. Biol. Chem.* **274,** 15487–15492.

Pauling, L. (1964). ''College Chemistry.'' 3rd ed. W. H. Freeman and Co., San Francisco, CA.

Ridd, J. H. (1978). Diffusion control and pre-association in nitrosation, nitration and halogenation. *Adv. Phys. Org. Chem.* **16,** 1–49.

Riggs, A. F. (1961). The binding of *N*-ethylmaleimide by human hemoglobin and its effect upon the oxygen equilibrium. *J. Biol. Chem.* **236,** 1948–1954.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **269,** 26066–26075.

Rubbo, H., Darley-Usmar, V., and Freeman, B. A. (1996). Nitric oxide regulation of tissue free radical injury [review] [135 refs.]. *Chem. Res. Toxicol.* **9,** 809–820.

Shaw, A. W., and Vosper, A. J. (1977). Solubility of nitric oxide in aqueous and nonaqueous solvents. *J. Chem. Soc. Faraday Trans. I* **8,** 1239–1244.

Singel, D. J., and Lancaster, J. R., Jr. (1996). Electron paramagnetic resonance spectroscopy and nitric oxide biology. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. S. Stamler, eds.), pp. 341–356. Wiley, New York.

Stadler, J., Bergonia, H. A., Di Silvio, M., Sweetland, M. A., Billiar, T. R., Simmons, R. L., and Lancaster, J. R., Jr. (1993). Nonheme iron–nitrosyl complex formation in rat hepatocytes: Detection by electron paramagnetic resonance spectroscopy. *Arch. Biochem. Biophys.* **302,** 4–11.

Stamler, J. S. (1994). *S*-Nitrosothiols and the bioregulatory actions of nitrogen oxides through reactions with thiol groups. *Curr. Top. Microbiol. Immunol.* **196,** 19–36.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). *S*-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stamler, J. S., Jia, L., Eu, J. P., McMahon, T. J., Demchenko, I. T., Bonaventura, J., Gernert, K., and Piantadosi, C. A. (1997). Blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient. *Science* **276,** 2034–2037.

Subczynski, W. K., and Hyde, J. S. (1983). Concentration of oxygen in lipid bilayers using a spin-label method. *Biophys. J.* **41,** 283–286.

Subczynski, W. K., Lomnicka, M., and Hyde, J. S. (1996). Permeability of nitric oxide through lipid bilayer membranes. *Free Radical Res.* **24,** 343–349.

Vanderkooi, J. M., Wright, W. W., and Erecinska, M. (1994). Nitric oxide diffusion coefficients in solutions, proteins and membranes determined by phosphorescence. *Biochim. Biophys. Acta* **1207,** 249–254.

Vanin, A. F. (1991). Endothelium-derived relaxing factor is a nitrosyl iron complex with thiol ligands. *FEBS Lett.* **289,** 1–3.

Vaughn, M. W., Kuo, L., and Liao, J. C. (1998). Effective diffusion of nitric oxide in the microcirculation. *Am. J. Physiol.* **274,** H1705–H1714.

Wilcox, D. E., and Smith, R. P. (1995). Detection and quantification of nitric oxide using electron magnetic resonance spectroscopy. *Methods (San Diego)* **7,** 59–70.

Williams, D. L. H. (1988). ''Nitrosation.'' Cambridge Univ. Press, Cambridge.

Wolzt, M., MacAllister, R. J., Davis, D., Feelisch, M., Moncada, S., Vallance, P., and Hobbs, A. J. (1999). Biochemical characterization of *S*-nitrosohemoglobin. Mechanisms underlying synthesis, NO release, and biological activity. *J. Biol. Chem.* **274,** 28983–28990.

Wood, J., and Garthwaite, J. (1994). Models for the diffusional spread of nitric oxide: Implications for neuronal nitric oxide signalling and its pharmacological properties. *Neuropharmacology* **33,** 1235–1244.

CHAPTER 14

Membrane Transport of L-Arginine and Cationic Amino Acid Analogs

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THE CATIONIC AMINO ACID L-ARGININE, THE SUBSTRATE FOR NITRIC OXIDE SYNTHASES (NOS), IS CONSIDERED A SEMIESSENTIAL AMINO ACID IN MOST MAMMALS. CONSEQUENTLY, MAMMALIAN CELLS MUST BE CAPABLE OF EXCHANGING CATIONIC AMINO ACIDS (CAA) WITH THE ENVIRONMENT. THE BILAYER OF THE PLASMA MEMBRANE IS IMPERMEABLE TO POLAR MOLECULES, AND THUS SPECIALIZED CARRIER PROTEINS WITH DISTINCT SUBSTRATE SPECIFICITY TRANSPORT HYDROPHILIC SOLUTES SUCH AS AMINO ACIDS. L-ARGININE SHARES THE SAME TRANSPORT SYSTEMS WITH OTHER CAA AND THEIR ANALOGS. IN ADDITION, SOME CAA TRANSPORT SYSTEMS ALSO INTERACT WITH NEUTRAL AMINO ACIDS (NAA). HENCE, CHANGES IN THE EXPRESSION AND/OR ACTIVITY OF THESE TRANSPORT SYSTEMS WILL AFFECT NOT ONLY TRANSPORT OF L-ARGININE, BUT ALSO TRANSPORT OF OTHER CAA, NAA, AND CAA ANALOGS, INCLUDING SOME NOS INHIBITORS. CDNAS ENCODING SPECIFIC CAA CARRIER PROTEINS HAVE ONLY RECENTLY BEEN CLONED. THE DEDUCED PROTEINS BELONG TO THREE DIFFERENT GENE FAMILIES (SOLUTE CARRIER FAMILIES 3, 6, AND 7) AND MEDIATE TRANSPORT ACTIVITIES RESEMBLING SYSTEMS y^+, y^+L, $b^{0,+}$, OR $B^{0,+}$ WHEN OVEREXPRESSED IN *XENOPUS LAEVIS* OOCYTES OR IN MAMMALIAN CELLS. SYSTEM Y^+ SEEMS TO BE THE MOST WIDELY EXPRESSED TRANSPORT ACTIVITY IN CELLS PRODUCING NITRIC OXIDE (NO), AND HENCE THE CARRIER PROTEINS MEDIATING Y^+ ACTIVITY (CATIONIC AMINO ACID TRANSPORTER-1, -2B, AND -3) ARE LIKELY TO PLAY AN IMPORTANT ROLE IN SUPPLYING L-ARGININE FOR NOS. IN THIS CHAPTER WE BRIEFLY REVIEW THE CURRENT KNOWLEDGE OF THE MECHANISMS AND REGULATION OF CAA CARRIER PROTEINS AND DISCUSS THE POTENTIAL INVOLVEMENT OF EACH TRANSPORTER IN SUPPLYING L-ARGININE FOR NO BIOSYNTHESIS.

Introduction

The cationic amino acid L-arginine is the physiological precursor of nitric oxide (NO) synthesized by nitric oxide synthases (NOS). In most mammals, L-arginine is considered a semiessential amino acid, reflecting the fact that *de novo* synthesis of L-arginine does not seem to sustain adequate supply, especially under conditions of high demand such as growth and wound healing (for reviews, see Barbul, 1986; Baron, 1994; Reyes *et al.,* 1994; Jenkinson *et al.,* 1996). L-arginine is involved in the synthesis of proteins, creatine, urea, agmatine, and polyamines, and it modulates the delivery of hormones and the synthesis of pyrimidine bases (Fig. 1; Reyes *et al.,* 1994). L-Arginine can be synthesized from L-ornithine via L-citrulline by enzymes of the urea cycle. L-Ornithine can be produced from L-arginine by the action of arginases, but it can also be synthesized from L-glutamate and L-proline (for review, see Wu and Morris,

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1998). L-Arginine synthesized by the liver is metabolized locally and does not contribute significantly to circulating plasma L-arginine levels, which are derived mainly from dietary intake of L-arginine (1–2 g/day) and synthesis of L-citrulline and L-arginine by the small intestine and proximal tubules of the kidney, respectively.

In most other cell types, L-arginine synthesis plays a minor role, and consequently this amino acids needs to be taken up from the extracellular space. The plasma membrane is impermeable to polar molecules such as amino acids, sugars, and nucleosides, and thus specialized carrier proteins exhibiting distinct substrate specificity transport these hydrophilic solutes. The carriers for L-arginine can also transport other cationic amino acids (CAA), such as the essential amino acid L-lysine and the nonproteinogenic amino acid L-ornithine, a substrate for polyamine, L-proline, and L-glutamate biosynthesis (Fig. 1). In addition, some CAA transport systems also interact with neutral amino acids (NAA). The existence of specific transport activities for CAA has been recognized for many years, and these have been designated as ''transport systems'' to emphasize the concept that a complex of different proteins rather than a single carrier protein may mediate a distinct transport activity. Several cDNAs encoding proteins involved in CAA transport have been cloned (see later). Overexpression of these proteins in *Xenopus laevis* oocytes or mammalian cells leads to an increase in the transport of CAA. In some cases [e.g., cationic amino acid transporter proteins (CATs) and the Na^+- and Cl^--dependent carrier ($ATB^{0,+}$)] the expression of a single protein is sufficient to confer full transport activity, whereas in other cases (e.g., heterodimeric amino acid transporters such as 4F2hc/y^+LAT, 4F2hc/4F2-lc6, and rBAT/$b^{0,+}$AT) coexpression of two proteins is required. Interactions between these overexpressed proteins with other, as yet unidentified cellular proteins cannot be excluded. Therefore, the term ''transport system'' is still used to define a specific transport activity in a given cell membrane, whereas the term ''carrier'' is used to refer to specific proteins that have been characterized at the molecular level. To date it remains unclear which of the specific transport processes for CAA are directly involved in supplying L-arginine for NO biosynthesis. In this chapter, we critically examine the evidence that specific transport systems and carrier proteins are involved in mediating the influx of CAA in mammalian cells. Although we focus principally on CAA transport processes potentially involved in modulating NO production, we also describe transport processes that might not be directly involved in supplying L-arginine for NOS.

Carrier Systems for Cationic Amino Acids in NO-Producing Cells or Tissues

In most cell types, transport of CAA is energy independent, with facilitated exchange of substrate between extracellular and intracellular compartments modulated by the membrane potential (Fig. 2A; White, 1985; Bogle *et al.,* 1991; Bussolati *et al.,* 1993; Sobrevia *et al.,* 1995, 1996; Zharikov *et al.,* 1997). Only one energy-dependent transport system coupling entry of Na^+ to the influx of CAA has been described (system $B^{0,+}$, Fig. 2B; Van Winkle *et al.,* 1988).

Na^+-independent membrane transport of CAA was originally assigned to the classic Na^+-independent system y^+

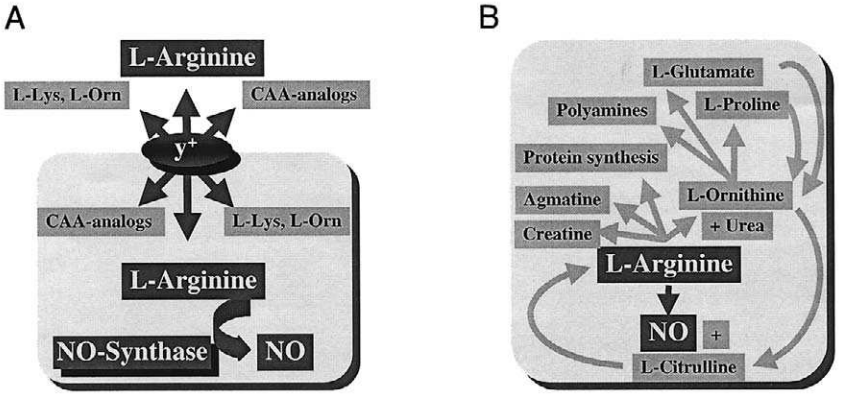


Figure 1 Factors influencing L-arginine availability for nitric oxide synthases in intact mammalian cells. (A) Transport through the plasma membrane. In NO-producing cells, transport of L-arginine is predominantly mediated via facilitated diffusion through cationic amino acid (CAA)-preferring carrier proteins (system y^+). Consequently, other CAA and CAA analogs compete with L-arginine for transport via these carrier proteins. As CAA are exchanged with one another, the intracellular concentration of L-arginine will depend not only on its extracellular concentration, but also on the concentration of the other CAA. (B) Utilization and synthesis of L-arginine. L-arginine is consumed by metabolic pathways other than NO synthases (indicated by straight arrows), for example, protein and urea synthesis, the latter giving rise to L-ornithine, an important precursor for polyamines, L-proline, and L-glutamate. Some cells, on the other hand, can recycle L-arginine (indicated by curved arrows), from L-citrulline or even L-ornithine and L-glutamate.

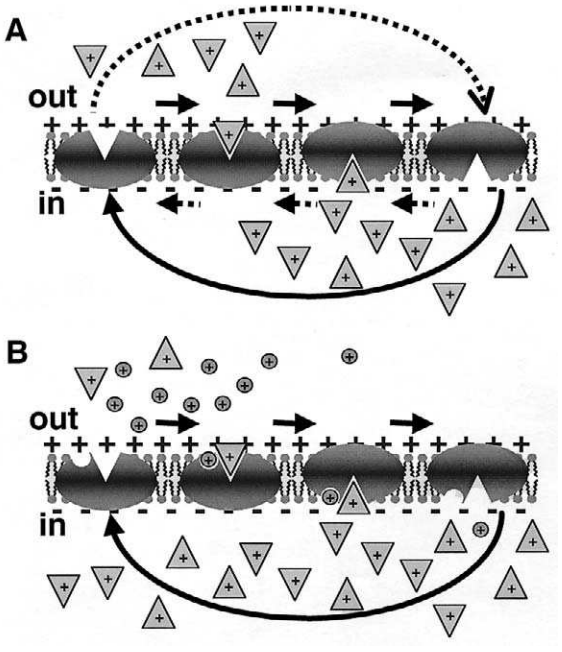


Figure 2 Scheme of carrier-mediated transport of cationic amino acids through the plasma membrane. (A) Facilitated diffusion. Cationic amino acid (CAA) fluxes through energy-independent carriers occur in both directions, into the cell (solid straight arrows) and out of the cell (dashed straight arrow). Net transport of substrate (shaded triangles) is only achieved when an unloaded carrier returns to its starting position after undergoing a conformational change (curved arrows). Although there is no direct coupling of solute transport with transport of an ion down its concentration gradient, CAA are still concentrated intracellularly due to the negative membrane potential of the cell. (B) Energy-dependent transport. These carrier proteins couple solute transport to the cotransport of one or several ions, using the ion gradient as the driving force to allow a greater concentration of substrate in the cell. The ion gradient directs this transport process in one direction (solid arrows).

characterized in fibroblasts (see review by White, 1985). Accumulating evidence indicates that L-arginine shares at least six different transport systems with other CAA (e.g., L-lysine and L-ornithine; see review by Deves and Boyd, 1998, and Van Winkel and Campione, 1990) and cationic NOS inhibitors (Baydoun and Mann, 1994; Schmidt *et al.,* 1994; McDonald *et al.,* 1997a). Five of these systems are Na^+ independent with respect to CAA and differ only in their interaction with NAA. CAA transport via system y^+ is saturable [Michaelis–Menten constant (K_m) of ~80–100 μM], pH independent, and stimulated by substrate at the *trans*-side of the plasma membrane (*trans*-stimulation). In the presence of extracellular Na^+, small NAA can inhibit system y^+-mediated transport activity (e.g., L-homoserine but not L-leucine). Inhibition is not observed when Na^+ is replaced by K^+, suggesting that Na^+ binds to the site that normally accepts the cationic side chain of a CAA (see review by White, 1985). The transport activity of system y^+ is increased during membrane hyperpolarization, implying that in the vascular endothelium most vasoactive agonists will stimulate entry of L-arginine (Bogle *et al.,* 1991; Bussolati *et al.,* 1993; Sobrevia *et al.,* 1995; Zharikov *et al.,* 1997). With the exception of the liver, system y^+ is expressed ubiquitously, and the majority of studies in endothelial, smooth muscle, and macrophage cell types have confirmed that L-arginine and other cationic analogs and NOS inhibitors are predominantly transported via a Na^+-independent system with characteristics of the classic system y^+ (Bogle *et al.,* 1991, 1992a; Greene *et al.,* 1993; Wu and Meininger, 1993; Baydoun and Mann, 1994; Schmidt *et al.,* 1994; Block *et al.,* 1995; Sobrevia *et al.,* 1995, 1996; Wileman *et al.,* 1995; Bogle *et al.,* 1996; Sobrevia and Mann, 1997). Interestingly, in human endothelial cells system y^+ transport activity is increased in cells obtained from gestational diabetic pregnancies or exposed to elevated D-glucose for 3–12 hours (Sobrevia *et al.,* 1996, 1997; Sobrevia and Mann, 1997). System y^+-like transport activity has also been described in astrocytes and cultured neuronal cells (Westergaard *et al.,* 1993; Schmidlin and Wiesinger, 1995; Schmidt *et al.,* 1995; Wayte *et al.,* 1996). As in the macrophage cell lines J774 and RAW 264.7 and in endothelial cells (Baydoun and Mann, 1994; Schmidt *et al.,* 1994; Bogle *et al.,* 1995), studies in a neuroblastoma/glioma hybrid cell line have confirmed that CAA and N^G-monomethyl-L-arginine (L-NMMA) are transported via system y^+, whereas N^G-nitro-L-arginine (L-NNA) is taken up by NAA transport systems (Schmidt *et al.,* 1995). As described below, the role of other distinct Na^+-independent CAA transport systems in NOS expressing cells has been characterized far less rigorously.

Transport of CAA via systems b_1^+ and b_2^+ has been described in preimplantation mouse conceptus (Van Winkel and Campione, 1990). Neither system is inhibitable by L-homoserine or L-leucine even in the presence of Na^+. Both systems demonstrate a higher affinity for L-arginine relative to L-lysine; the preference for L-arginine is most pronounced for system b_2^+, which exhibits a 100-fold lower K_m for L-arginine (about 80 μM) than for L-lysine. In addition, both systems recognize L-arginine with a higher affinity than L-homoarginine, in direct contrast to the classic system y^+. As systems b_1^+ and b_2^+ have only been described in mouse preimplantation conceptus, these CAA transport systems most likely will not play a major role in modulating the L-arginine transport in NO-producing cells.

System y^+L, originally identified in human erythrocytes and subsequently in intestine and placenta (Deves *et al.,* 1992; Harvey *et al.,* 1993; Fei *et al.,* 1995), exhibits a much higher affinity for CAA (K_m for lysine ~10 μM) than any other cationic transport system (see review by Deves and Boyd, 1998). System y^+L mediates Na^+-independent CAA and Na^+-dependent NAA transport. An additional difference between this system and systems y^+, b_1^+, and b_2^+ is the similar affinity of system y^+L for CAA and NAA (e.g., L-leucine, L-methionine, or L-glutamine) in the presence of Na^+ or Li^+. Entry of L-lysine through this system is unaffected by removal of Na^+, whereas the affinity of system y^+L for CAA is markedly decreased on substitution of extracellular Na^+ by K^+ (Deves *et al.,* 1992). *N*-Ethylmaleimide (200 μM) has been used as a relatively selective inhibitor of system y^+ in erythrocytes and peripheral blood mononuclear

cells, permitting resolution of cationic amino acid influx via systems y^+L and y^+ (Deves *et al.,* 1993; Hanssen *et al.,* 1998). Moreover, as L-leucine does not interact with system y^+, b_1^+, or b_2^+ this amino acid can be used to selectively inhibit CAA transport by system y^+L in the presence of Na^+ (see review by Deves and Boyd, 1998). In all tissues where system y^+L activity has been observed, maximal rates of transport mediated by this system are low, indicating either a low capacity and/or a low expression of the carrier protein(s). In epithelial cells such as the small intestine and the renal proximal tubule, system y^+L is preferentially localized to the basolateral membrane and serves as an efflux pathway for CAA reabsorbed across the brush border membrane (Novak *et al.,* 1997; see also review by Deves and Boyd, 1998). Although the role of system y^+L in nonepithelial cells such as erythrocytes, peripheral blood mononuclear cells, and platelets is less well defined, an inwardly directed Na^+ gradient under physiological conditions would favor efflux of intracellular CAA in exchange for entry of NAA and Na^+. Experiments *in vitro* have characterized influx and efflux of CAA via system y^+L (Deves *et al.,* 1992, 1993; Mendes Ribeiro *et al.,* 1997, 1999; Hanssen *et al.,* 1998; for review, see Deves and Boyd, 1998), and experiments in human platelets have established that L-arginine transport via system y^+L is stimulated in cells from patients with chronic renal failure (Mendes Ribeiro *et al.,* 1999). Unfortunately, virtually no information is available on the potential role of this transport system in NO-producing cells (see later).

System $b^{0,+}$ initially described in early mouse embryos has also been described in small intestinal and renal tubule epithelia, where it localizes to the apical membrane (Van Winkle *et al.,* 1988; Magagnin *et al.,* 1992; see review by Deves and Boyd, 1998). Transport of both CAA and NAA via system $b^{0,+}$ is Na^+ independent, with CAA and large α- and β-C atom unbranched NAA as the preferred substrates. Under physiological conditions, system $b^{0,+}$ recognizes CAA and NAA with similar affinity; however, in nonionic solutions (e.g., sucrose) the K_m for L-lysine is 10-fold lower than in isotonic salt solutions (about 60 μ*M*), whereas the K_m for L-leucine is similar in both solutions (90–140 μ*M*). Limited system $b^{0,+}$ transport activity has been observed in cultured vascular endothelial cells (Greene *et al.,* 1993; Pan *et al.,* 1995). In human umbilical vein endothelial cells (HUVEC), system y^+ represents the predominant entry pathway for CAA, since addition of L-leucine (10 m*M*) inhibits total Na^+-independent L-arginine uptake by only 15–20% (Pan *et al.,* 1996; Sobrevia *et al.,* 1996, 1997; Sobrevia and Mann, 1997). Similar studies in cultured porcine pulmonary artery endothelial cells have confirmed system y^+ as the key Na^+-independent transport pathway for CAA, although a Na^+-independent uptake mechanism for L-arginine resembling system $b^{0,+}$ but with lower affinity for CAA has also been described (Greene *et al.,* 1993).

To date, system $B^{0,+}$, as originally described in blastocytes (Van Winkel *et al.,* 1985), represents the only Na^+-dependent transport system described for CAA, and its activity has usually been identified in the same tissues as system $b^{0,+}$. The substrate specificity of the two systems is similar; however, system $B^{0,+}$ also accepts small NAA (L-alanine, L-serine) and NAA that are branched at the α and β carbon atom {e.g., 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH)}. System $B^{0,+}$ and $b^{0,+}$ transport activities have been described in porcine cultured pulmonary artery endothelial cells (Greene *et al.,* 1993; Block *et al.,* 1995). In the latter study, hypoxia was shown to decrease L-arginine transport by both system y^+ and $B^{0,+}$, yet the authors did not investigate whether decreased L-arginine transport via these transport systems limited NO production. To our knowledge, the involvement of systems $B^{0,+}$ and $b^{0,+}$ in modulating NO synthesis remains to be investigated.

Activation of Cationic Amino Acid Transport in Inflammation

Numerous studies have implicated NO as a cytokine-induced vasodilator in bacterial sepsis (see review by Knowles and Moncada, 1994). A Ca^{2+}-independent isoform of NO synthase (NOS II), as first described in macrophages (Hibbs *et al.,* 1988), has been identified in endothelial cells exposed to tumor necrosis factor α (TNF-α) and γ-interferon (IFN-γ) or bacterial lipopolysaccharide (LPS). Induction of NOS II occurs with a lag period reaching a maximum usually between 6 and 12 hours, is inhibited by glucocorticoids, and is dependent on a supply of extracellular L-arginine (Hibbs *et al.,* 1987; Bogle *et al.,* 1992b; for review, see Knowles and Moncada, 1994).

Experiments in the human endothelial cell line SGHEC-7 have shown that treatment with LPS for 6 hours caused parallel increases in CAA transport and production of nitrite, the stable breakdown product of NO (Bogle *et al.,* 1995). This report confirmed findings from similar studies in J774 macrophages and rat aortic smooth muscle cells (Baydoun *et al.,* 1993; Wileman *et al.,* 1995), where activation of transport of L-arginine and the cationic arginine analog L-NMMA by LPS was blocked by cycloheximide but unaffected by dexamethasone. In contrast, studies in rat pulmonary endothelial cells concluded that dexamethasone inhibited Na^+-independent L-arginine transport (Pan *et al.,* 1996), although the inhibitory effects of dexamethasone on NO production were not assessed in parallel. In other endothelial cells types, LPS activated system y^+ through an autocrine release of the cytokines TNF-α and interleukin-1 (IL-1), since pretreatment of cells with either an anti-TNF antibody or a IL-1 receptor antagonist significantly inhibited LPS-stimulated L-arginine transport (Cendan *et al.,* 1996). In this study, activation of L-arginine influx required protein synthesis and was apparently mediated via the Na^+-independent systems y^+ and the Na^+-dependent system $B^{0,+}$, with little or no change detected in system $b^{0,+}$ activity.

Earlier studies in cultured macrophages established that L-arginine supply was rate limiting for sustained NO synthesis in cytokine-activated cells (Hibbs *et al.,* 1987). More

recent reports based on studies in arterial ring preparations or cultured J774 macrophages and vascular smooth muscle cells have confirmed this observation, and they implicated L-arginine transport as a rate-limiting factor for NO production (Bogle *et al.,* 1992b; Schott *et al.,* 1993; Wileman *et al.,* 1995; Durante *et al.,* 1996). However, over shorter time intervals (2 min), NO production in the macrophage cell line J774 has been shown to be independent of extracellular L-arginine (Closs *et al.,* 1999).

Carrier Proteins Identified on the Molecular Level

Although the existence of specific carrier systems in the plasma membrane of mammalian cells has been documented for many years, identification of the corresponding proteins mediating the respective transport activities remains a challenge. It is only relatively recently that cDNAs encoding specific CAA carrier proteins have been cloned. Some of these have been cloned by serendipity or as a result of their homology to other carrier proteins, whereas others were identified by expression cloning. The proteins encoded by these cDNAs belong to three different families [solute carrier families (SLC) 3, 6, and 7, see Table I] and mediate transport activities resembling systems y^+, y^+L, $b^{0,+}$, or $B^{0,+}$ when overexpressed in *Xenopus laevis* oocytes or in mammalian cells. The molecular identification of these carrier proteins revealed an even greater diversity than had been anticipated from radiotracer flux experiments. The protein(s) responsible for the activity of system(s) b^+ has as yet not been identified.

Cationic Amino Acid Transporters

STRUCTURE AND FUNCTION OF THE CAT PROTEINS

The CAT proteins belong to the first amino acid carriers discovered in mammalian cells. Albritton and co-workers (1989) obtained a cDNA encoding the first family member when they identified the protein responsible for susceptibility to infection by murine ecotropic leukemia viruses (MuLV). Similarities between the secondary structure of the MuLV receptor and L-histidine and L-arginine permeases from *Saccharomyces cerevisiae* suggested that this protein may also function as a solute transporter. Expression of the ecotropic MuLV receptor in *Xenopus laevis* oocytes and subsequent transport studies demonstrated that this receptor mediates Na^+-independent transport of cationic amino acids (Kim *et al.,* 1991; Wang *et al.,* 1991). Thus, the virus receptor was renamed mCAT-1 (mouse cationic amino acid transporter). mCAT-1 is an integral membrane protein with 14 putative transmembrane domains and intracellular N- and C-termini (Fig. 3A). Four additional related proteins, named CAT-2A, -2B, -3, and -4, have since been identified in different mammalian species including humans (see Table I and references therein), with CAT-2A and -2B being splice variants that differ only in a stretch of 42 amino acids (Closs *et al.,* 1993a, 1997a; Kavanaugh *et al.,* 1994). The amino acid sequences of CAT-2A, -2B, and -3 are more closely related to each other than to CAT-4 (Table II). All known CAT proteins exhibit quite similar hydrophobicity plots, suggesting that their structure in the membrane is also similar. Consensus sequences for N-linked glycosylation can be found in each isoform but at different locations (see Fig. 3). CAT-1, -2A, and -2B have been shown to be glycosylated, indicating that these carriers are in fact located in the plasma membrane (Closs *et al.,* 1993b,c; Kim and Cunningham, 1993).

As evidenced by studies to date, all CAT proteins mediate Na^+-independent transport of CAA (see Table I and references therein). However, they differ in their transport properties. Mouse, rat, and human CAT-1, -2A, and -2B isoforms have been characterized in greatest detail. Their transport properties have been investigated in *Xenopus laevis* oocytes, where each carrier can be expressed individually and its activity studied against a low background of endogenous CAA transport. In this model system transport activity has been assayed either using radiolabeled amino acids or measuring amino acid-induced membrane currents by whole cell voltage clamp. Transfection of mammalian cells with expression vectors encoding the various CAT isoforms has also been employed to determine CAT activity. However, in general only a moderate increase in the transport rate of CAA has been observed in mammalian cells, even when using a strong promoter for the expression of CAT encoding cDNAs. It is worth noting that cells cultured *in vitro* usually express at least CAT-1, and hence it is not always feasible to unambiguously distinguish exogenous and endogenous transport activities.

Apparent K_m values for influx of L-arginine reported by different groups for CAT proteins expressed in *Xenopus laevis* oocytes are 70–250 μM for CAT-1, 2–5 mM for CAT-2A, and 38–380 μM for CAT-2B (Kim *et al.,* 1991; Wang *et al.,* 1991; Closs *et al.,* 1993a,b, 1997a; Kakuda *et al.,* 1993; Kavanaugh *et al.,* 1994). K_m values for the cationic amino acids L-lysine and L-ornithine are similar to those reported for L-arginine, suggesting that these three substrates be recognized with similar affinity. However, for CAT-2B an about two- to threefold lower affinity to L-ornithine as compared to L-arginine and L-lysine has been reported (Kakuda *et al.,* 1993; Kavanaugh *et al.,* 1994). The selectivity of CAT-1 for transport of dibasic amino acids is shown by the pH dependence of L-histidine transport (Kim *et al.,* 1991). At pH 7.4, L-histidine is a poor substrate for CAT-1, most likely because at neutral pH most L-histidine is a dipolar amino acid. In contrast, at pH 5.5, when L-histidine is largely protonated, transport rates for L-histidine via CAT-1 are similar to rates measured for L-arginine. Mouse and rat CAT-3 have also been shown to mediate Na^+-independent transport of CAA with the apparent K_m for L-arginine ranging from 40 to 120 μM (Hosokawa *et al.,* 1997; Ito and Groudine, 1997). However, the maximal transport activity (V_{max}) of CAT-3 expressed in *Xenopus laevis* oocytes is considerably lower than that of the aforementioned isoforms (0.02–0.06 versus 1–8 nmol/oocyte/hour; Ito and Groudine, 1997). The reason for this low activity is not readily apparent. In addition, the

Table I Carrier Proteins Involved in the Transport of L-Arginine[a]

Name of protein or cDNA	Gene name	Full-length cDNAs	Transport system[b]	Transport of CAA	Transport of NAA	Reference
CAT proteins						
CAT-1	SLC7A1 (ATRC1)[c]	Human, mouse, rat	y^+	Na^+ independent	Histidine,[d] homoserine, cysteine, Na^+ dependent, very low affinity	Closs *et al.* (1997a), reviews by Closs (1996), MacLeod (1996)
CAT-2A (CAT-2, CAT-2α)[c]	SLC7A2 (ATRC2)[c]	Human, mouse	Low affinity CAA transport, no trans-stimulation	Na^+ independent	n.d.	
CAT-2B (CAT-2, CAT-2β)[c]	SLC7A2 (ATRC2)[c]	Human, mouse	y^+	Na^+ independent	Histidine,[d] homoserine, cysteine, Na^+ dependent, very low affinity	
CAT-3	SLC7A3?	Mouse, rat	y^+ ?	Na^+ independent	?[e]	Hosokawa *et al.* (1997), Ito and Groudine (1997)
CAT-4	SLC7A4	Human	n.d.	(Arg)	n.d.	Sperandeo *et al.* (1998)
Glycoproteins involved in CAA transport activity						
4F2hc (CD98)[c]	SLC3A2	Human, mouse, rat	Dependent on coexpressed associated AT	See associated AT	See associated AT	Review by Palacin *et al.* (1998)
rBAT (NBAT, D2)[c]	SLC3A1	Human, rat, rabbit	$b^{0,+}$ (with $b^{0,+}$AT)	Na^+ independent[f]	Na^+ independent, broad substrate specificity including cysteine, obligatory exchange[f]	
4F2hc (CD98)-associated carriers						
y^+LAT1	SLC7A7	Human, mouse	y^+L	Na^+ independent	E.g., leucine and other large NAA, high affinity, Na^+ dependent, obligatory exchange	Torrents *et al.* (1998), Pfeiffer *et al.* (1999)
y^+LAT2	SLC7A6	Human, mouse	y^+L	Na^+ independent	E.g., leucine and other large NAA, high affinity, Na^+ dependent	Torrents *et al.* (1998), Pfeiffer *et al.* (1999)
4F2-lc6	SLC7A?	Rabbit	$b^{0,+}$	Na^+ independent	High affinity for NAA and cysteine, Na^+ independent	Rajan *et al.* (1999)
rBAT (NBAT, D2)-associated carriers						
$b^{0,+}$AT	SLC7A9	Human	$b^{0,+}$			Feliubadalo *et al.* (1999)
Na^+- and Cl^--dependent carriers						
$ATB^{0,+}$	SLC6A?	Human	$B^{0,+}$	Na^+ and Cl^- dependent	Na^+ and Cl^- dependent, broad substrate specificity, low affinity for proline	Sloan and Mager (1999)

[a] This table lists the carrier proteins identified to date at the molecular level that are involved in the transport of CAA. These carrier proteins belong to three different gene families and were largely characterized in transport studies in *Xenopus laevis* oocytes expressing the respective carrier protein.

[b] For references for the transport systems, see review by Deves and Boyd (1998).

[c] Names given in parentheses refer to alternative names also found in the literature.

[d] Histidine transport by CAT-1 and CAT-2B is only partially Na^+ dependent, because a small fraction of this amino acid is protonated at neutral pH and therefore behaves like CAA.

[e] Interaction of NAA with CAT-3 has been investigated by inhibition studies of L-arginine uptake conducted in COS cells and *Xenopus laevis* oocytes. Hosokawa *et al.* (1997, using $\sim 10^4$-fold excess competitor) found no cross-inhibition by any of the neutral or acidic amino acids except for L-citrulline. In contrast, Ito and Groudine (1997), using $\sim 10^6$-fold excess competitor, detected Na^+-independent inhibition by L-methionine, L-cysteine, as well as L-aspartic and L-glutamic acid.

[f] Transport properties have only been characterized for rBAT expressed in *Xenopus laevis* oocytes where it forms heterodimers with an endogenous protein. Transport studies on rBAT coexpressed with the newly identified $b^{0,+}$AT have not yet been reported.

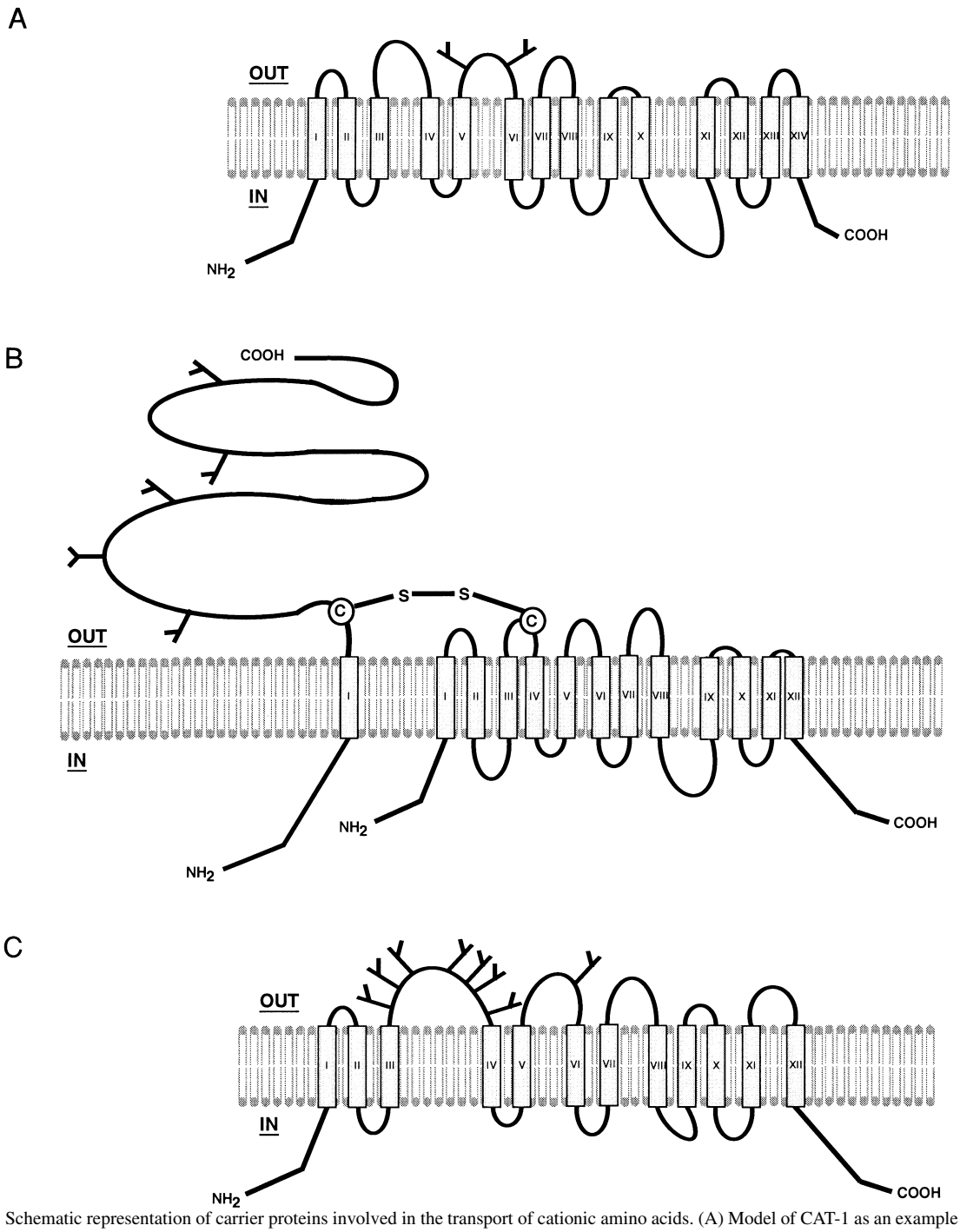


Figure 3 Schematic representation of carrier proteins involved in the transport of cationic amino acids. (A) Model of CAT-1 as an example for the CAT proteins. The members of the CAT family are glycosylated proteins with 14 putative transmembrane domains (TMs). Alternative models suggesting 12 or 13 TMs have also been proposed. Two asparagine residues in the third extracellular loop (indicated as branched lines) have been shown to be glycosylated in mCAT-1. These sites are conserved in CAT-2A and 2-B. In addition, there is one putative glycosylation site in the second extracellular loop of CAT-2A and 2-B. In contrast, only one consensus site for N-glycosylation located in the third extracellular loop is found in CAT-3. The three putative glycosylation sites of CAT-4 are located in the second, third, and sixth extracellular loop. (B) Model of 4F2hc and y^+LAT-1 as an example for the heterodimeric cationic amino acid carriers. 4F2hc and rBAT are distantly related glycoproteins. Only one typical α-helical TM is found in 4F2hc and rBAT, suggesting they are type II membrane proteins with an intracellular N terminus and an extracellular C terminus. An alternative model with three additional amphipathic TMs and an intracellular C terminus has been proposed for rBAT (Mosckovitz *et al.,* 1994). Cys^{109} in human 4F2hc has been shown to form a sulfur bridge with y^+LAT-1. This residue is conserved in rBAT. The associated carrier proteins (y^+LAT-1, -2, and 4F2-lc6 for 4F2hc and $b^{0,+}$AT for rBAT, respectively) are predicted to span the membrane 12 times. Although they are distantly related to the CAT proteins, these carrier proteins are not glycosylated and require an association with glycoproteins to translocate to the plasma membrane. A cysteine residue in the second extracellular loop conserved in y^+LAT-1, -2, and $b^{0,+}$AT has been proposed to form a sulfur bridge with 4F2hc and rBAT, respectively. (C) Model of $ATB^{0,+}$. $ATB^{0,+}$ belongs to the family of Na^+/Cl^--dependent neurotransmitter transporters. It contains 12 putative TMs and eight consensus sites for N-glycosylation in the second and third putative extracellular loop.

Table II Sequence Comparison between the Human CAT Proteins[a]

	hCAT-1	hCAT-2A	hCAT-2B	hCAT-3[b]	hCAT-4
hCAT-1	—	**60**	**62**	**61**	**41**
hCAT-2A	54	—	**96**	**58**	**42**
hCAT-2B	83	50	—	**59**	**41**
hCAT-3	71	52	71	—	**42**
hCAT-4	51	52	43	43	—

[a] This table compares the amino acid sequences of the five human CAT isoforms identified to date. Boldface numbers list the percent identity after an optimal alignment of the complete amino acid sequence. The numbers not in bold face list the percent identity after an optimal alignment of the short amino acid sequence that differs between CAT-2A and CAT-2B. Sequences were aligned using the NCBI BLASTP program.

[b] hCAT-3 sequence from N. Vékony and E. I. Closs, unpublished data.

substrate selectivity of CAT-3 seems to differ somewhat from that reported for the other CAT isoforms. The transport properties of CAT-4 have not yet been investigated.

In addition to the Na^+-independent CAA transport mediated by CAT-1 and CAT-2B, these carriers also mediate Na^+-dependent transport of some NAA, but with rather low affinity (Wang *et al.,* 1991; Kakuda *et al.,* 1993). CAT-3-mediated L-arginine transport is competitively inhibited not only by other CAA but also by L-citrulline, D-arginine (Hosokawa *et al.,* 1997), L-methionine, L-cysteine, and even L-aspartate and L-glutamate, but not by L-homoserine (Ito and Groudine, 1997). In contrast to CAT-1 and -2B, recognition of NAA by CAT-3 has been found to be Na^+ independent. However, in these studies potential competitor amino acids were used at 10^4 to 10^6 higher concentrations than the substrate, and transport inhibition was only 50–75%, raising the question whether these putative inhibitor interactions were specific.

The CAT proteins differ also in their sensitivity to *trans*-stimulation. Whereas the activities of CAT-1 and -2B are stimulated by physiological concentrations of substrate at the *trans*-side of the plasma membrane (100–250 μM), transport mediated by CAT-2A is largely independent of the presence of substrate at the *trans*-side of the membrane (Closs *et al.,* 1993b, 1997b). The most pronounced *trans*-stimulation has been observed for CAT-1. In the case of mCAT-3, *trans*-stimulation was about twofold at a *trans*-membrane substrate concentration of 10 mM compared to zero substrate concentration (Ito and Groudine, 1997).

For mCAT-1 a voltage dependence of CAA transport has been described; membrane hyperpolarization increases the V_{max} and decreases the apparent K_m for influx, and it decreases the V_{max} and increases the apparent K_m for efflux (Kavanaugh, 1993). Changes in the membrane potential may thus result in changes in the transport rate for L-arginine, as evidenced by numerous radiotracer flux studies in cultured cells (Bogle *et al.,* 1991; Bussolati *et al.,* 1993; Sobrevia *et al.,* 1995; Zharikov *et al.,* 1997). The voltage dependence of the other CAT isoforms has not yet been investigated.

The transport properties of CAT-1, -2B, and -3 are consistent with those originally attributed to the classic system y^+. In contrast, CAT-2A is a low-affinity carrier for CAA that is relatively insensitive to *trans*-stimulation. It is remarkable that CAT-2A and -2B demonstrate such divergent transport properties, even though their amino acid sequences are 96% identical and differ only in a stretch of 42 amino acids. According to the 14 transmembrane model, this protein domain is located in the fourth intracellular loop. Replacement of the corresponding region of mCAT-1 by that of mCAT-2A or -2B and vice versa leads to chimeric proteins with transport properties of the donor of that region (including the apparent affinity for L-arginine and sensitivity to *trans*-stimulation; Closs *et al.,* 1993a). Interestingly, in that region the three isoforms exhibiting similar transport properties (CAT-1, -2B, and -3) also show the highest percentage of amino acid sequence identity (Table II).

CAT Expression in NO-Producing Cells

System y^+ seems to be the major transport system for L-arginine in most NO-producing cells. In addition, the activity of system y^+ has been found to increase in response to exogenous stimuli such as LPS, IL-1β, TNF-α, insulin, angiotensin II, and bradykinin in a variety of cell types (Bogle *et al.,* 1991, 1992b; Lind *et al.,* 1993; Durante *et al.,* 1995, 1996; Pan *et al.,* 1995; Schmidlin and Wiesinger, 1995; Wileman *et al.,* 1995; Cendan *et al.,* 1996). Recent findings suggest that this is, at least in part, due to a differential induction of CAT gene expression in these cells (see Table III and references therein). In most studies the expression of CAT-1, -2A, and -2B mRNA has been examined, whereas only little information is available for the more recently discovered isoforms CAT-3 and -4. CAT-1 expression has been found in all cell types investigated, which is consistent with the presence of system y^+ transport activity deduced from radiotracer flux experiments (Table III and references therein). CAT-2B expression has been shown in macrophages and glial cells activated with LPS or LPS and IFN-γ (Closs *et al.,* 1993b, 1999; Stevens *et al.,* 1996; Kakuda *et al.,* 1999), in vascular smooth muscle cells (VSMC) induced

Table III Expression of the Carrier Proteins Involved in the Transport of L-Arginine[a]

Carrier	Constitutive expression	Upregulation of expression	References
CAT proteins			
CAT-1	Tissues: ubiquitous, except for liver, less abundant in skeletal and heart muscle, lung, enriched in microvessels of the blood–brain barrier Cells in culture: ubiquitous, including primary hepatocytes	Liver: dexamethasone, insulin, arginine, low protein, regeneration (transient) Many cell types: cell proliferation Vascular smooth muscle cells (VSMC): angiotensin II, PDGF, LPC, LPS + IFN-γ Cardiac myocytes: insulin, IL-1β + IFN-γ	Kim *et al.* (1991), Yoshimoto *et al.* (1992), Closs *et al.* (1993c), Stoll *et al.* (1993), Wu *et al.* (1994), Low and Grigor (1995), Durante *et al.* (1996, 1997, 1998), Simmons *et al.* (1996), Baydoun *et al.* (1999), Hattori *et al.* (1999)
CAT-2A (CAT-2, CAT-2α)[b]	Tissues: liver, less abundant in skeletal muscle, skin[c] Cells in culture: hepatoma cells, primary hepatocytes, cardiac myocytes, VSMC	Skeletal muscle: splenectomy, hepatectomy, starvation VSMC: LPS + IFN-γ Cardiac myocytes: IL-1β + IFN-γ	Closs *et al.* (1993c), Simmons *et al.* (1996), Kakuda *et al.* (1998), Baydoun *et al.* (1999)
CAT-2B (CAT-2, CAT-2β)	Tissues: skin,[c] uterus,[c] testis,[c] lung[c] Cells in culture: SL12.4 T-lymphoma, fibroblasts, cardiac myocytes, VSMC, HUVEC, and others	T lymphocytes: concanavalin A Macrophages: LPS Astroglia: LPS + IFN-γ VSMC: platelet-derived growth factor,[d] IL-1β + TNF-α,[d] LPC,[d] thrombin,[d] LPS + IFN-γ HUVEC: TNF-α	MacLeod *et al.* (1990), Closs *et al.* (1993a), Durante *et al.* (1996, 1997, 1998), Gill *et al.* (1996), Stevens *et al.* (1996), Baydoun *et al.* (1999), Hattori *et al.* (1999), Kakuda *et al.* (1999), Irie *et al.* (1997), Mann *et al.* (1998)
CAT-3	Tissues: early embryo, mesoderm; mid streak stage embryo, ubiquitous; adult, brain	n.d.[e]	Hosokawa *et al.* (1997), Ito and Groudine (1997)
CAT-4	Tissues: brain, testis, placenta	n.d.	Sperandeo *et al.* (1998)
Glycoproteins involved in CAA transport activity			
4F2hc (CD98)[b]	Tissues: ubiquitous	Placenta: highest in last third of gestation	Parmacek *et al.* (1989), Novak *et al.* (1997)
rBAT (NBAT, D2)[b]	Tissues: kidney (epithelial cells of proximal straight tubules), small intestine (microvilli) Cells in culture: OK renal tubular proximal cells	Lymphocytes and other cell types: cell proliferation Onset of expression: postnatal	Kanai *et al.* (1992), Furriols *et al.* (1993), Pickel *et al.* (1993), Mora *et al.* (1996)
4F2hc (CD98)[b]-associated carrier			
y^+LAT1	Tissues: kidney cortex, small intestine, lung, less abundant in liver, placenta, spleen Cells: peripheral blood leukocytes	n.d.	Torrents *et al.* (1998), Pfeiffer *et al.* (1999)
y^+LAT2	n.d.	n.d.	
4F2-lc6	Tissues: kidney, small intestine	n.d.	Rajan *et al.* (1999)
rBAT (NBAT, D2)[b]-associated carrier			
$b^{0,+}$AT	Tissues: kidney, liver, small intestine, placenta	n.d.	Feliubadalo *et al.* (1999)
Na^+- and Cl^+-dependent carrier			
$ATB^{0,+}$	Tissues: lung, salivary gland, mammary gland, pituitary gland, stomach	n.d.	Sloan and Mager (1999)

[a] This table summarizes data based on studies of the expression of the respective mRNAs and where available the protein.
[b] Names given in parentheses refer to alternative names reported in the literature.
[c] mRNA was only detected by RT-PCR and not by less sensitive methods like RNase protection analysis or Northern blots (MacLeod *et al.,* 1990).
[d] In these studies, no distinction between CAT-2A and CAT-2B expression has been made. We assume here that it is the high affinity isoform (CAT-2B) that was induced.
[e] n.d.: not determined.

either with IL-1β and TNF-α (Gill *et al.,* 1996), with lysophosphatidylcholine (LPC; Durante *et al.,* 1997), or with LPS and IFN-γ (Baydoun *et al.,* 1999; Hattori *et al.,* 1999), in cardiac myocytes and cardiac microvascular endothelial cells (CMEC) exposed to IL-1β and IFN-γ (Simmons *et al.,* 1996), and in HUVEC stimulated with TNF-α (Irie *et al.,* 1997). In most cell types a significant expression of CAT-2B is found only after cytokine or LPS treatment. CAT-2B has therefore been referred to as the inducible CAT isoform. It is generally coexpressed with the more constitutive CAT-1 isoform. However, expression of CAT-1 can also be induced in response to external stimuli such as angiotensin II (Low and Grigor, 1995), platelet-derived growth factor (Durante *et al.,* 1996), LPS combined with IFN-γ (Baydoun *et al.,* 1999; Hattori *et al.,* 1999), and it can be induced moderately by LPC (Durante *et al.,* 1997) in VSMC, by insulin, IL1-β, and IFN-γ in cardiac myocytes, by amino acid starvation or glucocorticoids and insulin in hepatoma cells (Hyatt *et al.,* 1997; Liu and Hatzoglou, 1998), and in hepatocytes *in vivo* by dexamethasone, insulin, L-arginine, low protein diet, and regeneration of the liver (Wu *et al.,* 1994; Aulak *et al.,* 1996). In addition, CAT-1 expression has been shown to increase with cell proliferation in a variety of cell types (Yoshimoto *et al.,* 1992). The induction of CAT-1 mRNA has been shown to occur at both the transcriptional and posttranscriptional levels. In amino acid-starved FAO hepatoma cells and in regenerating liver, CAT-1 mRNA is induced without change of the transcription rate (Aulak *et al.,* 1996; Hyatt *et al.,* 1997), whereas the induction of CAT-1 by glucocorticoids and insulin in quiescent liver is due to both an increase in the transcription rate and stabilization of the mRNA (Liu and Hatzoglou, 1998).

The expression of CAT-2B is often induced together with NOS II, the inducible isoform of NO synthase. The signaling pathways leading to induction of CAT expression are at least in part distinct from those leading to induction of NOS II (see Fig. 4). In VSMC activation of p38 mitogen-activated protein kinase (p38 MAPK) represents an important signaling mechanism, regulating both enhanced L-arginine transport and induced NO synthesis, whereas protein tyrosine kinases (PTK) appear to be involved in the induction only of NOS II and not of CAT expression (Baydoun *et al.,* 1999). In RAW 264 macrophages, LPS but not IFN-γ induces CAT-2B expression, whereas NOS II is induced with similar efficacy by both compounds (E. I. Closs, unpublished observation). Surprisingly, expression of the low-affinity carrier CAT-2A, in addition to CAT-1 and -2B, has been found in cardiomyocytes and CMEC stimulated with IL-1β and IFN-γ (Simmons *et al.,* 1996), as well as in VSMC treated with LPS and IFN-γ (Baydoun *et al.,* 1999). The physiological role of a carrier with a K_m for cationic amino acids ≥2 m*M* is not evident in cells that are normally exposed to plasma with a CAA concentration of about 0.2 m*M*. The expression of CAT-3 is found in mesoderm and in many developing tissues of mid streak mouse embryos, but it seems to be confined to the brain in adult animals (Hosokawa *et al.,* 1997; Ito and Groudine, 1997). Studies in rat brain provide evidence for the localization of CAT-3 mRNA in specific neurons but not glial or endothelial cells (Hosokawa *et al.,* 1999; Braissant *et al.,* 1999). In contrast, CAT-1 presents ubiquitous neuronal and glial expression (Braissant *et al.,* 1999), and a study in neuronal cultures derived from rat hypothalamus/brain stem attributed basal rates of L-arginine transport principally to CAT-1 activity (Stevens and Vo, 1998). Similar to CAT-3, CAT-4 is expressed in the brain but also in testis and placenta (Sperandeo *et al.,* 1998). The cell type(s) expressing CAT-4 in these tissues has not yet been identified.

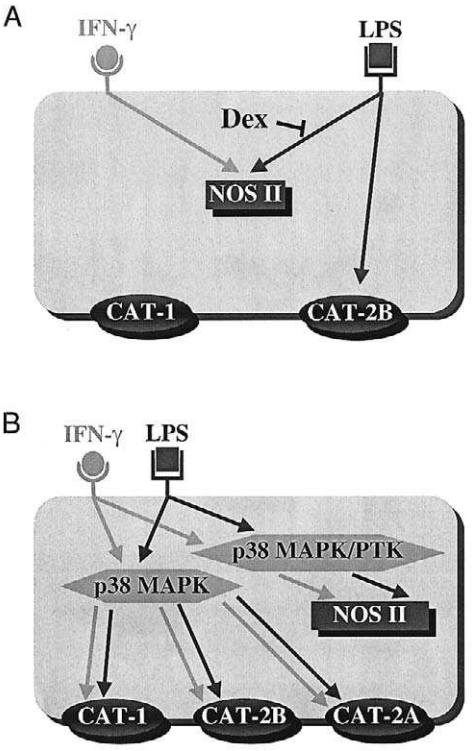


Figure 4 Differential expression of cationic amino acid transporters and NOS II in different cell types. (A) Macrophages. In RAW and J774 murine macrophages exposure to lipopolysaccharide (LPS), but not γ-interferon (IFN-γ), leads to an increase in L-arginine transport activity (Bogle *et al.,* 1992b; Baydoun and Mann, 1994) and an induction of CAT-2B expression (Closs *et al.,* 1993a, 1999; Kakuda *et al.,* 1999). In contrast, both LPS and IFN-γ induce the expression of NOS II. Dexamethasone (Dex) inhibits the induction of NOS II but not of CAT-2B. CAT-1 expression is detected in unstimulated macrophages and does not change in response to LPS or IFN-γ. (B) Vascular smooth muscle cells. In rat cultured vascular smooth muscle cells CAT-1, -2A, and -2B are expressed under basal conditions. The expression of all three isoforms, as well as NOS II, is increased on exposure of the cells to LPS and IFN-γ, with a concomitant increase in L-arginine transport and NO release (Wileman *et al.,* 1995; Baydoun *et al.,* 1999). Activation of p38 mitogen-activated protein kinase (p38 MAPK) represents an important signaling mechanism, regulating both enhanced CAT and NOS II expression, whereas protein tyrosine kinases (PTK) are involved in the induction of NOS II but not of CAT expression (Baydoun *et al.,* 1999).

In contrast to mRNA expression, little information is available concerning protein expression of the CAT. In most cell types investigated to date the increase in L-arginine transport in response to external stimuli is considerably less than the observed increase in CAT mRNA expression. This suggests either that the mRNA is not translated into protein

or that a large portion of the newly synthesized carriers is not active. Evidence for a silent system y^+-like carrier comes from studies in rabbit and rat peritoneal macrophages, where the transport rate for L-arginine can be increased up to 10-fold by a treatment with the protein kinase C (PKC) activating phorbol ester [phorbol-12-myristate-13-acetate (PMA)] without the need for protein synthesis (Racke *et al.,* 1998). We have found that the activity of CAT-1 decreased in response to PMA with no change in the protein expression both in *Xenopus laevis* oocytes and in human endothelial cells, suggesting that different PKC isoforms might have a differential effect on the CAT activity (P. Gräf, H. Li, U. Förstermann, and E. I. Closs, unpublished data). To address the question of whether CAT activity is under posttranslational control, the expression and subcellular localization, as well as possible posttranslational modifications of the CAT-proteins, will need to be investigated.

Substrate Supply for NOS by the CAT Proteins

As CAT-2B is often induced together with NOS II, it has generally been assumed that CAT-2B specifically delivers L-arginine to NOS II. However, no direct evidence has been provided in support of this hypothesis. A specific role for CAT-1 in providing substrate for the endothelial isoform of NOS (NOS III) has been proposed, based on immunostaining of porcine endothelial cells, which suggests a colocalization of CAT-1, NOS III, and caveolin-1 in endothelial caveoli (McDonald *et al.,* 1997b). In support of this hypothesis, we found that the NOS III activity was decreased in human endothelial cells after PMA-induced downregulation of L-arginine transport (P. Gräf, H. Li, U. Förstermann, and E. I. Closs, unpublished data). However, the effect of PMA on transport was much greater than its effect on NOS activity, demonstrating that NOS III is still active, even when L-arginine transport was reduced to 5 to 10% of the control transport rate. In contrast to inducing CAT-1 and CAT-2B mRNA expression and L-arginine transport in smooth muscle cells, LPC has been reported to inhibit the high-affinity component of the L-arginine transport (presumably CAT-1) in aortic endothelial cells (Kikuta *et al.,* 1998). LPC also inhibited NO release induced by ADP in these cells, and this inhibition could be attenuated by excess extracellular L-arginine, suggesting that the impairment of CAT-1 might account for the inhibition of NOS III by LPC. Functional knockouts of individual CAT isoforms will be necessary to elucidate the function of each isoform in supplying the substrate L-arginine for NOS. A germ-line null mutation has been introduced into the mCAT-1 gene by targeted mutagenesis of embryonic stem cells (Perkins *et al.,* 1997). The homozygous knockout mice die on day 1 after birth, exhibit a 25% reduction in size compared to the wild-type littermates, and suffer from severe anemia. Unfortunately, no information is available on the activity of NOS in the different cell types of these mice.

Besides transporting L-arginine, CAT proteins also mediate the transport of some L-arginine analogs. In agreement with earlier studies on system y^+ (Baydoun and Mann, 1994; Schmidt *et al.,* 1994), the substrate specificity of the CAT proteins for L-arginine analogs differs markedly from that of the inducible NOS II (Closs *et al.,* 1997b). Thus, there are some NOS inhibitors that are recognized and transported by the CAT proteins [e.g., L-NMMA, L-N^5-(1-iminoethyl)-ornithine (L-NIO) or asymmetrical N^G,N'^G-dimethyl-L-arginine (L-ADMA)], whereas others such as L-NNA and its methyl ester N^G-nitro-L-arginine methyl ester (L-NAME) are not. The latter neutral arginine analogs enter most cell types via broad-specificity NAA transport systems (Baydoun and Mann, 1994; Schmidt *et al.,* 1994). In contrast, some L-arginine analogs are transported by the CAT proteins [e.g., symmetrical N^G,N^G-dimethyl-L-arginine (L-SDMA), and α-amino-δ-isothioureidovaleric acid (AITV)], but they are not recognized by NOS and do not inhibit NOS activity directly. The different behavior of these arginine derivatives with respect to CAT-mediated transport is most likely due to differences in the charge of their side chain. Because of *trans*-stimulation, L-arginine analogs that are CAT substrates not only compete with L-arginine for influx, but also stimulate efflux of intracellular L-arginine and other cationic substrates. Therefore, compounds such as L-SDMA or AITV, which do not interfere with the NOS enzyme directly but limit substrate availability, may indirectly inhibit NOS activity in intact cells (see review by Closs and Gräf, 1999). However, as circulating levels of L-SDMA are only within the micromolar range, their inhibitory effects may well be restricted to system y^+L.

Heterodimeric Carrier Proteins for CAA

Structure and Function of the Heterodimeric Carrier Proteins

Transport activities resembling systems $b^{0,+}$ and y^+L are mediated by heterodimeric proteins consisting of two unrelated moieties: a glycosylated protein with a structure not typical for carrier proteins and a typical multimembrane spanning carrier protein (see Fig. 3). The first glycoprotein identified to be involved in amino acid transport was rBAT (related to $b^{0,+}$ amino acid transporter; for alternative names, see Table I and review by Palacin *et al.,* 1998). cDNAs encoding rBAT have been cloned by different groups from rabbit, rat, and human by functional expression of kidney mRNA in *Xenopus laevis* oocytes (see review by Palacin *et al.,* 1998, and references therein). rBAT expression in oocytes leads to a significant increase in the Na^+-independent high-affinity transport of CAA, NAA, and cystine, a transport activity resembling system $b^{0,+}$, except that the latter does not mediate the transport of cystine. Mutations in the human rBAT gene leading to a defective or missing rBAT protein have been shown to be the cause for cystinuria type I, a recessive inherited disease with defect in renal and intestinal reabsorption of cystine and CAA (see review by Palacin *et al.,* 1998). The involvement of a Na^+-independent transporter in epithelial reabsorption seems surprising. It can be explained, however, by the fact that the transport activity induced by rBAT is an obligatory exchanger of NAA and CAA (see Fig. 5). It has therefore been postulated that system $b^{0,+}$/rBAT represents a tertiary active transport in the

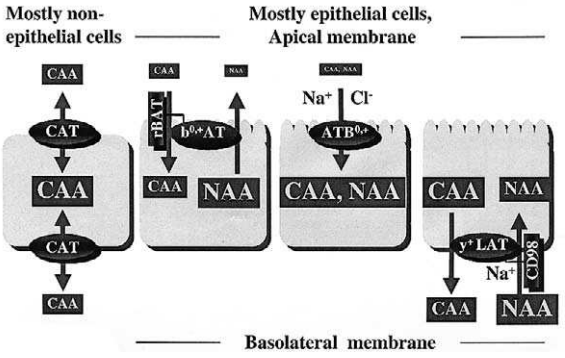


Figure 5 Transport characteristics of carrier proteins for cationic amino acids. The CAT proteins mediate facilitated diffusion of CAA with very low affinity for neutral amino acids (NAA, not shown). CAT-1, -2B, and -3 exhibit a high affinity for CAA and are sensitive to *trans*-stimulation (system y^+-like carriers). In contrast, CAT-2A is a low-affinity CAA carrier that is relatively insensitive to *trans*-stimulation. One or more CAT isoforms are expressed in nearly all cell types. rBAT most likely forms a heterodimer with $b^{0,+}$AT, mediating the obligatory exchange of CAA, cystine, and NAA, reminiscent of the transport activity assigned to system $b^{0,+}$. Owing to the negative membrane potential and the high intracellular concentration of NAA (which is due to Na^+-dependent NAA carriers), it is proposed that CAA (and cystine, not shown) are preferentially transported into the cell in exchange for NAA (tertiary active transport). rBAT and presumably $b^{0,+}$AT are localized at the apical membrane of epithelial cells in the kidney and small intestine. $ATB^{0,+}$ transports CAA and NAA in a Na^+- and Cl^--dependent manner (system $B^{0,+}$-like activity), and it is predominantly expressed in lung and salivary gland, but also in mammary gland, pituitary gland, and stomach. System $B^{0,+}$ has been shown to be located in the apical membrane of epithelial cells, and thus an apical localization also seems likely for $ATB^{0,+}$. 4F2hc builds a heterodimer with y^+LAT-1 and y^+LAT-2 to form a transport system (y^+L) that mediates Na^+-independent transport of CAA and Na^+-dependent transport of NAA. Similar to system $b^{0,+}$, y^+L is also an obligatory exchanger. As a result of the Na^+ gradient encountered under physiological conditions, NAA are preferentially transported into the cell across the basolateral membrane in exchange for intracellular CAA. 4F2hc has been shown to be located at the basolateral membrane of epithelial cells in the kidney and small intestine. Therefore, system y^+L seems to function as an export pathway for CAA, allowing the *trans*-epithelial flux of CAA in concert with transport systems operative in the apical membrane mediating accumulative uptake into the cell. The 4F2hc/4F2-lc6 heterodimer, which is also expressed in small intestine and kidney and mediates $b^{0,+}$-like activity (Rajan *et al.,* 1999), is not shown.

epithelial cells, where NAA are concentrated by Na^+-dependent carriers (secondary active transport), leading to high intracellular concentrations of NAA that are then exchanged against extracellular CAA and cystine (Busch *et al.,* 1994; Chillaron *et al.,* 1996). In addition, the uptake of CAA and cystine is favored because of the negative membrane potential and the intracellular reduction of cystine to cysteine.

Owing to homology to rBAT, the amino acid transport activity of a second glycoprotein (4F2hc or CD98), originally identified as a tumor antigen on lymphoblastoid cells, was assayed (Bertran *et al.,* 1992; Wells *et al.,* 1992). Expression of 4F2hc in *Xenopus laevis* oocytes leads to an increase in high-affinity transport of CAA (Na^+-independent) and NAA (Na^+-dependent), resembling transport activity attributed to system y^+L. Interestingly, Bröer and coworkers (1995) demonstrated that in rat C6 glioma cells 4F2hc is an essential component of system L, a Na^+-independent system for large NAA, suggesting that 4F2hc might be involved in different transport activities.

Although both rBAT and 4F2hc induced amino acid transport activity when expressed alone in *Xenopus laevis* oocytes, there is convincing evidence that neither serves as a carrier protein but that each associates with a second protein to build the functional transporter. First, both proteins are predicted to span the membrane only once or, in an alternative model, four times (Mosckovitz *et al.,* 1994); both configurations are unusual structures for a carrier protein. Second, the transport activity of rBAT in *Xenopus laevis* oocytes does not correlate with the amount of protein expressed in the plasma membrane, suggesting that a second protein may be limiting (Estevez *et al.,* 1998). Third, both proteins have been shown to build heterodimers with non-glycosylated smaller proteins, referred to as light chains of the glycoproteins (see review by Palacin *et al.,* 1998). cDNAs encoding these smaller proteins have now been cloned. All proteins of this newly identified group are predicted to have 12 transmembrane domains (TMs) (see Fig. 3) and are distantly related to the CAT proteins; with amino acid sequences aligned optimally, identity lies in the range of 20%. Other than the CAT proteins, these are not glycosylated, and they localize to the plasma membrane and exhibit transport activity only when coexpressed with the respective glycoprotein. A conserved cysteine residue in the second putative extracellular loop is likely to form a disulfide bond with a conserved cysteine residue in rBAT and 4F2hc (Cys^{109} and Cys^{103} of human and mouse 4F2hc, Torrents *et al.,* 1998; Nakamura *et al.,* 1999). The first light chain (named LAT-1) was identified by independent groups in human, rat, and mouse. It mediates a system L-like activity when coexpressed with 4F2hc (Kanai *et al.,* 1998; Mastroberardino *et al.,* 1998; Nakamura *et al.,* 1999; Prasad *et al.,* 1999). More recently, five additional light chains were reported to mediate the activity of system L (LAT-2), y^+L (y^+LAT-1 and y^+LAT-2), x_C^- (xCT, an exchanger for anionic amino acids, e.g., cystine and glutamate), and surprisingly $b^{0,+}$ when associated with 4F2hc (Torrents *et al.,* 1998; Pfeiffer *et al.,* 1999; Pineda *et al.,* 1999; Sato *et al.,* 1999; Segawa *et al.,* 1999; Rajan *et al.,* 1999). Mutations in the human y^+LAT-1 gene (SLC7A7) segregate with lysinuric protein intolerance (LPI), a heritable disorder associated with a defect of CAA transport in the basolateral membrane of epithelial cells in small intestine and renal tubules (Borsani *et al.,* 1999; Torrents *et al.,* 1999). Transport by 4F2hc/y^+LAT-1 is strongly *trans*-stimulated, suggesting that this transport agency is also an obligatory exchanger. As the transport of NAA is Na^+ dependent, NAA can only be transported into the cell due to the Na^+ gradient (Fig. 5). Very recently, a light chain associating with rBAT, named $b^{0,+}$AT, has been reported (Feliubadalo *et al.,* 1999). Consistent with system $b^{0,+}$, coexpression of rBAT/$b^{0,+}$AT leads to a Na^+-independent transport activity for NAA and CAA. Therefore, both rBAT and 4F2hc seem to mediate system $b^{0,+}$ activity when associated

with the respective light chain (rBAT/$b^{0,+}$AT and 4F2hc/4F2-lc6, respectively). Mutations in the human gene for $b^{0,+}$AT (SLC7A9) segregate with non-type I cystinuria.

Expression and Physiological Role of the Heterodimeric Carrier Proteins Involved in CAA Transport

Much more information is available concerning the expression and function of the glycoproteins rBAT and 4F2hc than of the only recently discovered proteins that associate with them. rBAT mRNA has been shown to be expressed in small intestine and the proximal straight tubules of the kidney (see review by Palacin *et al.,* 1998). Ultrastructural analysis showed that rBAT localizes to the brush-border membrane of epithelial cells, which is consistent with its role in reabsorption of CAA and cystine (Pickel *et al.,* 1993). Furthermore, the observation that mutations of both rBAT and its associated protein $b^{0,+}$AT cause cystinuria (rBAT, type I cystinuria; $b^{0,+}$AT, non-type I cystinuria) is consistent with this function. Transcripts hybridizing with rBAT cDNA probes but larger than the mRNAs found in kidney and small intestine have been detected in brain and with lower abundance in many other tissues. It is not clear whether these transcripts code for rBAT or for a homologous protein. However, in these tissues, antibodies directed against rBAT react with structures located intracellularly rather than in the plasma membrane. As rBAT immunoreactivity has been localized to neurons that also express the neuronal isoform of NOS (NOS I), a role of rBAT in providing substrate for NOS I has been postulated (Pickel *et al.,* 1999). However, there are no functional data available to support this hypothesis. It would be interesting to establish whether there is an impairment of NOS I activity in patients with cystinuria as a result of restricted substrate availability.

In contrast to rBAT, 4F2hc is expressed in many cell types, including NO-producing cells. Like CAT-1, expression of 4F2hc is increased in proliferating cells and activated T cells (see review by Parmacek *et al.,* 1989). In addition to amino acid transport, a role of 4F2hc in other processes such as cell fusion and aggregation as well as cell survival has been postulated (see review by Palacin *et al.,* 1998). As the function of 4F2hc as an amino acid transporter is determined by its associated carrier, the identification of the respective light chain can provide the only insight into which transport system it might form in a given cell. In order to transport CAA, 4F2hc has to couple to y^+LAT-1 or y^+LAT-2 (to form system y^+L), or to 4F2-lc6 (to form system $b^{0,+}$). y^+LAT-1 is preferentially expressed in the renal cortex but also in small peripheral blood mononuclear cells, intestine, lung, and to a lesser extent in liver, placenta, and spleen. 4F2hc/y^+LAT-1 is assumed to serve preferentially as an export mechanism for CAA at the basolateral membrane of epithelial cells (see Fig. 5). Consistent with this hypothesis is the basolateral localization of 4F2hc in renal epithelial cells and the involvement of y^+LAT-1 mutations in LPI. The role of 4F2hc/y^+LAT-1 or 4F2hc/y^+LAT-2 in nonepithelial, for example, NO-producing cells, is not yet understood. The investigation of NO synthesis in cells from LPI patients should shed light on whether 4F2hc/y^+LAT-1 is important for providing substrate for NOS enzymes in specific cell types. 4F2-lc6 is predominantly expressed in kidney and small intestine and it has been suggested that it might be a new candidate gene for cystinuria (Rajan *et al.,* 1999). However, the subcellular localization of 4F2hc/4F2-lc6 in epithelial cells has not yet been investigated.

$ATB^{0,+}$

A cDNA encoding a Na^+- and Cl^--dependent carrier ($ATB^{0,+}$) capable of transporting CAA has been isolated on the basis of sequence homology with neurotransmitter transporters (Sloan and Mager, 1999). $ATB^{0,+}$ also mediates the Na^+/Cl^--dependent transport of NAA with broad substrate specificity and exhibits high affinity for both CAA and NAA. $ATB^{0,+}$ belongs to the family of Na^+ and Cl^--dependent neurotransmitter transporters, including the transporter for betaine, taurine, serotonin, dopamine, glycine, and proline. Like all members of the family, it is predicted to span the membrane 12 times (see Fig. 3). $ATB^{0,+}$ contains multiple consensus sites for N-linked glycosylation. Its expression is most abundant in lung and salivary gland, but it can also be found in mammary gland, stomach, and pituitary gland (Sloan and Mager, 1999). As CAA transport in NO-producing cells is mostly Na^+-independent, $ATB^{0,+}$ is not likely to play an important role in modulating substrate supply for NOS enzymes.

Conclusions

Mammalian cells are able to exchange cationic amino acids with the environment, and hence inhibition of CAA transport processes by competitor substrates may well affect intracellular metabolism of CAA, such as synthesis of NO. As L-arginine shares the same carrier proteins with other CAA, changes in carrier expression and/or activity will affect the transport of not only L-arginine and other CAA such as L-lysine and L-ornithine, but also cationic L-arginine analogs, including the naturally occurring symmetrical and asymmetrical dimethyl-L-arginine and some NOS inhibitors. Even substrates that have no direct effect on the activity of NOS may influence NOS activity in intact cells by competing with L-arginine for the same carrier proteins and changing the intracellular L-arginine concentration. Thus, the design of L-arginine analogs, targeted to individual CAA carrier proteins, could be exploited to specifically inhibit NOS in a given cell type where the respective carrier protein is crucial for the substrate supply for NO synthesis, without affecting the activity of NOS in other cell types. System y^+ seems to be the major transport agency for L-arginine in NO-producing cells, and the carrier proteins mediating system y^+ activity (CAT-1, -2B, and -3) are differentially expressed in these cells. Hence, the CAT proteins seem to be good candidates for the manipulation of substrate supply for NOS.

However, the involvement of other specific CAA carrier systems (b^+, y^+L, $b^{0,+}$, $B^{0,+}$) in providing the substrate for NOS remains to be elucidated. Furthermore, as our understanding of the role that amino acid metabolism plays in modulating intracellular availability of L-arginine is limited, future research could investigate the potential of reversibly inhibiting intracellular pathways involved in metabolism of L-arginine.

Advances in our molecular understanding of amino acid transporters have provided important insights into the potential mechanisms regulating CAA transport in mammalian cells (see reviews by Closs, 1996, MacLeod, 1996, Deves and Boyd, 1998, Palacin *et al.,* 1998, and Closs and Gräf, 1999). Knockout experiments aimed at suppressing the expression of individual carrier isoforms or the development of selective pharmacological inhibitors will prove invaluable in determining the physiological relevance of specific CAA carrier proteins in regulating NOS activity in cells capable of releasing NO basically or in response to inflammatory stimuli.

Acknowledgments

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References

Albritton, L. M., Tseng, L., Scadden, D., and Cunningham, J. M. (1989). A putative murine ectropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* **57,** 659–666.

Aulak, K. S., Liu, J., Wu, J., Hyatt, S. L., Puppi, M., Henning, S. J., and Hatzoglou, M. (1996). Molecular sites of regulation of expression of the rat cationic amino acid transporter gene. *J. Biol. Chem.* **271,** 29799–29806.

Barbul, A. (1986). Arginine: Biochemistry, physiology, and therapeutic implications. *J. Parenter. Enteral. Nutr.* **10,** 227–238.

Baron, A. D. (1994). Hemodynamic actions of insulin. *Am. J. Physiol.* **267,** E187–E202.

Baydoun, A. R., and Mann, G. E. (1994). Selective targeting of nitric oxide synthase inhibitors to system y^+ in activated macrophages. *Biochem. Biophys. Res. Commun.* **200,** 726–731.

Baydoun, A. R., Bogle, R. G., Pearson, J. D., and Mann, G. E. (1993). Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells. *Br. J. Pharmacol.* **110,** 1401–1406.

Baydoun, A., Wileman, S. M., Wheeler-Jones, C. P. D., Marber, M. S., Mann, G. E., Pearson, J. D., and Closs, E. I. (1999). Transmembrane signalling mechanisms regulating expression of cationic amino acid transporters and inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. J.* **344,** 265–272.

Bertran, J., Magagnin, S., Werner, A., Markovich, D., Biber, J., Testar, X., Zorzano, A., Kuhn, L. C., Palacin, M., and Murer, H. (1992). Stimulation of system y^+-like amino acid transport by the heavy chain of human 4F2 surface antigen in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5606–5610.

Block, E. R., Herrera, H., and Couch, M. (1995). Hypoxia inhibits L-arginine uptake by pulmonary artery endothelial cells. *Am. J. Physiol.* **13,** L574–L580.

Bogle, R. G., Coade, S. B., Moncada, S., Pearson, J. D., and Mann, G. E. (1991). Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **180,** 926–932.

Bogle, R. G., Moncada, S., Pearson, J. D., and Mann, G. E. (1992a). Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br. J. Pharmacol.* **105,** 768–770.

Bogle, R. G., Baydoun, A. R., Pearson, J. D., Moncada, S., and Mann, G. E. (1992b). L-arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.* **284,** 15–18.

Bogle, R. G., Macallister, R. J., Whitley, G. S., and Vallance, P. (1995). Induction of N^G-monomethyl-L-arginine uptake: A mechanism for differential inhibition of NO synthases? *Am. J. Physiol.* **38,** C750–C756.

Bogle, R. G., Baydoun, A. R., Pearson, J. D., and Mann, G. E. (1996). Regulation of L-arginine transport and nitric oxide release in superfused porcine aortic endothelial cells. *J. Physiol.* **490,** 229–241.

Borsani, G., Bassi, M. T., Sperandeo, M. P., De Grandi, A., Buoninconti, A., Riboni, M., Manzoni, M., Incerti, B., Pepe, A., Andria, G., *et al.* (1999). SLC7A7, encoding a putative permease-related protein, is mutated in patients with lysinuric protein intolerance. *Nat. Genet.* **21,** 297–301.

Braissant, O., Gotoh, T., Loup, M., Mori, M., and Bachmann, C. (1999). L-arginine uptake, the citrulline–NO cycle and arginase II in the rat brain: An in situ hybridization study. *Brain Res. Mol. Brain Res.* **70,** 231–241.

Bröer, S., Bröer, A., and Hamprecht, B. (1995). The 4F2hc surface antigen is necessary for expression of system L-like neutral amino acid-transport activity in C6-BU-1 rat glioma cells: Evidence from expression studies in *Xenopus laevis* oocytes. *Biochem. J.* **312,** 863–870.

Busch, A. E., Herzer, T., Waldegger, S., Schmidt, F., Palacin, M., Biber, J., Markovich, D., Murer, H., and Lang, F. (1994). Opposite directed currents induced by the transport of dibasic and neutral amino acids in *Xenopus oocytes* expressing the protein rBAT. *J. Biol. Chem.* **269,** 25581–25586.

Bussolati, O., Sala, R., Astorri, A., Rotoli, B. M., Dall'Asta, V., and Gazzola, G. C. (1993). Characterization of amino acid transport in human endothelial cells. *Am. J. Physiol.* **265,** C1006–C1014.

Cendan, J. C., Topping, D. L., Pruitt, J., Snowdy, S., Copeland, E. M., and Lind, D. S. (1996). Inflammatory mediators stimulate arginine transport and arginine-derived nitric oxide production in a murine breast cancer cell line. *J. Surg. Res.* **60,** 284–288.

Chillaron, J., Estevez, R., Mora, C., Wagner, C. A., Suessbrich, H., Lang, F., Gelpi, J. L., Testar, X., Busch, A. E., Zorzano, A., *et al.* (1996). Obligatory amino acid exchange via systems $b^{0,+}$-like and y^+L-like. A tertiary active transport mechanism for renal reabsorption of cystine and dibasic amino acids. *J. Biol. Chem.* **271,** 17761–17770.

Closs, E. I. (1996). CATs, a family of three distinct mammalian cationic amino acid transporters. *Amino Acids* **11,** 193–208.

Closs, E. I., and Gräf, P. (1999). Cationic amino acid transporters (CATs)-targets for the manipulation of NO synthase activity? *In* "Membrane Transporters as Drug Targets" (G. L. Amidon and W. Sadee, eds.), pp. 229–249. Kluwer Academic/Plenum, New York.

Closs, E. I., Lyons, C. R., Kelly, C., and Cunningham, J. M. (1993a). Characterization of the third member of the mCAT family of cationic amino acid transporters—identification of a domain that determines the transport properties of the mCAT proteins. *J. Biol. Chem.* **268,** 20796–20800.

Closs, E. I., Albritton, L. M., Kim, J. W., and Cunningham, J. M. (1993b). Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. *J. Biol. Chem.* **268,** 7538–7544.

Closs, E. I., Rinkes, I. H., Bader, A., Yarmush, M. L., and Cunningham, J. M. (1993c). Retroviral infection and the expression of cationic amino acid transporters in rodent hepatocytes. *J. Virol.* **67,** 2097–2102.

Closs, E. I., Gräf, P., Habermeier, A., Cunningham, J. M., and Förstermann, U. (1997a). Human cationic amino acid transporters hCAT-1, hCAT-2A, and hCAT-2B: Three related carriers with distinct transport properties. *Biochemistry* **36,** 6462–6468.

Closs, E. I., Basha, F. Z., Habermeier, A., and Förstermann, U. (1997b). Interference of L-arginine analogues with L-arginine transport mediated by the y^+ carrier hCAT-2B. *Nitric Oxide Biol. Chem.* **1,** 67–73.

Closs, E. I., Scheld, J.-S., Sharafi, M., and Förstermann, U. (2000). Substrate supply for NO synthase in macrophages and endothelial cells: The role of cationic amino acid transporters (CATs). *Mol. Pharm.* **57,** 68–74.

Deves, R., and Boyd, C. A. R. (1998). Transporters for cationic amino acids in animal cells: Discovery, structure, and function. *Physiol. Rev.* **78,** 487–545.

Deves, R., Chavez, P., and Boyd, C. A. (1992). Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J. Physiol.* **454,** 491–501.

Deves, R., Angelo, S., and Chavez, P. (1993). *N*-ethylmaleimide discriminates between two lysine transport systems in human erythrocytes. *J. Physiol.* **468,** 753–766.

Durante, W., Liao, L., and Schafer, A. I. (1995). Differential regulation of L-arginine transport and inducible NOS in cultured vascular smooth muscle cells. *Am. J. Physiol.* **37,** H1158–H1164.

Durante, W., Liao, L., Iftikhar, I., Cheng, K., and Schafer, A. I. (1996). Platelet-derived growth factor regulates vascular smooth muscle cell proliferation by inducing cationic amino acid transporter gene expression. *J. Biol. Chem.* **271,** 11838–11843.

Durante, W., Liao, L., Peyton, K. J., and Schafer, A. I. (1997). Lysophosphatidylcholine regulates cationic amino acid transport and metabolism in vascular smooth muscle cells—Role in polyamine biosynthesis. *J. Biol. Chem.* **272,** 30154–30159.

Durante, W., Liao, L., Peyton, K. J., and Schafer, A. I. (1998). Thrombin stimulates vascular smooth muscle cell polyamine synthesis by inducing cationic amino acid transporter and ornithine decarboxylase gene expression. *Circ. Res.* **83,** 217–223.

Estevez, R., Camps, M., Rojas, A. M., Testar, X., Deves, R., Hediger, M. A., Zorzano, A., and Palacin, M. (1998). The amino acid transport system y^+L/4F2hc is a heteromultimeric complex. *FASEB J.* **12,** 1319–1329.

Fei, Y. J., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (1995). The amino acid transport system y^+L induced in *Xenopus laevis* oocytes by human choriocarcinoma cell (JAR) mRNA is functionally related to the heavy chain of the 4F2 cell surface antigen. *Biochemistry* **34,** 8744–8751.

Feliubadalo, L., Font, M., Purroy, J., Rousaud, F., Estivill, X., Nunes, V., Golomb, E., Centola, M., Aksentijevich, I., Kreiss, Y., *et al.* (1999). Non-type I cystinuria caused by mutations in SLC7A9, encoding a subunit ($b^{0,+}$AT) of rBAT. *Nat. Genet.* **23,** 52–57.

Furriols, M., Chillaron, J., Mora, C., Castello, A., Bertran, J., Camps, M., Testar, X., Vilaro, S., Zorzano, A., and Palacin, M. (1993). rBAT, related to L-cysteine transport, is localized to the microvilli of proximal straight tubules, and its expression is regulated in kidney by development. *J. Biol. Chem.* **268,** 27060–27068.

Gill, D. J., Low, B. C., and Grigor, M. R. (1996). Interleukin-1β and tumor necrosis factor-α stimulate the cat-2 gene of the L-arginine transporter in cultured vascular smooth muscle cells. *J. Biol. Chem.* **271,** 11280–11283.

Greene, B., Pacitti, A. J., and Souba, W. W. (1993). Characterization of L-arginine transport by pulmonary artery endothelial cells. *Am. J. Physiol.* **264,** L351–L356.

Hanssen, H., Brunini, T. M., Conway, M., Banning, A. P., Roberts, N. B., Mann, G. E., Ellory, J. C., and Mendes Ribeiro, A. C. (1998). Increased L-arginine transport in human erythrocytes in chronic heart failure. *Clin. Sci. Colch.* **94,** 43–48.

Harvey, C. M., Muzyka, W. R., Yao, S. Y., Cheeseman, C. I., and Young, J. D. (1993). Expression of rat intestinal L-lysine transport systems in isolated oocytes of *Xenopus laevis*. *Am. J. Physiol.* **265,** G99–G106.

Hattori, Y., Kasai, K., and Gross, S. S. (1999). Cationic amino acid transporter gene expression in cultured vascular smooth muscle cells and in rats. *Am. J. Physiol.* **276,** H2020–H2028.

Hibbs, J. B., Taintor, R. R., and Vavrin, Z. (1987). Macrophage cytotoxicity: Role for L-arginine deiminase activity and imino nitrogen oxidation to nitrite. *Science* **235,** 473–476.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94.

Hosokawa, H., Sawamura, T., Kobayashi, S., Ninomiya, H., Miwa, S., and Masaki, T. (1997). Cloning and characterization of a brain-specific cationic amino acid transporter. *J. Biol. Chem.* **272,** 8717–8722.

Hosokawa, H., Ninomiya, H., Sawamura, T., Sugimoto, Y., Ichikawa, A., Fujiwara, K., and Masaki, T. (1999). Neuron-specific expression of cationic amino acid transporter 3 in the adult rat brain. *Brain Res.* **838,** 158–165.

Hyatt, S. L., Aulak, K. S., Malandro, M., Kilberg, M. S., and Hatzoglou, M. (1997). Adaptive regulation of the cationic amino acid transporter-1 (Cat-1) in Fao cells. *J. Biol. Chem.* **272,** 19951–19957.

Irie, K., Tsukahara, F., Fujii, E., Uchida, Y., Yoshioka, T., He, W. R., Shitashige, M., Murota, S., and Muraki, T. (1997). Cationic amino acid transporter-2 mRNA induction by tumor necrosis factor-α in vascular endothelial cells. *Eur. J. Pharmacol.* **339,** 289–293.

Ito, K., and Groudine, M. (1997). A new member of the cationic amino acid transporter family is preferentially expressed in adult mouse brain. *J. Biol. Chem.* **272,** 26780–26786.

Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D. (1996). Comparative properties of arginases. *Comp. Biochem. Physiol.* **114,** 107–132.

Kakuda, D. K., Finley, K. D., Dionne, V. E., and MacLeod, C. L. (1993). Two distinct gene products mediate y^+ type cationic amino acid transport in *Xenopus oocytes* and show different tissue expression patterns. *Transgene* **1,** 91–101.

Kakuda, D. K., Finley, K. D., Maruyama, M., and MacLeod, C. L. (1998). Stress differentially induces cationic amino acid transporter gene expression. *Biochim. Biophys. Acta—Biomembr.* **1414,** 75–84.

Kakuda, D. K., Sweet, M. J., MacLeod, C. L., Hume, D. A., and Markovich, D. (1999). CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages. *Biochem. J.* **340,** 549–553.

Kanai, Y., Stelzner, M. G., Lee, W. S., Wells, R. G., Brown, D., and Hediger, M. A. (1992). Expression of mRNA (D2) encoding a protein involved in amino acid transport in S3 proximal tubule. *Am. J. Physiol.* **263,** F1087–F1092.

Kanai, Y., Segawa, H., Miyamoto, K., Uchino, H., Takeda, E., and Endou, H. (1998). Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). *J. Biol. Chem.* **273,** 23629–23632.

Kavanaugh, M. P. (1993). Voltage dependence of facilitated arginine flux mediated by the system y^+ basic amino acid transporter. *Biochemistry* **32,** 5781–5785.

Kavanaugh, M. P., Wang, H., Zhang, Z., Zhang, W., Wu, Y. N., Dechant, E., North, R. A., and Kabat, D. (1994). Control of cationic amino acid transport and retroviral receptor functions in a membrane protein family. *J. Biol. Chem.* **269,** 15445–15450.

Kikuta, K., Sawamura, T., Miwa, S., Hashimoto, N., and Masaki, T. (1998). High-affinity arginine transport of bovine aortic endothelial cells is impaired by lysophosphatidylcholine. *Circ. Res.* **83,** 1088–1096.

Kim, J. W., and Cunningham, J. M. (1993). *N*-linked glycosylation of the receptor for murine ecotropic retroviruses is altered in virus-infected cells. *J. Biol. Chem.* **268,** 16316–16320.

Kim, J. W., Closs, E. I., Albritton, L. M., and Cunningham, J. M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* **352,** 725–728.

Knowles, R. G., and Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.* **298,** 249–258.

Lind, D. S., Copeland, E. M., Souba, W. W., Baker, C. C., and Billiar, T. (1993). Endotoxin stimulates arginine transport in pulmonary artery endothelial cells. *Surgery* **114,** 199–205.

Liu, J., and Hatzoglou, M. (1998). Control of expression of the gene for the arginine transporter Cat-1 in rat liver cells by glucocorticoids and insulin. *Amino Acids* **15,** 321–337.

Low, B. C., and Grigor, M. R. (1995). Angiotensin II stimulates system y^+ and cationic amino acid transporter gene expression in cultured vascular smooth muscle cells. *J. Biol. Chem.* **270,** 27577–27583.

McDonald, K. K., Rouhani, R., Handlogten, M. E., Block, E. R., Griffith, O. W., Allison, R. D., and Kilberg, M. S. (1997a). Inhibition of endothelial cell amino acid transport System y^+ by arginine analogs that inhibit nitric oxide synthase. *Biochim. Biophys. Acta* **21,** 133–141.

McDonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997b). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the ''arginine paradox.'' *J. Biol. Chem.* **272,** 31213–31216.

MacLeod, C. L. (1996). Regulation of cationic amino acid transporter (CAT) gene expression. *Biochem. Soc. Trans.* **24,** 846–852.

MacLeod, C. L., Finley, K., Kakuda, D., Kozak, C. A., and Wilkinson, M. F. (1990). Activated T cells express a novel gene on chromosome 8 that is closely related to the murine ecotropic retroviral receptor. *Mol. Cell. Biol.* **10,** 3663–3674.

Magagnin, S., Bertran, J., Werner, A., Markovich, D., Biber, J., Palacin, M., and Murer, H. (1992). Poly(A)+ RNA from rabbit intestinal mucosa induces $b^{0,+}$ and y^+ amino acid transport activities in *Xenopus laevis* oocytes. *J. Biol. Chem.* **267,** 15384–15390.

Mann, G. E., Siow, R. C. M., Closs, E. I., and Sobrevia, L. (1998). Expression of human cationic amino acid transporters (hCAT) and nitric oxide synthase in human fetal endothelial cells: Modulation by elevated D-glucose. *In* ''The Biology of Nitric Oxide'' (S. Moncada, N. Toda, H. Maeda, and E. A. Higgs, eds.), p. 171. Portland Press, London.

Mastroberardino, L., Spindler, B., Pfeiffer, R., Skelly, P. J., Loffing, J., Shoemaker, C. B., and Verrey, F. (1998). Amino-acid transport by heterodimers of 4F2hc/CD98 and members of a permease family. *Nature* **395,** 288–291.

Mendes Ribeiro, A. C., Hanssen, H., Kiessling, K., Roberts, N. B., Mann, G. E., and Ellory, J. C. (1997). Transport of L-arginine and the nitric oxide inhibitor N^G-monomethyl-L-arginine in human erythrocytes in chronic renal failure. *Clin. Sci. Colch.* **93,** 57–64.

Mendes Ribeiro, A. C., Brunini, T. M. C., Yaqoob, M., Aronson, J. K., Mann, G. E., and Ellory, J. C. (1999). Identification of system y^+L as the high-affinity transporter for L-arginine in human platelets: Up-regulation of L-arginine influx in uraemia. *Pfluegers Arch. Eur. J. Physiol.* **438,** 573–575.

Mora, C., Chillaron, J., Calonge, M. J., Forgo, J., Testar, X., Nunes, V., Murer, H., Zorzano, A., and Palacin, M. (1996). The rBAT gene is responsible for L-cystine uptake via the $b^{0,+}$-like amino acid transport system in a ''renal proximal tubular'' cell line (OK cells). *J. Biol. Chem.* **271,** 10569–10576.

Mosckovitz, R., Udenfriend, S., Felix, A., Heimer, E., and Tate, S. S. (1994). Membrane topology of the rat kidney neutral and basic amino acid transporter. *FASEB J.* **8,** 1069–1074.

Nakamura, E., Sato, M., Yang, H. L., Miyagawa, F., Harasaki, M., Tomita, K., Matsuoka, S., Noma, A., Iwai, K., and Minato, N. (1999). 4F2 (CD98) heavy chain is associated covalently with an amino acid transporter and controls intracellular trafficking and membrane topology of 4F2 heterodimer. *J. Biol. Chem.* **274,** 3009–3016.

Novak, D. A., Matthews, J. C., Beveridge, M. J., Yao, S. Y., Young, J., and Kilberg, M. S. (1997). Demonstration of system y^+L activity on the basal plasma membrane surface of rat placenta and developmentally regulated expression of 4F2hc mRNA. *Placenta* **18,** 643–648.

Palacin, M., Estevez, R., Bertran, J., and Zorzano, A. (1998). Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev.* **78,** 969–1054.

Pan, M., Wasa, M., Lind, D. S., Gertler, J., Abbott, W., and Souba, W. W. (1995). TNF-stimulated arginine transport by human vascular endothelium requires activation of protein kinase C. *Ann. Surg.* **221,** 590–601.

Pan, M., Wasa, M., Ryan, U., and Souba, W. (1996). Lipopolysaccharide and tumor necrosis factor stimulate lung microvascular arginine uptake, a response attenuated by dexamethasone. *J. Parenter. Enteral. Nutr.* **20,** 50–55.

Parmacek, M. S., Karpinski, B. A., Gottesdiener, K. M., Thompson, C. B., and Leiden, J. M. (1989). Structure, expression and regulation of the murine 4F2 heavy chain. *Nucleic Acids Res.* **17,** 1915–1931.

Perkins, C. P., Mar, V., Shutter, J. R., delCastillo, J., Danilenko, D. M., Medlock, E. S., Ponting, I. L., Graham, M., Stark, K. L., Zuo, Y., Cunningham, J. M., and Bosselman, R. A. (1997). Anemia and perinatal death result from loss of the murine ecotropic retrovirus receptor mCAT-1. *Gene Dev.* **11,** 914–925.

Pfeiffer, R., Rossier, G., Spindler, B., Meier, C., Kuhn, L., and Verrey, F. (1999). Amino acid transport of y^+L-type by heterodimers of 4F2hc/CD98 and members of the glycoprotein-associated amino acid transporter family. *EMBO J.* **18,** 49–57.

Pickel, V. M., Nirenberg, M. J., Chan, J., Mosckovitz, R., Udenfriend, S., and Tate, S. S. (1993). Ultrastructural localization of a neutral and basic amino acid transporter in rat kidney and intestine. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7779–7783.

Pickel, V. M., Pohorille, A., Chan, J., Tate, S. S., and Nirenberg, M. J. (1999). Regional and subcellular distribution of a neutral and basic amino acid transporter in forebrain neurons containing nitric oxide synthase. *J. Comp. Neurol.* **404,** 459–472.

Pineda, M., Fernandez, E., Torrents, D., Estevez, R., Lopez, C., Camps, M., Lloberas, J., Zorzano, A., and Palacin, M. (1999). Identification of a membrane protein, LAT-2, that co-expresses with 4F2 heavy chain, an L-type amino acid transport activity with broad specificity for small and large zwitterionic amino acids. *J. Biol. Chem.* **274,** 19738–19744.

Prasad, P. D., Wang, H. P., Huang, W., Kekuda, R., Rajan, D. P., Leibach, F. H., and Ganapathy, V. (1999). Human LAT1, a subunit of system L amino acid transporter: Molecular cloning and transport function. *Biochem. Biophys. Res. Commun.* **255,** 283–288.

Racke, K., Hey, C., Mossner, J., Hammermann, R., Stichnote, C., and Wessler, I. (1998). Activation of L-arginine transport by protein kinase C in rabbit, rat and mouse alveolar macrophages. *J. Physiol.* **511,** 813–825.

Rajan, D. P., Kekuda, R., Huang, W., Wang, H. P., Devoe, L. D., Leibach, F. H., Prasad, P. D., and Ganapathy, V. (1999). Cloning and expression of a $b^{0,+}$-like amino acid transporter functioning as a heterodimer with 4F2hc instead of rBAT—A new candidate gene for cystinuria. *J. Biol. Chem.* **274,** 29005–29010.

Reyes, A. A., Karl, I. E., and Klahr, S. (1994). Role of arginine in health and in renal disease. *Am. J. Physiol.* **267,** F331–F346.

Sato, H., Tamba, M., Ishii, T., and Bannai, S. (1999). Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem.* **274,** 11455–11458.

Schmidlin, A., and Wiesinger, H. (1995). Stimulation of arginine transport and nitric oxide production by lipopolysaccharide is mediated by different signaling pathways in astrocytes. *J. Neurochem.* **65,** 590–594.

Schmidt, K., Klatt, P., and Mayer, B. (1994). Uptake of nitric oxide synthase inhibitors by macrophage RAW 264.7 cells. *Biochem. J.* **301,** 313–316.

Schmidt, K., List, B. M., Klatt, P., and Mayer, B. (1995). Characterization of neuronal amino acid transporters: Uptake of nitric oxide synthase inhibitors and implication for their biological effects. *J. Neurochem.* **64,** 1469–1475.

Schott, C. A., Vetrovsky, P., and Stoclet, J. C. (1993). Cationic amino acids inhibit the effects of L-arginine in rat aorta exposed to lipopolysaccharide. *Eur. J. Pharmacol.* **236,** 155–157.

Segawa, H., Fukasawa, Y., Miyamoto, K., Takeda, E., Endou, H., and Kanai, Y. (1999). Identification and functional characterization of a Na^+-independent neutral amino acid transporter with broad substrate selectivity. *J. Biol. Chem.* **274,** 19745–19751.

Simmons, W. W., Closs, E. I., Cunningham, J. M., Smith, T. W., and Kelly, R. A. (1996). Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes Regulation of L-arginine transport and no production by CAT-1, CAT-2A, and CAT-2B. *J. Biol. Chem.* **271,** 11694–11702.

Sloan, J. L., and Mager, S. (1999). Cloning and functional expression of a human Na^+ and Cl^--dependent neutral and cationic amino acid transporter B^{0+}. *J. Biol. Chem.* **274,** 23740–23745.

Sobrevia, L., and Mann, G. E. (1997). Dysfunction of the endothelial nitric oxide signalling pathway in diabetes and hyperglycaemia. *Exp. Physiol.* **82,** 423–452.

Sobrevia, L., Cesare, P., Yudilevich, D. L., and Mann, G. E. (1995). Diabetes-induced activation of system y^+ and nitric oxide synthase in human endothelial cells: Association with membrane hyperpolarization. *J. Physiol.* **489,** 183–192.

Sobrevia, L., Nadal, A., Yudilevich, D. L., and Mann, G. E. (1996). Activation of L-arginine transport (system y^+) and nitric oxide synthase by elevated glucose and insulin in human endothelial cells. *J. Physiol.* **490,** 775–781.

Sobrevia, L., Yudilevich, D. L., and Mann, G. E. (1997). Activation of A(2)-purinoceptors by adenosine stimulates L-arginine transport (system y^+) and nitric oxide synthesis in human fetal endothelial cells. *J. Physiol.* **499,** 135–140.

Sperandeo, M. P., Borsani, G., Incerti, B., Zollo, M., Rossi, E., Zuffardi, O., Castaldo, P., Taglialatela, M., Andria, G., and Sebastio, G. (1998). The gene encoding a cationic amino acid transporter (SLC7A4) maps to the region deleted in the velocardiofacial syndrome. *Genomics* **49,** 230–236.

Stevens, B. R., and Vo, C. B. (1998). Membrane transport of neuronal nitric oxide synthase substrate L-arginine is constitutively expressed with CAT1 and 4F2hc, but not CAT2 or rBAT. *J. Neurochem.* **71,** 564–570.

Stevens, B. R., Kakuda, D. K., Yu, K., Waters, M., Vo, C. B., and Raizada, M. K. (1996). Induced nitric oxide synthesis is dependent on induced alternatively spliced CAT-2 encoding L-arginine transport in brain astrocytes. *J. Biol. Chem.* **271,** 24017–24022.

Stoll, J., Wadhwani, K. C., and Smith, Q. R. (1993). Identification of the cationic amino acid transporter (System y^+) of the rat blood–brain barrier. *J. Neurochem.* **60,** 1956–1959.

Torrents, D., Estevez, R., Pineda, M., Fernandez, E., Lloberas, J., Shi, Y. B., Zorzano, A., and Palacin, M. (1998). Identification and characterization of a membrane protein (y^+L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity y^+L. A candidate gene for lysinuric protein intolerance. *J. Biol. Chem.* **273,** 32437–32445.

Torrents, D., Mykkanen, J., Pineda, M., Feliubadalo, L., Estevez, R., de Cid, R., Sanjurjo, P., Zorzano, A., Nunes, V., Huoponen, K., *et al.* (1999). Identification of SLC7A7, encoding y^+LAT-1, as the lysinuric protein intolerance gene. *Nat. Genet.* **21,** 293–296.

Van Winkel, L. J., and Campione, A. L. (1990). Functional changes in cation-preferring amino acid transport during development of preimplantation mouse conceptuses. *Biochim. Biophys. Acta* **1028,** 165–173.

Van Winkel, L. J., Christensen, H. N., and Campione, A. L. (1985). Na^+-dependent transport of basic, zwitterionic, and bicyclic amino acids by a broad scope system in mouse blastocytes. *J. Biol. Chem.* **260,** 12118–12123.

Van Winkle, L. J., Campione, A. L., and Gorman, J. M. (1988). Na^+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. *J. Biol. Chem.* **263,** 3150–3163.

Wang, H., Kavanaugh, M. P., North, R. A., and Kabat, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature* **352,** 729–731.

Wayte, J., Buckingham, J. C., and Cowell, A. M. (1996). [^{3}H]L-arginine transport and nitric oxide synthase activity in foetal hypothalamic cultures. *Neuroreport* **8,** 267–271.

Wells, R. G., Lee, W.-S., Kanai, Y., Leiden, J. M., and Hediger, M. A. (1992). A 4F2 antigen heavy chain induces uptake of neutral and dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **267,** 15285–15288.

Westergaard, N., Beart, P. M., and Schousboe, A. (1993). Transport of L-[^{3}H]arginine in cultured neurons: Characteristics and inhibition by nitric oxide synthase inhibitors. *J. Neurochem.* **61,** 364–367.

White, M. F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta* **822,** 355–374.

Wileman, S. M., Mann, G. E., and Baydoun, A. R. (1995). Induction of L-arginine transport and nitric oxide synthase in vascular smooth muscle cells: Synergistic actions of proinflammatory cytokines and bacterial lipopolysaccharide. *Br. J. Pharmacol.* **116,** 3243–3250.

Wu, G., and Meininger, C. J. (1993). Regulation of L-arginine synthesis from L-citrulline by L-glutamine in endothelial cells. *Am. J. Physiol.* **265,** H1965–H1971.

Wu, G. Y., and Morris, S. M. (1998). Arginine metabolism: Nitric oxide and beyond. *Biochem. J.* **336,** 1–17.

Wu, J. Y., Robinson, D., Kung, H. J., and Hatzoglou, M. (1994). Hormonal regulation of the gene for the type C ecotropic retrovirus receptor in rat liver cells. *J. Virol.* **68,** 1615–1623.

Yoshimoto, T., Yoshimoto, E., and Meruelo, D. (1992). Enhanced gene expression of the murine ecotropic retroviral receptor and its human homolog in proliferating cells. *J. Virol.* **66,** 4377–4381.

Zharikov, S. I., Herrera, H., and Block, E. R. (1997). Role of membrane potential in hypoxic inhibition of L-arginine uptake by lung endothelial cells. *Am. J. Physiol.* **16,** L78–L84.

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CHAPTER 15

The Respiratory Cycle: A Three-Gas System

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THE BIOLOGY OF NITRIC OXIDE (NO) HAS BEEN DEFINED BY THE INTERACTIONS OF NO WITH HEMOGLOBIN (HB). SPECIFICALLY, THE ''ADDITION REACTION'' IN WHICH NO PURPORTEDLY BINDS IRREVERSIBLY TO UNLIGANDED HEMES IN HB, AND THE ''OXIDATION REACTION'' IN WHICH NITRATE AND METHEMOGLOBIN (METHB) ARE PRODUCED FROM THE INTERACTION OF NO WITH OXYHEMOGLOBIN (OXYHB), HAVE BEEN WIDELY VIEWED AS THE PRINCIPAL FATES OF NO *IN VIVO.* THESE REACTION PATHWAYS, HOWEVER, ARE DIFFICULT TO RECONCILE WITH THE ACTIVITY OF NO AS ENDOTHELIUM-DERIVED RELAXING FACTOR AND, IF DOMINANT, DICTATE THAT NO CONSUMPTION WOULD FAR EXCEED NO PRODUCTION *IN VIVO,* IN VIOLATION OF A MASS-BALANCE CONSTRAINT. HERE WE REVIEW RECENT WORK FROM OUR LABORATORY SHOWING THAT, IN FACT, THE NO ADDITION AND OXIDATION REACTIONS, AS OPERATIONALLY DEFINED, ARE OF SECONDARY IMPORTANCE UNDER PHYSIOLOGICAL CONDITIONS. INSTEAD, HB RECRUITS ALTERNATIVE REACTION PATHWAYS WITH NO THAT PRESERVE ITS BIOACTIVITY. IN PARTICULAR, REACTIONS THAT S-NITROSYLATE THE PROTEIN ARE FAVORED IN THE R (OR OXYGENATED) STRUCTURE. FOR EXAMPLE, THE OXYGENATION-INDUCED ALLOSTERIC TRANSITION IN HB [FROM THE T (OR DEOXYGENATED) TO THE R STRUCTURE] IN THE LUNG TRIGGERS THE MIGRATION OF NO FROM HEMES TO CYS-β93, THEREBY FORMING *S*-NITROSOHEMOGLOBIN (SNO-HB). CONVERSELY, THE DEOXYGENATION-LINKED ALLOSTERIC TRANSITION IN SNO-HB (FROM THE R TO THE T STRUCTURE) PROMOTES THE RELEASE OF NO BIOACTIVITY IN THE TISSUES TO AUGMENT BLOOD FLOW. BECAUSE O_2 AFFINITY IS INCREASED IN SNO-HB, S-NITROSYLATION HAS THE EFFECT OF LIMITING THE EXCESSIVE OR UNTIMELY RELEASE OF NO GROUPS. THESE DATA SHOW THAT HB CONSERVES ITS NO WHILE DISPENSING IT SELECTIVELY TO MAXIMIZE O_2 DELIVERY IN THE RESPIRATORY CYCLE.

Introduction

The chemistry of nitric oxide (NO) interactions with hemoglobin (Hb) has been critical to our understanding of NO biology. The landmark identification of NO bioactivity with endothelium-derived relaxing factor (EDRF), for example, relied in part on observations that NO and EDRF were each seemingly inactivated by Hb (Ignarro *et al.,* 1987; Palmer *et al.,* 1987). Similarly, NO biology in the specific realm of the cardiovascular system has been predicated on two classic tenets: (1) NO binding to unliganded (deoxygenated) hemes in Hb is noncooperative and effectively irreversible [the NO addition reaction; Eq. (1)], and (2) that NO reaction with oxyhemoglobin (oxyHb) yields methemoglobin (metHb) and nitrate [the NO oxidation reaction; Eq. (2)] (Eich *et al.,* 1996; Gow *et al.,* 1999; Gross and Lane, 1999).

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$$Hb[Fe(II)] + NO \rightleftharpoons Hb[Fe(II)]NO \quad (1)$$

$$Hb[Fe(II)]O_2 + NO \rightarrow Hb[Fe(III)] + NO_3^- \quad (2)$$

These reactions with hemoglobin have been viewed both as a way of mitigating NO vasoactivity and as a route of NO elimination from the body. However, assuming these reactions to be the principal fate of endothelium-derived NO, the amount of NO synthesized daily is calculated to be several orders of magnitude too low to sustain the level of NO bioactivity that is required to dilate blood vessels (Castillo *et al.,* 1996; Gow *et al.,* 1999). Thus, biological models built on these assumptions not only are unable to explain how EDRF can dilate blood vessels *in vivo,* when Hb scavenges NO and constricts blood vessels *in vitro,* but also present what we have termed a mass-balance constraint: NO consumption would far exceed production. Gross and Lane (1999) have eluded to the additional problem that tissue oxygenation would be compromised by the inexorable accumulation of iron nitrosylHb (Hb[Fe(II)]NO) and metHb, were additional reaction pathways not available for NO.

The major failings of the classic model are twofold. First, whereas EDRF was originally identified with free NO, we have come to understand that a significant component of EDRF-related activity exists in the form of *S*-nitrosothiols (SNOs) (Creager *et al.,* 1997; Day *et al.,* 1996; Lancaster, 1994; Stamler, 1996), which are not subject to inactivation by hemoglobin (Gaston *et al.,* 1994; Jia *et al.,* 1996). Second, it is now clear that alternative NO reaction pathways with Hb are available, which preserve its bioactivity (Gow *et al.,* 1999; Gow and Stamler, 1998). These reaction channels serve two functions: they recycle NO (to obviate the mass-balance constraint) (Gow and Stamler, 1998; Gross and Lane, 1999), and they dispense it for regulation of O_2 delivery in the respiratory cycle (Gross and Lane, 1999; Stamler *et al.,* 1997). More specifically, Hb assumes the R conformation in O_2-rich environments, enabling NO to bind cooperatively to vacant hemes [Eq. (3)]. Additional S-nitrosylation pathways are then available to secure NO groups [Eq. (4), see discussion], which are thereby transported to the tissues. There, *S*-nitrosohemoglobin (SNO-Hb) undergoes an O_2-linked allosteric transition from the R to the T structure that triggers the delivery of vasodilator SNOs. In this revised picture SNO-Hb is the principal NO reservoir in the circulation, functioning as an allosterically controlled NO buffer, dispensing NO groups to tissues in need (Jia *et al.,* 1996; Stamler *et al.,* 1997). SNO-Hb does not transition to the T structure, however, if tissue PO_2 rises (as in O_2 delivery exceeding tissue demand). The net result in this case is NO sequestration [Eq. (3)] and consequently vasoconstriction. Accordingly, SNO-Hb participates in the classic physiological responses of hypoxic vasodilation and hyperoxic vasoconstriction.

$$Hb[Fe(II)(O_2)_3][Fe(II)] + NO \rightleftharpoons Hb[Fe(II)(O_2)_3][Fe(II)NO] \quad (3)$$

$$S^-\text{-}Hb[Fe(II)(NO)][Fe(II)(O_2)_3] + 2\,O_2 \rightleftharpoons SNO{-}Hb[Fe(II)(O_2)_4] + O_2^- \quad (4)$$

Oxygen affinity is greater in SNO-Hb than in unmodified Hb (Bonaventura *et al.,* 1999; McMahon *et al.,* 2000; Patel *et al.,* 1999; Wolzt *et al.,* 1999). Given that the fraction of Hb molecules carrying SNO is ~1/1000, this augmented O_2 affinity is, however, unlikely to influence the aggregate O_2 affinity of blood. Rather, evidence suggests that by shifting the equilibrium toward the R structure, S-nitrosylation serves to limit the excessive release of NO groups from SNO-Hb (McMahon *et al.,* 2000). Here we review the results of experiments that show how SNO-Hb senses hypoxia to regulate blood flow and hence O_2 delivery within the respiratory cycle, and yet tightly controls what would otherwise be a deleterious, untimely, or excessive release of vasodilator (S)NO. In other words, Hb conserves the vast majority of its NO.

Nitric Oxide Reactions with Hemoglobin, and Allosterically Related Bioactivity: Selected Methods and Results

The Oxyhemoglobin and NO Addition Reactions

Difference absorption spectroscopy was used to assess the formation of metHb and nitrosylHb on the addition of NO to Hb under aerobic conditions (Gow *et al.,* 1999). Standard difference spectra of authentic metHb, deoxyhemoglobin (deoxyHb), and iron nitrosylHb versus oxyHb are shown in Fig. 1A. Submicromolar concentrations of NO were titrated against 33 μM Hb in room air (O_2 saturation, 99%), and the difference spectra versus oxyHb are shown in Fig. 1B. Assuming a model of simple competition between the production of nitrosylHb (the addition reaction) and that of metHb (the oxyhemoglobin reaction), the expected difference spectrum at 99% O_2 saturation under these conditions would largely resemble that of metHb. Instead, however, the difference spectra primarily show the formation of nitrosylHb (i.e., the difference spectrum can be mostly accounted for by the deoxyHb minus oxyHb standard spectrum, Fig. 1A). Furthermore, photolysis–chemiluminescence analysis of the reaction products under similar conditions reveals yields of SNO-Hb exceeding those of Hb[Fe(II)]NO (Fig. 1C). Thus, under physiological conditions (and, in particular, when the NO to heme ratio is $<$1/100), the direct oxidation of oxyHb by NO does not predominate. Rather, NO binding to the unliganded hemes in oxyHb is cooperative, and Hb recruits additional reaction pathways, including S-nitrosylation, in order to preserve NO.

Auto S-Nitrosylation of Hemoglobin on Oxygenation

The yield of SNO was measured in partially nitrosylated hemoglobin solutions following rapid exposure to air (which induces the allosteric transition from the T to the R conformation) (Gow and Stamler, 1998). When ratios of NO to Hb were in the physiological range (1:100 or less), intramolecular NO group transfer from heme to thiol accounted for

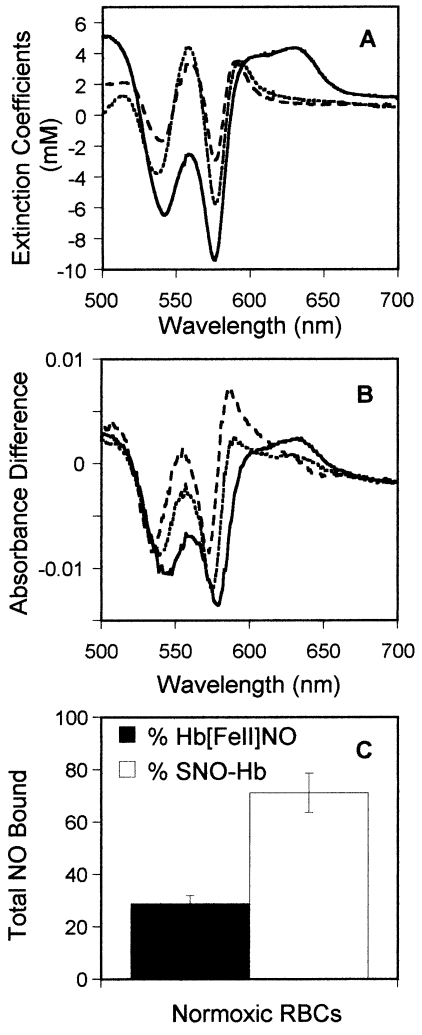


Figure 1 The oxyhemoglobin reaction of NO does not account for its fate under physiological conditions. (A) Difference spectra for standard solutions of metHb (solid line), deoxyHb (dotted line), and nitrosylHb (dashed line) versus oxyHb. (B) Difference spectra generated from the exposure of NO to normoxic (~99% O_2 saturated) Hb. NO was added to Hb in 100 m*M* phosphate (solid line), 10 m*M* phosphate (dotted line), or 10 m*M* phosphate plus 0.05% borate. NitrosylHb and some metHb forms in 10 m*M* phosphate (conditions which favor the R structure); mainly nitrosylHb forms in the added presence of borate; whereas mainly metHb is formed in 100 m*M* phosphate (which favors the T structure among the partially liganded molecules). (C) SNO-Hb and Hb[Fe(II)]NO formed by exposure of oxygenated Hb (~99% saturation, 48 μM) to NO (1.2 μM). SNO (open bar) and Fe(II)NO (solid bar) were measured by photolysis–chemiluminescence (Jia *et al.,* 1996; Stamler *et al.,* 1997). Data shown are the average of 12 experiments ± standard error.

~80% of the initial NO added (Fig. 2). These findings indicate that Hb itself promotes the formation of SNO. Molecular O_2 in this context serves both to trigger the conformational change in Hb and to accept an electron in the S-nitrosylation reaction (Gow *et al.,* 1997). As the NO:Hb ratio is raised, however, the SNO yield drops to near zero (i.e., NO becomes increasingly unavailable to form SNO, Fig. 2). SNO formation increases again at higher ratios of NO:Hb (1:1 and greater) but reaches a maximum of ~5% of

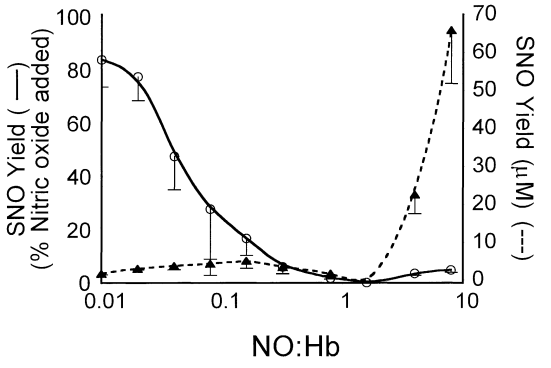


Figure 2 Auto S-nitrosylation of Hb. Varying amounts of NO were added to deoxyHb, the mixture was immediately oxygenated by rapid exposure to air, and the yield of *S*-nitrosothiol was measured. SNO yield is expressed both in absolute terms (μM) and relative to the initial NO added. The means ± SEM of 4–27 experiments are shown.

the NO added; this reaction is not likely to have physiological relevance (Fig. 2).

Hemoglobin-Bound NO in Mammalian Blood

The allosteric regulation of the distribution of NO binding sites was studied in rat blood (Stamler *et al.,* 1997). SNO-Hb and Hb[Fe(II)]NO levels were measured before and during elimination of the longitudinal O_2 gradient (the difference in O_2 saturation from artery to arteriole and from artery to vein) in hyperbaric chambers by the application of 3 ATA (atmospheres of absolute pressure) and the administration of 100% O_2. Hb[Fe(II)]NO predominated in venous blood from rats breathing 21% O_2, whereas SNO-Hb was present in significant amounts in arterial blood drawn simultaneously (Fig. 3). When the O_2 gradient was then eliminated in these rats (i.e., arterial and venous blood were 100 and 93% O_2 saturated, respectively), SNO-Hb was the predominant Hb–NO adduct in both arterial and venous blood (Fig. 3). Thus, SNO-Hb appears to form endogenously in the R

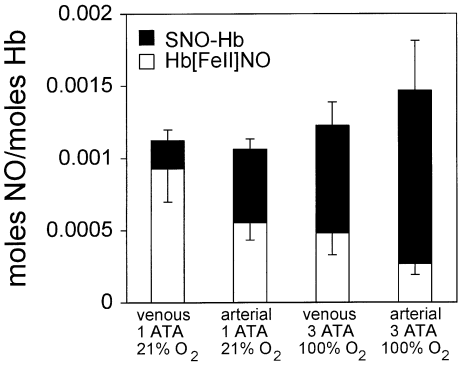


Figure 3 Hb-bound NO in mammalian blood: role of O_2 tension. SNO-Hb and Hb[Fe(II)]NO levels in arterial and venous blood were measured in rats exposed first to room air (RA) at 1 atmosphere (ATA) and then to 100% O_2 and 3 ATA. O_2 saturations were as follows: venous/RA, 69%; arterial/RA, 93%; venous/100% O_2 + 3 ATA, 93%; arterial/100% O_2 + 3 ATA, 100%.

structure, whereas the release of SNO *in vivo* is favored in the T structure. Remarkably, total levels of Hb-bound NO appear to remain relatively constant from artery to vein in both the presence and absence of an O_2 gradient. Thus, at least some of the (S)NO released when Hb undergoes the allosteric transition appears to be autocaptured at the hemes, preserving NO signaling equivalents.

O_2 Affinity of *S*-Nitrosohemoglobin

The O_2 affinities of Hb and SNO-Hb (~1.8 SNO per Hb tetramer) were compared using standard tonometric techniques (McMahon *et al.,* 2000). The O_2 binding curve, expressed as a Hill plot, for SNO-Hb was shifted to the left relative to that of unmodified Hb (Fig. 4). The leftward shift was asymmetric in that differences in affinity between Hb and SNO-Hb were greatest at low PO_2 values (Fig. 4). These results suggest that the increased O_2 affinity in SNO-Hb is largely attributable to a destabilization of the T structure. Reciprocal relationships between the binding of O_2 and NO to hemes and thiols, respectively, required by thermodynamics (Wyman, 1964), thus dictate that deoxygenation promotes NO group release from thiols.

S-Nitrosohemoglobin Vasoactivity: Allosteric Control and Potentiation by Low-Mass Thiols

The influence of the oxygen tension and thiol abundance on the bioactivity of SNO-Hb was studied in vascular ring bioassays (McMahon *et al.,* 2000). Rings of rabbit thoracic aorta were mounted in tissue baths filled with Krebs buffer solution and bubbled with varied gas mixtures. In 21% O_2, vessel rings incubated with glutathione (GSH) at either 10^{-7} or 10^{-5} *M* contracted in response to SNO-Hb (Fig. 5) in a concentration-dependent manner. These responses are consistent with the sequestration of endothelium-derived NO and are comparable to responses to unmodified Hb (data not shown). In the presence of 10^{-3} *M* GSH, however, vasorelaxation was seen in response to SNO-Hb. In hypoxia (<1% O_2), SNO-Hb elicited vasorelaxation in the presence of 10^{-5}M GSH; i.e., its potency was increased 100-fold (Fig. 5). Thus, SNO-Hb bioactivity is linked to the allosteric transition in Hb: in the R structure, responses primarily reflect scavenging at the heme centers, whereas in the T structure, (S)NO group release and vasorelaxation are facilitated. The low-molecular-weight thiol GSH accelerates the release of (S)NO from SNO-Hb *in vitro,* in keeping with Eqs. (4) and (5).

Blood Flow Regulation by *S*-Nitrosohemoglobin: Allosteric Control

Anesthetized adult rats were intubated and mechanically ventilated so as to maintain a PCO_2 of 35–45 mm Hg (Stamler *et al.,* 1997). The inspired O_2 concentration and the atmospheric pressure were adjusted in order to change the arterial PO_2. Regional blood flow was measured using H_2-sensitive platinum microelectrodes implanted in various regions in the brain. Changes in local blood flow in response to intravenous Hb or SNO-Hb (1 μmol/kg i.v. over 3 min) were compared (Stamler *et al.,* 1997). Hb reduced blood

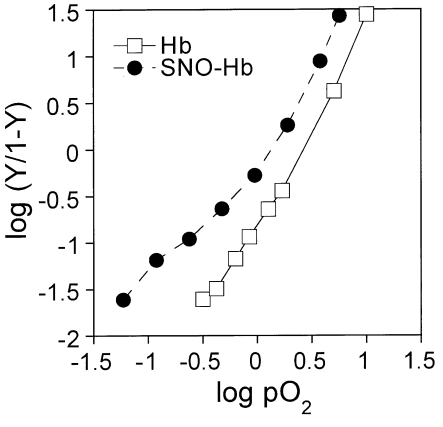


Figure 4 O_2 affinity is increased in SNO-Hb. The O_2-binding curves for Hb and partially S-nitrosylated Hb (SNO/Hb tetramer ratio ~1.4) were compared using a standard tonometric technique and are expressed as Hill plots (the $\log[Y/(1 - Y)]$ versus log pO_2, where Y is the fractional O_2 saturation). The leftward shift for SNO-Hb is asymmetric in that differences are greatest at low PO_2 values.

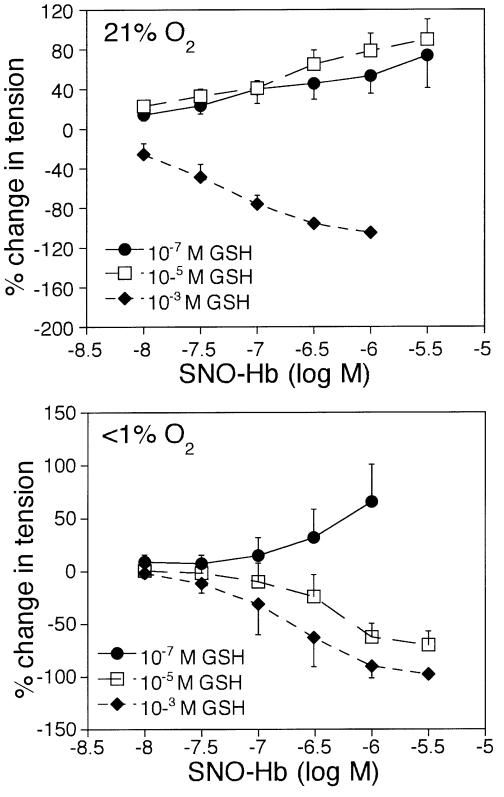


Figure 5 Control of SNO-Hb vasoactivity by the allosteric state and thiol abundance. Isometric changes in tension in response to SNO-Hb were measured in isolated rabbit aortic rings in either 21% O_2 (A) or <1% O_2 (B) and in the presence of varying concentrations of GSH (10^{-7}, 10^{-5}, or 10^{-3} *M*).

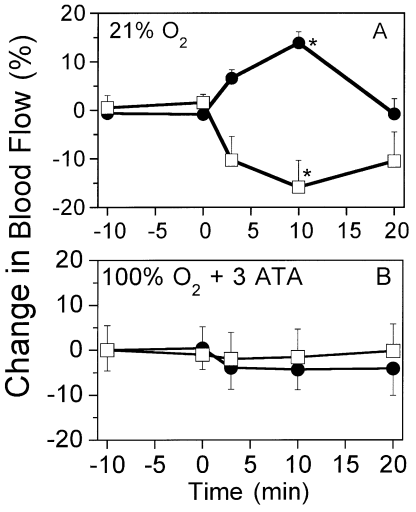


Figure 6 Regulation of blood flow *in vivo* by *S*-nitrosohemoglobin. Changes in blood flow following the administration of either Hb or SNO-Hb were measured in the cerebral cortex of rats either when breathing 21% O_2 at 1 ATA (A) or when the physiological O_2 gradient was eliminated by the administration of 100% O_2 and the application of 3 ATA (B). $*p < 0.05$ by ANOVA for SNO-Hb versus Hb ($n = 7$).

flow, whereas SNO-Hb raised blood flow in rats breathing 21% O_2 (normoxia, Fig. 6A). In contrast, decreases in blood flow were seen in response to both Hb and SNO-Hb when the physiological O_2 gradient was eliminated by the administration of 100% O_2 in hyperbaric chambers adjusted to 3 ATA (Fig. 6B).

Discussion

Our understanding of NO biology has been shaped by the viewpoint that hemoglobin eliminates NO bioactivity by oxidizing it to nitrate [Eq. (2)]. Previous studies, however, had been carried out with NO concentrations in excess of Hb (Eich *et al.,* 1996), whereas the NO concentration *in vivo* is only a fraction of the Hb (0.01%). Moreover, the population of vacant hemes in oxygenated blood (typically 1% of total Hb is desaturated) always exceeds that of free NO. This excess, together with the cooperativity of NO binding to oxyHb, enables the binding of NO to outcompete the oxidative pathway [Eq. (2)] even (indeed, especially) at high O_2 saturations [Eq. (3)] (Gow *et al.,* 1999). NO bound to hemes in oxygenated Hb is oxidatively transferred to Cys-β93, thereby preserving NO bioactivity in the form of SNO-Hb (Gow *et al.,* 1999; Gow and Stamler, 1998; Jia *et al.,* 1996) [Eq. (4)]. Such S-nitrosylated proteins exist in equilibrium with peptide and amino acid SNOs in all biological systems [Eq. (5)] (Gaston *et al.,* 1998; Jia *et al.,* 1996). NO channeling through interactions with Hb thus provides a means to account for the equilibrium population of SNOs in blood, which constitute the vast majority of NO bioactivity *in vivo* (Jia *et al.,* 1996; Stamler *et al.,* 1992, 1997).

$$\text{SNO}-\text{Hb[Fe(II)]O}_2 + \text{GSH} \rightleftharpoons \text{GSNO} + \text{S}^-\text{-Hb[Fe(II)]O}_2 + \text{H}^+ \quad (5)$$

$$\text{Hb[Fe(II)]O}_2 \rightleftharpoons \text{Hb[Fe(II)]} + \text{O}_2 \quad (6)$$

More generally, our data speak to the existence of a redox-dependent equilibrium between NO groups bound to hemes of Hb in the T structure and thiols of Hb in the R structure [Eq. (7)]. Accordingly, the oxygenation-induced allosteric transition of partially nitrosylated Hb molecules is reciprocally coupled with NO migration from hemes (of T structure molecules) to Cys-β93 (in the R structure) (Gow and Stamler, 1998; McMahon *et al.,* 2000). In other words, SNO-Hb is also formed when partially nitrosylated Hb is exposed to O_2, as in the case of red blood cells entering the lung. Conversely, deoxygenation promotes NO group release from Cys-β93, as happens during arterial–venous transit (Jia *et al.,* 1996; McMahon *et al.,* 2000; Stamler *et al.,* 1997). It is important to recognize that molecular O_2 not only is involved in triggering the allosteric transition—a function that might be alternatively carried out by other allosteric effectors (PCO_2 and pH) and which determines the equilibrium position in Eq. (7)—but is also capable of serving as an electron acceptor in the synthesis of SNO (Gross and Lane, 1999).

$$\text{S}^-\text{-Hb[Fe(II)(NO)][Fe(II)]}_3 + 5\ \text{O}_2 \rightleftharpoons \text{SNO}-\text{Hb[Fe(II)(O}_2\text{)]}_4 + \text{O}_2^- \quad (7)$$

The binding and release of NO groups by the highly conserved Cys-β93 of Hb are controlled not only by the allosteric state of Hb but also by the abundance of ambient thiols capable of transducing the SNO signal [Eq. (5)]. Such transnitrosation reactions are likewise promoted by deoxygenation and heme oxidation (T structure, high spin) (Jia *et al.,* 1996; Pawloski *et al.,* 1998; Stamler *et al.,* 1997), in keeping with thermodynamic linkage relationships in Hb (Wyman, 1964). For example, SNO-Hb is also in equilibrium with low-mass SNOs (Ignarro *et al.,* 1987), such as *S*-nitrosoglutathione (GSNO) [Eq. (5)]. High concentrations of GSH thus shift the equilibrium in favor of the deoxy structure (Jia *et al.,* 1996) [Eq. (5) and (6)]. As is generally the case for SNOs, the mechanism of vasorelaxation by GSNO is independent of free NO (Gaston *et al.,* 1998; Kerr *et al.,* 1992), which would only be sequestered at the hemes of Hb.

Our findings motivate a revision of the respiratory cycle, which can now be viewed as a ''three-gas system'' (in which Hb transports NO, CO_2 and O_2) (Gross and Lane, 1999). In relatively deoxygenated central venous blood, Hb binds NO primarily at the hemes (Fig. 7). As Hb takes up oxygen in the lung, SNO-Hb is formed (in concert with the conformational switch from the T to the R structure in NO-carrying Hb molecules). The NO group is subsequently released from SNO-Hb in the peripheral microcirculation where PO_2 is low. Transnitrosation reactions with ambient thiols are implicated in the dilation of blood vessels and thereby couple regional blood flow with the metabolic needs of the tissues. However, not all of the NO/SNO released from Hb reaches targets in vascular smooth muscle. Rather, some NO is also

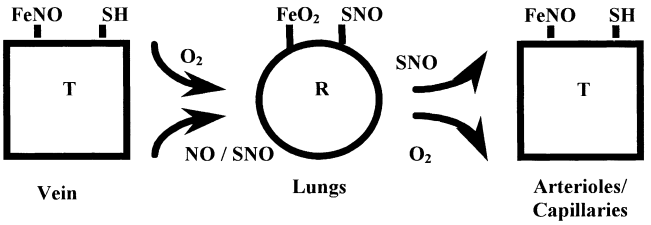


Figure 7 Transport and delivery of NO groups by Hb within the respiratory cycle.

recaptured at the hemes. In this way, Hb conserves its NO (Funai *et al.,* 1997; Gladwin *et al.,* 1999; Jia *et al.,* 1996; McMahon *et al.,* 2000).

This preservation of NO bioactivity and tight control of seemingly small concentrations of NO can be understood by appreciating a fundamental difference between NO and O_2 in biology: (S)NO acts principally as a signal at nanomolar concentrations (De Belder *et al.,* 1994; Feelisch *et al.,* 1994; MacAllister *et al.,* 1995), whereas O_2 supports aerobic metabolic activities at millimolar concentrations. Accordingly, the liberation of only a small fraction (<0.1%) of the micromolar concentrations of Hb-bound SNO across the peripheral microcirculation is sufficient to evoke substantial regional increases in blood flow. Moreover, release of larger amounts would not be compatible with life on two accounts. First, it would impose a metabolic burden on the organism: more NO would be turned over by Hb than is produced within the lining of the blood vessels. Second, release of micromolar SNO would cause hypotension and shunting of blood. Multiple mechanisms, in fact, operate *in vivo* to limit the flux of NO/SNO released from Hb. These include the autocapture of NO by the hemes of Hb (Ignarro *et al.,* 1987; McMahon *et al.,* 2000) and the augmented intrinsic O_2 affinity of SNO-Hb, which disfavors the deoxygenation-dependent release of SNO. By reinforcing the R structure in Hb (see Hill plots in Fig. 4), S-nitrosylation mitigates the untimely or excessive offloading of NO, which would be incompatible with life.

How NO–Hb interactions may regulate or perturb blood flow in pathophysiological settings is less clear. Hb[Fe(II)]NO levels are high in animal models of endotoxic (lipopolysaccharide-induced) sepsis (Kosaka *et al.,* 1997). In these experiments, NO was found to bind primarily to the α-hemes, shifting the aggregate O_2-binding curve slightly to the right (Kosaka *et al.,* 1997). It is unclear, however, that NO ever reaches sufficient concentrations to have meaningful effects on O_2 delivery through direct posttranslational effects on the protein. Indeed, the importance of NO binding to Hb lies not in its effect on the population of Hb at large, but on the functional behavior of the few molecules carrying NO. That is, NO binding to thiols of Hb limits the release of NO groups on deoxygenation of (S)NO-carrying molecules by shifting the equilibrium position in Eq. (5) to the left. In this way Hb tightly regulates the dispensing of NO groups. Because blood flow is regulated by nanomolar SNO targeted toward smooth muscle (Feelisch *et al.,* 1994; Jia *et al.,* 1996; MacAllister *et al.,* 1995), this tight regulation of the NO group is far more important for O_2 delivery than any NO-related change in O_2 saturation of Hb, which could only be effected by supraphysiological NO levels. The formation of five-coordinate α-nitrosyls in Hb is particularly well adapted to this end, as they serve to reinforce the T structure in Hb (Gow and Stamler, 1998; Yonetani *et al.,* 1998). Inasmuch as high concentrations of NO and low pH favor five-coordinate α-nitrosyls in Hb, this scenario may present a means to raise blood pressure in the septic animal. We are currently investigating Hb-bound NO dynamics in human sepsis.

Our findings have important implications for the development of experimental therapeutics designed to exploit NO–Hb interactions *in vivo.* For example, inhaled NO or alternative NO donor agents—once believed to be inactivated by erythrocytic Hb—might be administered for extrapulmonary effects (Fox-Robichard *et al.,* 1998; Kermarrec *et al.,* 1998; Kubes *et al.,* 1999; Troncy *et al.,* 1997), and thus may be utilized in the treatment of ischemic diatheses (e.g., angina, stroke, or sickle cell disease). Moreover, by regulating the NO channeling in Hb, one might anticipate both the promise of improved NO delivery functions by hemoglobins as well as the ability to divert NO toward storage pools or detoxification pathways that may ameliorate the undesirable effects of blood substitutes or the NO overproduction in septic shock.

Acknowledgments

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References

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368,** 850–853.

Bonaventura, C., Ferruzi, G., Tesh, S., and Stevens, R. D. (1999). Effects of S-nitrosation on oxygen binding by normal and sickle cell hemoglobin. *J. Biol. Chem.* **274,** 24742–24748.

Castillo, L., Beaumier, L., Ajami, A. M., and Young, V. R. (1996). Whole body nitric oxide synthesis in healthy men determined from [^{15}N]arginine-to-[^{15}N]citrulline labelling. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11460–11465.

Creager, M. A., Roddy, M.-A., Boles, K., and Stamler, J. S. (1997). *N*-Acetylcysteine does not influence the activity of endothelium-derived relaxing factor *in vivo. Hypertension* **29,** 668–672.

Day, B. J., Jia, L. J., Arnelle, D. J., Crapo, J. D., and Stamler, J. S. (1996). Transduction of nitric oxide synthase activity from endothelium to vascular smooth muscle: Involvement of *S*-nitrosothiols. *In* ''The Biology of Nitric Oxide,'' Part 5. Portland Press, London, 1996.

De Belder, A. J., MacAllister, R., Radomski, M. W., Moncada, S., and Vallance, P. J. T. (1994). Effects of *S*-nitrosoglutathione in the human forearm circulation: Evidence for selective inhibition of platelet activation. *Cardiovasc. Res.* **28,** 691–694.

Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr., and Olson, J. S. (1996). Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* **35,** 6976–6983.

Feelisch, M., te Poel, M., Zamora, R., Deussen, A., and Moncada, S. (1994). Understanding the controversy over the identity of EDRF. *Nature* **368,** 62–65.

Fox-Robichaud, A., Payne, D., Hasan, S., Ostrovsky, L., Fairhead, T., Reinhardt, T., and Kubes, P. (1998). Inhaled NO as a viable antiadhesive therapy for ischemia–reperfusion injury of distal microvascular beds. *J. Clin. Invest.* **101,** 2497–2505.

Funai, E. F., Davidson, A., Seligman, S. P., and Finlay, T. F. (1997). *S*-Nitrosohemoglobin in the fetal circulation may represent a cycle for blood pressure regulation. *Biochem. Biophys. Res. Commun.* **239,** 875–877.

Gaston, B., Drazen, J. M., Jansen, A., Sugarbaker, D. A., Loscalzo, J., Richards, W., and Stamler, J. S. (1994). Relaxation of human bronchial smooth muscle by *S*-nitrosothiols *in vitro*. *J. Pharmacol. Exp. Ther.* **268,** 978–984.

Gaston, B., Fry, E., Sears, S., Heroman, W. M., Ignarro, L. J., and Stamler, J. S. (1998). Umbilical arterial *S*-nitrosothiols in stressed newborns: Role in perinatal circulatory transition. *Biochem. Biophys. Res. Commun.* **253,** 899–901.

Gladwin, M. T., Schechter, A. N., Shelhamer, J. H., Pannell, L. K., Conway, D. A., Hrinczenko, B. W., Nichols, J. S., Pease-Fye, M. E., Noguchi, C. T., Rodgers, G. P., and Ognibene, F. P. (1999). Inhaled nitric oxide augments nitric oxide transport on sickle cell hemoglobin without affecting oxygen affinity. *J. Clin. Invest.* **104,** 937–945.

Gow, A. J., Luchsinger, B. P., Pawloski, J. R., Singel, D. J., and Stamler, J. S. (1999). The oxyhemoglobin reaction of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 9027–9032.

Gow, A. J., and Stamler, J. S. (1998). Nitric oxide/hemoglobin reactions reexamined under physiological conditions. *Nature* **391,** 169–173.

Gow, A. J., Buerk, D. D., and Ischiropoulos, H. (1997). A novel reaction mechanism for the formation of *S*-nitrosothiol *in vivo*. *J. Biol. Chem.* **272,** 2841–2845.

Gross, S. S., and Lane, P. (1999). Physiological reactions of nitric oxide and hemoglobin: A radical rethink. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 9967–9969.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996). *S*-Nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* **380,** 221–226.

Kermarrec, N., Zunic, P., Beloucif, S., Benessiano, J., Drouet, L., and Payen, D. (1998). Impact of inhaled nitric oxide on platelet aggregation and fibrinolysis in rats with endotoxic lung injury. Role of cyclic guanosine 5'-monophosphate. *Am. J. Respir. Crit. Care Med.* **158,** 833–839.

Kerr, S. W., Buchanan, L. V., Bunting, S., and Mathews, W. R. (1992). Evidence that *S*-nitrosothiols are responsible for the smooth muscle relaxing activity of the bovine retractor penis inhibitory factor. *J. Pharmacol. Exp. Ther.* **263,** 285–292.

Kosaka, H., and Seiyama, A. (1997). Increased oxygen dissociation by nitric oxide from RBC. *In* ''Oxygen Transport to Tissue XIX.'' Plenum, New York.

Kubes, P., Payne, D., Grisham, M. B., Jourd'heuil, D., and Fox-Robichaud, A. (1999). Inhaled nitric oxide impacts intravascular but not extravascular compartments in postischemic peripheral organs. *Am. J. Physiol.* **277,** H676–H682.

Lancaster, J. R. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8137–8141.

MacAllister, R. J., Calver, A. L., Riezebos, J., Collier, J., and Vallance, P. (1995). Relative potency and arteriovenous selectivity of nitrovasodilators on human blood vessels: An insight into the targeting of nitric oxide delivery. *J. Pharmacol. Exp. Ther.* **273,** 154–160.

McMahon, T. J., Stone, A. E., Bonaventura, J., Singel, D. J., and Stamler, J. S. (2000). Functional coupling of oxygen binding and vasoactivity in *S*-nitrosohemoglobin. *J. Biol. Chem.,* in press.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Patel, R. P., Hogg, N., Spencer, N. Y., Kalyanaraman, B., Matalon, S., and Darley-Usmar, V. D. M. (1999). Biochemical characterization of *S*-nitrosohemoglobin. *J. Biol. Chem.* **274,** 15487–15492.

Pawloski, J. R., Swaminathan, R. V., and Stamler, J. S. (1998). Cell-free and erythrocytic *S*-nitrosohemoglobin inhibits human platelet aggregation. *Circulation* **97,** 263–267.

Stamler, J. S. (1996). Alzheimer's disease. A radical vascular connection. *Nature* **380,** 108–111.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-Nitrosylation of proteins with nitric oxide. Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stamler, J. S., Jia, L., Eu, J. P., McMahon, T. J., Demchenko, I. T., Bonaventura, J., Gernert, K., and Piantadosi, C. A. (1997). Blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient. *Science* **276,** 2034–2037.

Troncy, E., Francoeur, M., Salazkin, I., Yang, F., Charbonneau, M., Leclerc, G., Vinay, P., and Blaise, G. (1997). Extra-pulmonary effects of inhaled nitric oxide in swine with and without phenylephrine. *Br. J. Anaesth.* **79,** 631–640.

Wolzt, M., MacAllister, R. J., Davis, D., Feelisch, M., Moncada, S., Vallance, P., and Hobbs, A. J. (1999). Biochemical characterization of *S*-nitrosohemoglobin. *J. Biol. Chem.* **274,** 28983–28990.

Wyman, J. (1964). Linked functions and reciprocal effects in hemoglobin: A second look. *Adv. Protein Chem.* **17,** 223–286.

Yonetani, T., Tsuneshige, A., Zhou, Y., and Chen, X. (1998). Electron paramagnetic resonance and oxygen binding studies of α-nitrosyl hemoglobin. A novel oxygen carrier having NO-assisted allosteric functions. *J. Biol. Chem.* **273,** 20323–20333.

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CHAPTER 16

Role of Nitric Oxide and Other Radicals in Signal Transduction

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FREE RADICALS DERIVED FROM NITROGEN AND OXYGEN HAVE BEEN FOUND TO HAVE A SIGNIFICANT ROLE IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL PROCESSES. ORIGINALLY, FREE RADICALS WERE BELIEVED TO BE ASSOCIATED EXCLUSIVELY WITH CELLULAR DAMAGE. FREE RADICALS ARE NOW WIDELY IMPLICATED IN CELLULAR SIGNALING, A FACT WHICH ATTACKS OUR CLASSIC NOTION OF BIOLOGICAL MEDIATORS UTILIZING RECEPTOR–LIGAND INTERACTIONS IN A ''LOCK AND KEY'' MANNER. FREE RADICAL SIGNALING IS MAINLY BASED ON REDOX CHEMISTRY, A UNIQUE MODE OF SIGNAL TRANSDUCTION. NITRIC OXIDE (NO), WHICH IS SYNTHESIZED IN MOST TISSUES, IS DIFFUSIBLE AND CAN TAKE ON SEVERAL CHEMICAL FORMS, EACH OF WHICH HAS ITS OWN REACTIVE SPECIFICITY. THE MAJOR CHEMICAL MODIFICATION PRODUCED BY THESE SPECIES IS NITROSATION OF THE TARGET, GENERALLY A PROTEIN IRON OR THIOL. THE DIRECT INTERACTION OF NITRIC OXIDE WITH THE PROTEIN TARGET MAY RESULT IN EITHER ACTIVATION, INACTIVATION, OR SWITCHING OF PROTEIN FUNCTION AND SUBSEQUENT MODULATION OF GENE EXPRESSION. SOME PHYSIOLOGICAL EVENTS REGULATED BY THIS TYPE OF SIGNALING INCLUDE VASODILATION, CYTOTOXICITY, INFLAMMATION, AND SYNAPTIC PLASTICITY. REACTIVE OXYGEN SPECIES AID IN PROPAGATING SIGNALS TRIGGERED BY GROWTH FACTORS, HORMONES, AND CYTOKINES. THESE SPECIES ALSO MODIFY THE TARGETS DIRECTLY. SOME OF THE PHYSIOLOGICAL EVENTS REGULATED BY REACTIVE OXYGEN SPECIES INCLUDE CELL GROWTH, CELL ADHESION, APOPTOSIS, ION TRANSPORT, NEUROMODULATION, AND TRANSCRIPTION. THIS CHAPTER ATTEMPTS TO EXPLAIN THE MECHANISTIC BASIS OF CELL SIGNALING BY NITROGEN- AND OXYGEN-DERIVED CHEMICAL SPECIES AND DISCUSSES THEIR CELLULAR TARGETS AND THE PHYSIOLOGICAL EVENTS THEY REGULATE.

Introduction

Multicellular organisms function in an organized manner by communicating within and between various organs and tissues. Cells communicate by using different kinds of signaling molecules such as proteins, small peptides, amino acids, nucleotides, steroids, retinoids, fatty acid derivatives, and gases such as nitric oxide (NO). Cells respond to this apparent babel of signals in an organized manner either by differentiating, by proliferating, by dying, or by performing some specialized function.

Depending on the type of signal, the target cell initiates a specific signaling cascade. This specificity is achieved by means of cellular receptors that are either on the cell surface or inside the target cell. Transmembrane receptors on the cell surface are essential for hydrophilic signaling molecules that cannot cross the lipid bilayer. The activated receptor transmits a signal across the membrane and generates an intracel-

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lular messenger. Hydrophobic compounds, such as steroid hormones, can pass through the plasma membrane, bind to specific intracellular receptors, and directly trigger gene transcription. Steroid hormones do not need any intervening signal transducers. Similarly, gaseous signaling molecules can also bypass surface receptors by diffusing through the target cell plasma membrane. Here we focus on how free radical species transmit signals. In order to appreciate the signaling mechanisms utilized by gaseous messengers like nitric oxide and reactive oxygen species, it is necessary to take an overview of classic cell signaling (Alberts *et al.,* 1994; Hardie, 1990).

How Is Classic Signal Transduction Achieved?

Because steroid hormones can permeate cells easily and bind to intracellular receptors that directly regulate gene expression, they do not generate many signaling events in the cell (Fig. 1). However, hydrophilic signaling molecules generate many molecular signaling events on interaction with surface receptors. Most cell surface receptors belong to one of three classes, depending on the transduction mechanism used: ion channel-linked receptors, G protein-linked receptors, and enzyme-linked receptors.

Ion Channel-Linked Receptors

Ion channel-linked receptors are mainly involved in inorganic ion transport. They specifically allow Na^+, K^+, Ca^{2+}, or Cl^- to diffuse through the lipid bilayer. Specificity of ion transport is achieved through size and charge selection by the channel. Gates of ion channels are regulated by either voltage potential across the membrane, mechanical stress, or a ligand binding to its receptor.

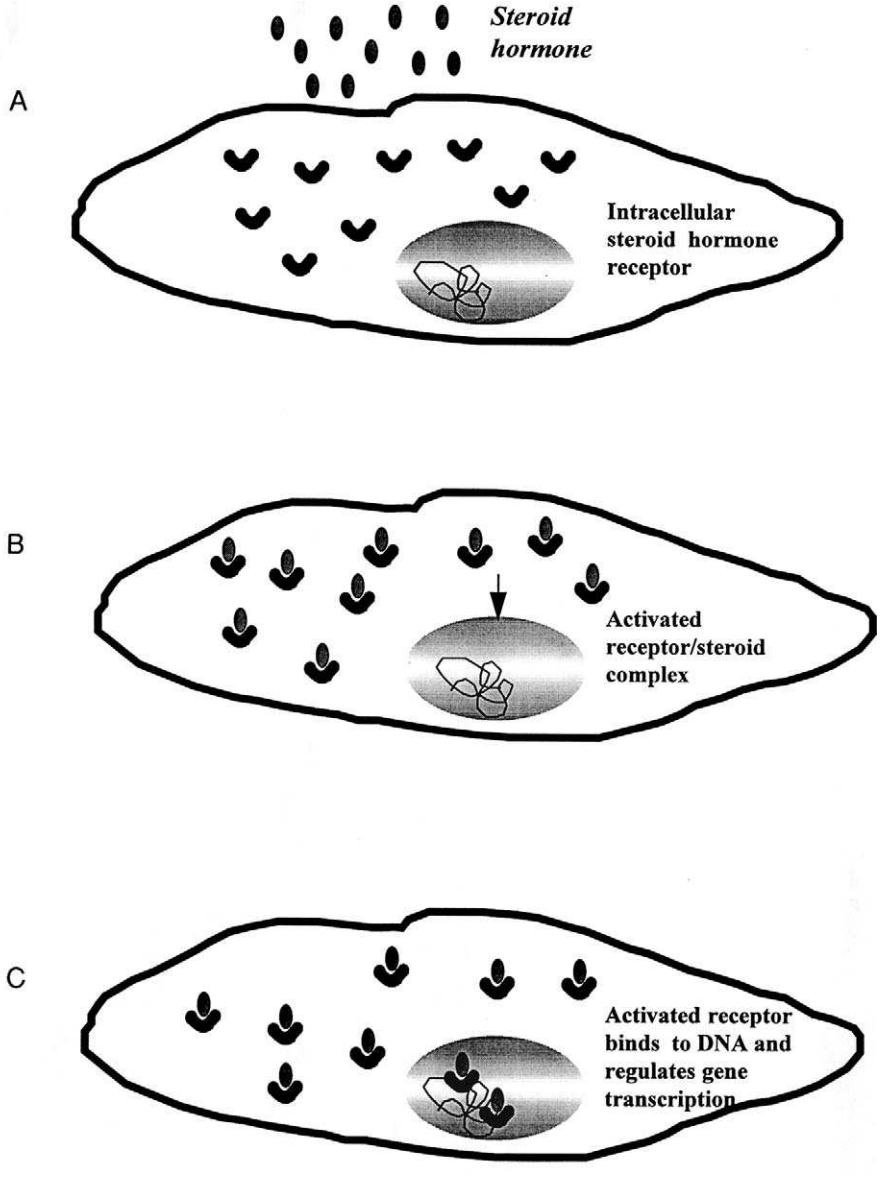


Figure 1 Interaction of steroid hormone with its intracellular receptor.

In the nervous system, neurotransmitters mediate signaling by ion channels. Acetylcholine released by nerve terminals binds to its receptors on the muscle cell plasma membrane, which triggers opening of the gated cation channels associated with it. This leads to a large influx of Na^+, resulting in membrane depolarization. As a result of local depolarization, more Na^+ enters due to opening of voltage-gated Na^+ channels, and the local depolarization spreads through the entire plasma membrane. This activates the voltage-gated Ca^{2+} channels of the muscle cell and finally opens the Ca^{2+} release channels of the sarcoplasmic reticulum. The Ca^{2+} released from the sarcoplasmic reticulum contracts the myofibrils of the muscle cell. Thus, the signaling cascade triggered by acetylcholine brings about a functional response in the target cell, namely, muscle contraction (Fig. 2).

G Protein-Linked Receptors

The G protein-linked receptors indirectly relay signals either to enzymes or to ion channels via their associated trimeric GTP-binding proteins (G proteins). The receptors in this superfamily have similar secondary structures with a single polypeptide chain that threads the lipid bilayer seven times. The G proteins associated with the receptors are known to conduct a variety of signals by hormones, neurotransmitters, and growth factors. G proteins do not signal when bound to GDP and trigger signaling when bound to GTP. Ligand binding to these receptors triggers GDP/GTP exchange on the associated G protein. This, in turn, activates a chain of intracellular events leading to alteration in second messenger levels (Fig. 3). For example, epinephrine, a stress hormone released by the adrenal gland, binds to its G protein-linked receptor and relays a signal via cyclic AMP. During stress, one of the messages conveyed by epinephrine is to mobilize glucose. It binds to the β-adrenergic receptors on muscle cells and activates a G protein and adenylyl cyclase. This in turn increases the level of cyclic AMP, which then activates cyclic AMP-dependent protein kinase. This enzyme phosphorylates two other enzymes: phosphorylase kinase and glycogen synthase. Phosphorylase kinase further phosphorylates glycogen phosphorylase and helps in releasing glucose by breaking down glycogen. Also, phosphorylation of glycogen synthase inhibits its activity and thus prevents glycogen synthesis. By this signaling pathway epinephrine makes glucose available in order to respond to stress.

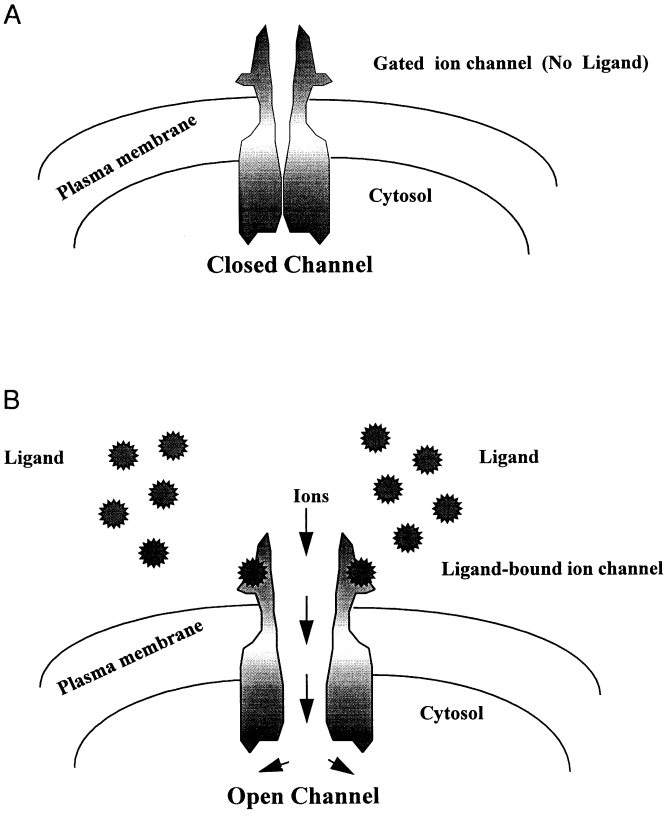


Figure 2 Ion channel-linked receptor (e.g., neurotransmitters).

Calcium ion is an important second messenger and is involved in signaling that leads to muscle contraction, neurotransmission, gene transcription, and cell growth. A Ca^{2+} gradient exists across the cell because the Ca^{2+} concentration of the cytosol is kept extremely low (10^{-7} *M*) as compared to cell exterior (10^{-3} *M*) and the endoplasmic reticulum. A stimulus increases the Ca^{2+} concentration in the cytosol by activating a Ca^{2+} channel either in the plasma membrane or in the endoplasmic reticulum. The increase in cytosolic Ca^{2+} subsequently activates Ca^{2+}-responsive proteins in the cell. These include troponin, some members of the protein kinase C family, Ca^{2+}–calmodulin kinases, calmodulin-dependent protein phosphatase, and nitric oxide synthase.

The receptors of ligands such as vasopressin, acetylcholine, and thrombin activate a G protein which modulates Ca^{2+} levels of the cytosol. Initially, GTP-bound G protein activates phospholipase C-β. This enzyme acts on phosphorylated phosphatidylinositol, a phospholipid present in the cell membrane. It generates two products, inositol trisphosphate and diacylglycerol. Inositol trisphosphate opens Ca^{2+} release channels on the endoplasmic reticulum and releases Ca^{2+} into the cytosol. Increased Ca^{2+} levels further regulate Ca^{2+}-responsive proteins. Diacylglycerol is cleaved into arachidonic acid, or it may directly activate various members of the protein kinase C family. Protein kinase C specifically phosphorylates serine and threonine residues on target proteins, which include transcription factors and other regulatory proteins, ultimately regulating the transcription of specific genes.

Enzyme-Linked Receptors

Enzyme-linked receptors are transmembrane proteins with an extracellular ligand binding domain and a cytosolic domain which either has intrinsic enzyme activity or is associated with an enzyme. These receptors can be further classified into five categories on the basis of their enzymatic activities (Fig. 4).

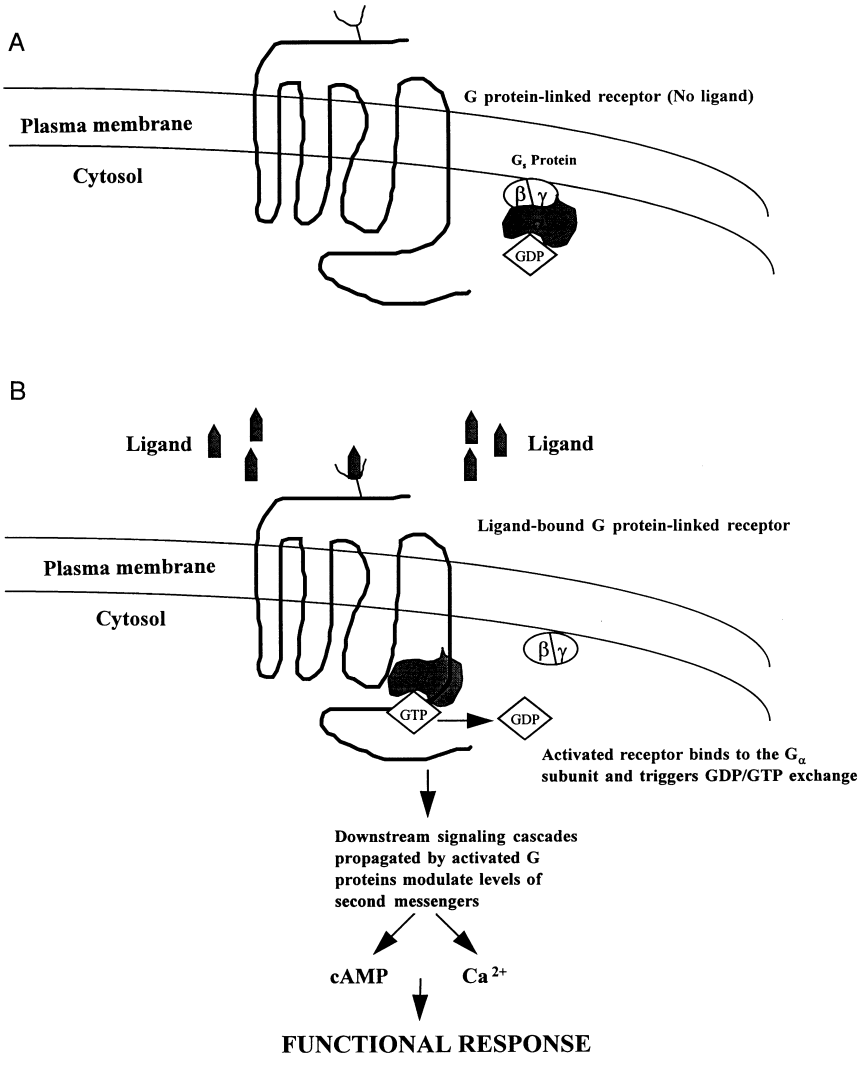


Figure 3 G-protein-linked receptors (e.g., epinephrine, vasopressin, thrombin).

RECEPTOR GUANYLYL CYCLASE

Guanylyl cyclase receptors directly convert GTP to cyclic GMP on ligand binding. Members of the atrial natriuretic peptide family utilize this mode of signaling.

RECEPTOR TYROSINE KINASES

Tyrosine kinase receptors auto- and *trans*-phosphorylate tyrosine residues on ligand binding. Ligands for these receptors include various growth factors such as epidermal growth factor, insulin, insulin-like growth factor, platelet-derived growth factor, nerve growth factor, and vascular endothelial growth factor.

TYROSINE KINASE-ASSOCIATED RECEPTOR

The tyrosine kinase-associated receptors mainly depend on associated nonreceptor tyrosine kinases for propagating the signal. This family includes receptors for cytokines, hormones, and antigen-specific receptors on T and B cell receptors. The associated nonreceptor tyrosine kinases mainly belong to either the Src or Janus family.

RECEPTOR TYROSINE PHOSPHATASE

Tyrosine phosphatase receptors propagate signals by removing phosphate groups from specific tyrosine residues of a particular protein. CD45 present on lymphocytes belongs to this receptor class.

RECEPTOR SERINE/THREONINE KINASES

Serine/threonine kinase receptors phosphorylate serine and threonine residues on target proteins. Receptors of the transforming growth factor β superfamily have this enzymatic property.

The receptors for most growth factors are transmembrane tyrosine protein kinases. Hence, the signaling cascade generated by this receptor class will be discussed briefly. Ligand–receptor interaction leads to autophosphorylation of the receptor on tyrosine. The phosphotyrosine residues serve as docking sites for intracellular signaling proteins including GTPase-activating proteins, phospholipase C-γ, and phosphatidylinositol 3'-kinase via their common SH2 domains.

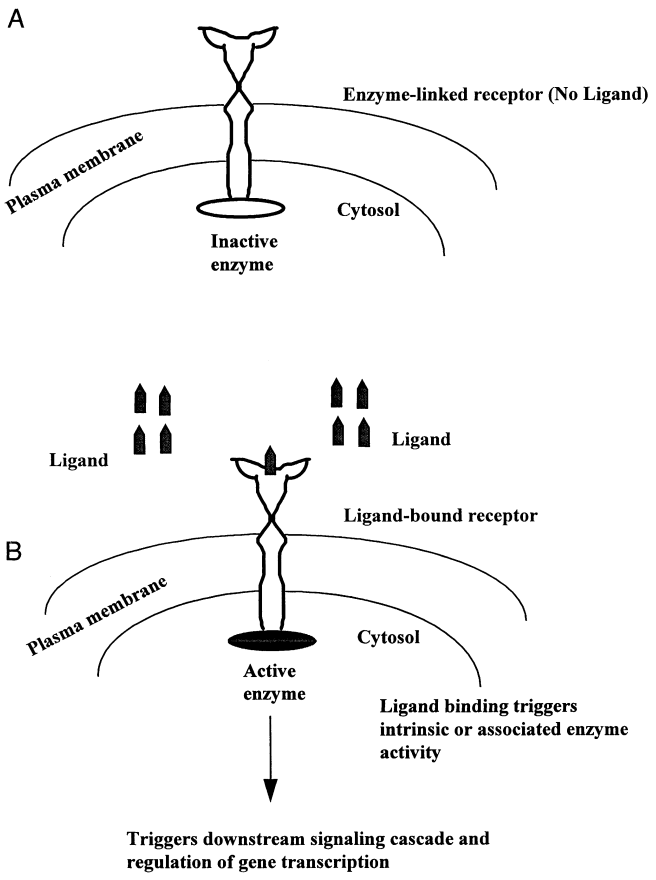


Figure 4 Enzyme-linked receptors (e.g., growth factor, cytokine, and the T and B cell receptors).

This association sets in motion the signaling cascade. Some of the key signaling components include the monomeric G protein Ras, Raf kinase, and the mitogen-activated protein (MAP) kinases.

The above overview of classic signaling events helps one appreciate how the cell has evolved intricate and well-controlled means of communication in order to respond specifically to environmental cues. A novel means of communication has been identified whereby cells utilize reactive free radical species derived from nitric oxide and oxygen to propagate signals. Unlike conventional receptor–ligand signaling that involves lock and key recognition, the basis of free radical signaling is chemistry. Reactive free radical species interact with different targets such as thiols and iron, depending on their redox potential. However, a major conceptual hurdle in redox signaling is understanding the means by which specificity is achieved. Its involvement in various crucial cellular events such as ion transport, neuromodulation, apoptosis, gene transcription, and growth factor signaling leaves little doubt that redox signaling has evolved into a specific and major signaling mechanism. How simple chemical events regulate biological processes will be the focus of next sections.

Nitric Oxide: How Does It Fit into Signal Transduction?

It Can Bypass Interactions at the Surface of the Cell

Nitric oxide can diffuse through cellular membranes with ease. Hence, a cell does not need a specific receptor to respond to nitric oxide signaling; rather, specificity of signaling is regulated at different levels. This is achieved by modulating the NO synthesis machinery, the redox milieu of the cell, the availability of protein binding sites, and the scavenger proteins that regulate the chemical form and effective concentration of NO. NO and its cogeners signal via mechanisms used by both protein kinases, which control function by covalent modification (i.e., phosphorylation), and reactive oxygen species, which signal through redox events and coordinative interactions with metals.

Target Specificity Is Achieved through Chemistry

Through its interactions with intracellular or extracellular moieties, NO can be quickly scavenged or stabilized, or it can transiently modify cellular targets. Interestingly, the endogenous scavenger gluthathione stabilizes and transports NO by forming adducts and converting itself into a NO donor. Depending on the redox milieu, NO can modify its target very quickly. The chemistry of NO plays a critical role in determining its cellular targets. NO has an unpaired electron, making it highly reactive, and its reaction with redox modulators yields many reactive species. Thus, the term NO does not reveal anything regarding its different redox forms. Hence, the term reactive nitrogen species (RNS) is used in this chapter to refer to those species whose origin is the free radical NO, but whose final chemical nature depends on its interaction with local redox modulators and the redox milieu of the cell (Lander, 1997).

NO can exist in three redox forms: nitrosonium cation (NO^+), nitric oxide ($\cdot NO$), and nitroxyl anion (NO^-) with nitrogen oxidation numbers +3, +2, and +1, respectively. In the presence of superoxide anion (O_2^-), NO combines to form peroxynitrite ($OONO^-$), a strong prooxidant species:

$$\cdot NO + O_2^{\cdot -} \rightarrow OONO^-$$

In aqueous aerobic solutions NO predominantly forms nitrite (NO_2^-). In the presence of oxyhemoglobin and oxymyoglobin, NO is completely oxidized to nitrate (NO_3^-) (Stamler *et al.,* 1992a; Ignarro *et al.,* 1993).

Covalent interactions of RNS with cellular macromolecules are responsible for its many physiological and pathological effects. Proteins containing iron and thiol groups are the major cellular targets of RNS. Extensive studies have been performed characterizing the RNS–iron interaction. The iron, as Fe^{2+} or Fe^{3+}, can be targeted when either in a heme group or in an iron–sulfur cluster (Ignarro, 1991; Bredt and Synder, 1994). The physiological significance of *S*-nitrosothiol adduction is now widely accepted. *S*-Nitroso-

thiols at critical active site thiol residues are reported to regulate the function of several proteins (Stamler, 1994):

$$RS^- + [NO^+] \rightarrow RSNO$$

Under more extreme conditions, like severe oxidative and nitrosative stress, RNS react at other targets such as amino groups on DNA and tyrosine residues on proteins. RNS modification of target proteins results in modulation of their functional properties and can propagate downstream signals (Fig. 5).

RNS serve as an extraordinarily widespread effector of cellular functions. Generally, RNS-responsive targets serve sensory and regulatory roles in signal transduction. The target recognizes RNS and transduces the chemical signal into a functional response. Some of the signaling pathways involving RNS are discussed below.

In most cases the ultimate fate of NO is generally its oxidation to nitrate. Two enzymes, SNO lyase and flavohemoglobin, have been identified in *Escherichia coli* and were found to denitrosylate S-nitrosylated proteins and oxidize NO to nitrate, respectively (Hausladen *et al.,* 1998).

Advantages of Utilizing NO and Its Congeners as Chemical Messengers

Signaling by RNS can be considered to be economical since cells do not use vesicular machinery to release it. Moreover, it uses receptor systems that are already found in the general regulatory machinery of cells, and NO can pass through and between cells with ease, due to its gaseous nature. Since RNS have many physiological actions in different tissues, their unstable nature is beneficial by allowing for truly local actions at the site of synthesis.

Another advantage is that the targets of RNS (heme iron, iron–sulfur clusters, and thiol groups) are commonly found in either allosteric or catalytic sites of the proteins. Thus, in a conducive chemical environment and at appropriate concentrations, RNS can modulate protein activity and trigger

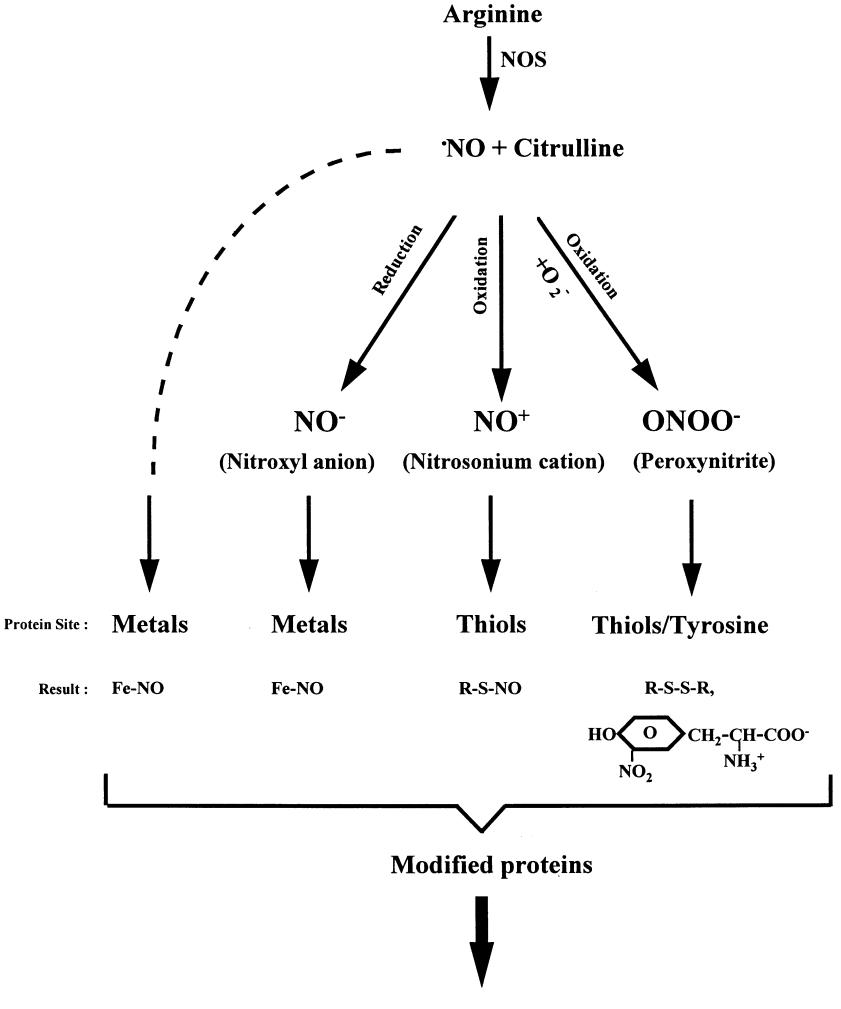


Figure 5 Initial molecular events of RNS signaling.

signaling. This mode of signaling is akin to phosphorylation of tyrosine, serine, and threonine residues.

What Is the Source of NO?

Endogenously, NO is derived enzymatically by the oxidation of one of the terminal guanidino nitrogen atoms of L-arginine by nitric oxide synthase (NOS), an enzyme which exists in three isoforms. nNOS (type I) (Bredt *et al.,* 1991) and eNOS (type III) (Lamas *et al.,* 1992), which were initially cloned from neuronal and endothelial cells, respectively, are Ca^{2+}–calmodulin dependent and expressed constitutively under most conditions. iNOS (type II), which was first identified in macrophages, is Ca^{2+} independent and inducible (Xie *et al.,* 1992). Most tissues have now been shown to express one or more of these isoforms.

The regulation of these enzymes is complex. The active enzyme requires five cofactors (FAD, FMN, heme, calmodulin, and tetrahydrobiopterin) and three cosubstrates (L-arginine, NADPH, and O_2) (Nathan and Xie, 1994). The enzymes are tightly controlled by substrate or cofactor availability, and by targeting of eNOS to plasmalemmal caveolae (small invaginations in the plasma membrane made up of caveolin protein), thus forming inactive complexes of eNOS and caveolin. This sequestration serves to prevent NO synthesis until the extracellular stimulus activates the complex. Moreover, because caveolae sequester many signaling molecules, including G proteins, receptors, and protein kinases, this microlocalization may aid in bringing NO and its target in close proximity.

Redox-regulated signaling involves multiple signaling cascades and concomitant regulation of several biological processes. We now highlight the cellular targets of NO, the major signaling pathways utilized, and examine their physiological relevance.

What Happens after RNS Modify Cellular Targets?

Interaction with Heme Iron

Metalloproteins contain redox-sensitive heme groups located at catalytic or allosteric sites. These are natural sensors of redox reactive species such as RNS because iron present in the heme group is a potent oxidizing agent. A variety of heme-containing proteins exhibit RNS-responsive control.

GUANYLYL CYCLASE

Guanosine 3',5'-monophosphate (cGMP) is utilized as an intracellular amplifier and second messenger by a wide spectrum of ligands to elicit diverse physiological responses. cGMP synthesis is catalyzed by multiple types of soluble and particulate guanylyl cyclase.

Soluble guanylyl cyclase is a family of heterodimeric heme proteins exhibiting a pyridine hemochrome visual absorption spectrum typical for ferroprotoporphyrin IX, and it contains copper and iron as transition metals. The activation of soluble guanylyl cyclase by RNS is the principal mechanism of action in various RNS-induced cellular events, such as smooth muscle relaxation (Ignarro and Kadowitz, 1985; Ignarro, 1991) and inhibition of platelet adhesion (Radomski *et al.,* 1987). The cellular consequences of RNS-induced guanylyl cyclase activation are listed in Table I. RNS bind to the heme moiety of guanylyl cyclase and, by disrupting the plane of the heme iron, induce a conformational change that allosterically activates the enzyme. The cGMP synthesized further modulates an array of mediators including ion channels, phosphodiesterases, and protein kinases. Activation of guanylyl cyclase was the first RNS-induced signaling mechanism reported. To date, it remains the most important physiological pathway by which RNS propagate cellular signals.

NITRIC OXIDE SYNTHASE

NOS is a homodimer with a molecular mass of approximately 300 kDa. Its regulation is complex, with checkpoints at transcriptional, posttranscriptional, and posttranslational levels. Interestingly, RNS can inhibit the enzyme activity of NOS itself by posttranslational modification. Unlike guanylyl cyclase, this enzyme utilizes the heme prosthetic group in catalysis. The binding of RNS to either Fe(II) or Fe(III) heme may interfere with the conversion of ferric to ferrous iron required for catalysis and result in attenuation of enzyme activity. Thus, negative feedback inhibition is achieved (Griscavage *et al.,* 1994). This is likely to be a critical event in turning off NO synthesis and to be crucial for maintaining redox homeostasis.

Interaction with Iron–Sulfur Proteins

Iron–sulfur proteins are polymetallic structures whose iron atoms are linked to inorganic sulfides and are usually liganded to proteins by cysteine thiolates. These proteins are sensitive to oxidoreduction and have long been known as targets of O_2^- and H_2O_2. In *in vitro* models, RNS were shown to yield complexes with [Fe–S] clusters. It is thought that peroxynitrite may react with iron–sulfur clusters, and, in contrast to a reversible reaction of RNS with heme, its reaction with iron–sulfur clusters results in the dissolution of the cluster.

ACONITASE

In addition to its participation in the citric acid cycle, the mammalian [4Fe–4S] cytoplasmic aconitase is also a regulator of iron homeostasis. RNS inhibit aconitase activity by disrupting [Fe–S] clusters. This disruption exposes its RNA binding site, permitting binding of the protein, now called the iron regulatory binding protein, to the iron-responsive element on transferrin receptor and ferritin mRNA. When bound to the iron-responsive element at the 3' end of transferrin receptor mRNA, it stabilizes the mRNA. However, when bound to the iron-responsive element at the 5' end of ferritin mRNA, it inhibits translation. Thus, RNS signal

Table I Cellular Events Regulated by the RNS/cGMP Signaling Pathway

Cell type	Cellular event
Smooth muscle	Vasodilation
Platelets	Antiaggregation
Sheep mitral cells	Olfactory memory formation by potentiation of glutamate release
Human neutrophils	Attenuates platelet-activating factor-induced release of elastase
Human myocardium	Negative inotropic effect
Human hepatocellular carcinoma cells	Hypoxic regulation of erythropoietin production
Human γδ T lymphocytes	CD95-induced apoptosis
Bovine chondrocytes	Disruption of focal adhesion signaling complex
Rat smooth muscle, myocytes, pinealocytes, alveolar epithelial	Inhibits spontaneous depolarization of L-type Ca^{2+} channels
Rat thyroid follicular, Rat-2 fibroblasts	Activation of transcription from AP-1 responsive promoters
Rat aorta	Inhibition of α_1-adrenergic receptor-induced c-*fos* and c-*jun* mRNA
Mouse splenic B cells	Inhibition of apoptosis by increasing expression of bcl-2
Rat hippocampus	Downregulation of prodynorphin mRNA, upregulation of proenkephalin mRNA
Rat pheochromocytoma PC12	Activation of transcription factor AP-1
Mouse hypothalamic GT1	Repression of hypothalamic gonadotropin-releasing hormone gene expression

through proteins involved in iron metabolism and regulates iron homeostasis (Drapier *et al.,* 1993; Jaffrey *et al.,* 1994).

SoxR

In response to microbial attack, activated macrophages produce various free radical species, including superoxide anion, hydrogen peroxide, and NO. However, bacteria possess several protective mechanisms against macrophage-induced oxidative and nitrosative stress. SoxR is a bacterial transcriptional activator requiring an iron–sulfur center for its activity. It is a homodimeric protein, and each monomer possess a [2Fe–2S] cluster. Oxidation or nitrosation reversibly activates the FeS centers of SoxR protein which triggers the *soxS* gene. The product of the *soxS* gene further activates transcription of approximately 12 regulon genes, which code for defense proteins such as manganese superoxide dismutase, the DNA repair enzyme endonuclease IV, and glucose-6-phosphate dehydrogenase. Activation of *soxRS* regulon genes confers resistance to attack by activated macrophages. Thus, redox-sensing FeS centers detect oxidative and nitrosative stress and help bacteria survive by triggering the coordinated expression of defense genes (Hidalgo and Demple, 1994).

Interaction with Thiol

Cysteine residues are known to be important for maintaining the native conformation of proteins; they are critical residues at the active sites of enzymes and are the most reactive residues to RNS at physiological pH. Furthermore, cysteine residues are sites for covalent attachment of other regulatory molecules, for example, lipid and ADP-ribose. RNS react with thiols to form a variety of oxidized thiol species including sulfenic acids, disulfides, mixed disulfides, nitrosothiol, and covalent NAD–thiol linkages.

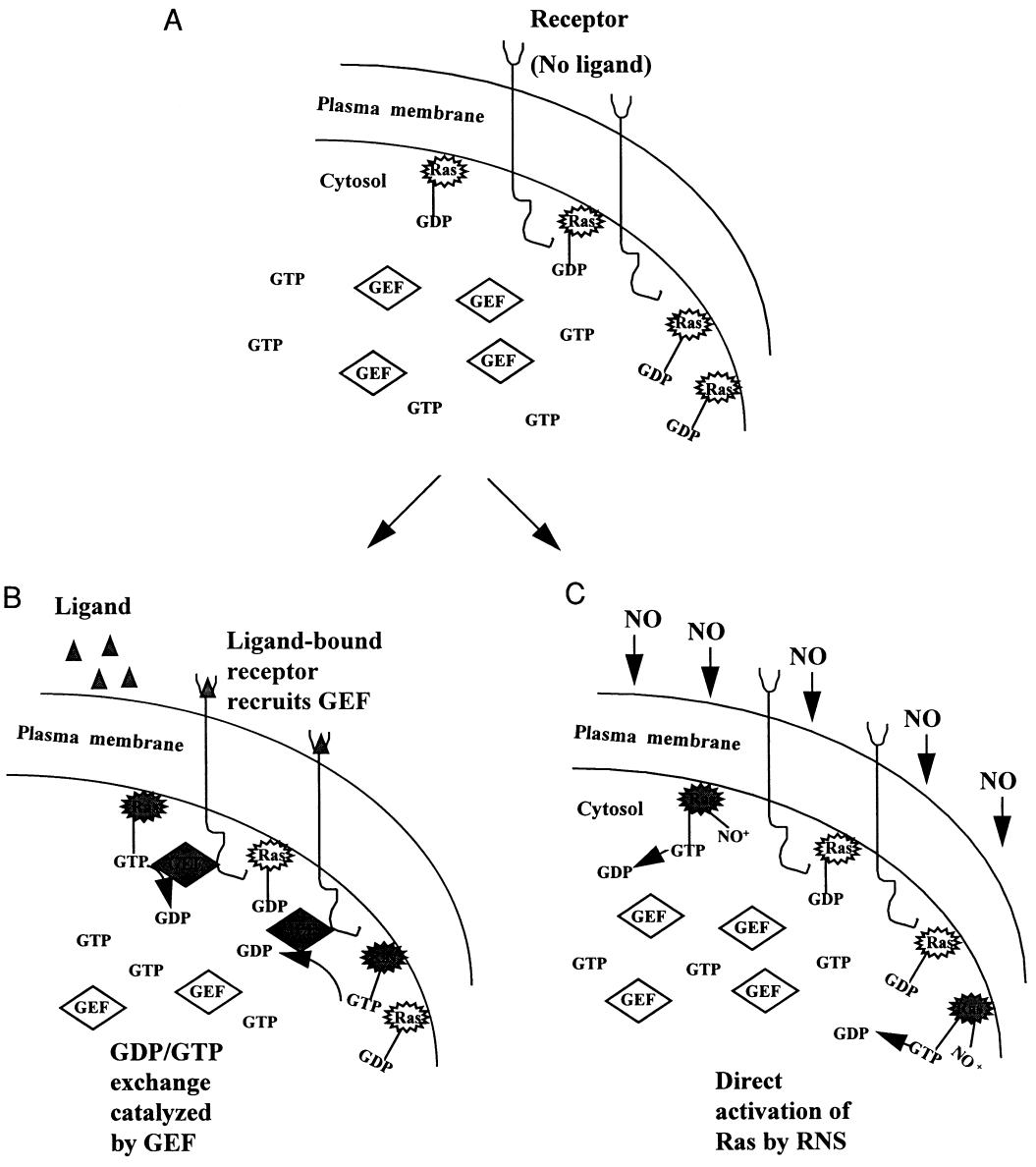
Under physiological aerobic conditions, oxidation of NO yields NO^+-like species that have a high affinity for nucleophilic centers such as thiols, forming nitrosothiol (RSNO). Thiol nitrosation may also be mediated by the oxidative activation of NO through binding to transition metals. Nitrosothiol formation by metal ion-mediated formation of NO^+ is likely to be faster than via reactions of NO and O_2. Also, peroxynitrite anion is capable of nitrosating thiols. Nitrosothiols have a longer half-life than free NO and are an important pool from which redox signals can be generated (Stamler *et al.,* 1992b; Stamler, 1994). The S-nitrosation of protein thiols is a form of posttranslational modification and may either activate or inactivate protein function.

Proteins Activated by *S*-Nitrosothiol Formation

Ras The monomeric G protein Ras is a key element of many signaling pathways. It is implicated in the regulation of proliferation and differentiation by tyrosine kinase and G-protein-coupled receptors. Activation of Ras involves guanine nucleotide exchange factor-mediated exchange of GDP for GTP and subsequent interaction with effector proteins. Effector proteins transduce signals via several pathways and induce cellular responses.

RNS were found to activate Ras in human T cells, rat pheochromocytoma cells, and human endothelial cells. Nitrosothiol formation at a single cysteine residue, Cys^{118} of Ras, was found to trigger GDP/GTP exchange (Lander *et al.,* 1996). Thus, RNS activate Ras by a mechanism akin to that of growth factors. However, it differs in that it bypasses the requirement for guanine nucleotide exchange factors by directly modifying Ras and triggering GDP/GTP exchange (Fig. 6).

Mitogen-activated protein kinases are important components of the Ras-dependent signal transduction pathway. S-Nitrosated Ras was found to trigger activation of three mitogen-activated protein kinases, extracellular signal-regulated kinase, c-Jun NH_2-terminal kinase, and p38 MAP kinase. Interestingly, c-Jun NH_2-terminal kinase and p38 MAP kinase are also activated by proinflammatory cytokines and environmental stress. Further downstream, RNS were found to activate the transcription factor NF-κB. This activation was observed in human peripheral blood mono-



GEF: Guanine nucleotide exchange factor.

Figure 6 RNS modification of Ras bypasses the classic requirement for guanine nucleotide exchange factor (GEF).

nuclear cells, T cells, and pheochromocytoma cells, and it depended on *S*-nitrosothiol formation at Cys^{118} residue of Ras. Identifying the signals immediate to RNS-induced Ras activation (which includes Ras effectors such as phosphoinositide 3-kinase, Raf-1, and protein kinase C-ζ) and downstream intermediate signals leading to NF-κB activation will help in deciphering the RNS-induced Ras pathway. Because Ras is also activated by other redox modulators such as hemin, mercuric chloride, and hydrogen peroxide, it may serve as a general sensor of cellular redox stress and enable the cell to respond appropriately to the external milieu.

Calcium Release Channel (Ryanodine Receptor) Ryanodine receptors belong to a multigene family of channel proteins. They are localized at the junctional sarcoplasmic reticulum in muscle and the endoplasmic reticulum in epithelial and neuronal cells. These receptors are sensitive to the muscle paralyzing alkaloid, ryanodine, and are responsible for release of Ca^{2+} from intracellular stores, which activates contraction. Ion channels are reported to be redox regulated via their sulfhydryl groups. RNS were found to induce ryanodine receptor channel opening and Ca^{2+} release from skeletal and cardiac sarcoplasmic reticulum into the cytoplasm. Polynitrosation of up to 12 free thiols of the cardiac

calcium release channel reversibly activates the channel, which then releases Ca^{2+} from the sarcoplasmic reticulum in response to a muscle action potential. Interestingly, in contrast to S-nitrosation, the oxidation of thiol groups has no effect on channel function, suggesting a specificity of nitrosothiol-induced activation (Xu *et al.,* 1998). Thus, direct interaction of RNS with thiols of the cardiac calcium release channel regulates force in the contrasting muscle and thereby controls excitation–contraction coupling.

Tissue-Type Plasminogen Activator The normal endothelium secretes cardioprotective mediators such as RNS and tissue-type plasminogen activator (t-PA). t-PA is involved in the activation of the fibrinolytic system. S-Nitrosation of t-PA at Cys^{83} endows the enzyme with vasodilatory and antiplatelet properties and enhances the catalytic efficiency of plasminogen activation in the presence of fibrin (Stamler *et al.,* 1992c). S-Nitrosated t-PA was also able to attenuate cardiac necrosis after myocardial ischemia–reperfusion and inhibited a neutrophil–endothelium interaction. This latter effect may involve a decrease in expression of the adhesion molecule, P-selectin. Hence, S-nitrosation of t-PA converts a simple protease into a pleiotropic antithrombotic agent.

Calcium-Dependent Potassium Channels The endothelium controls vascular smooth muscle tone by secreting relaxing and contracting factors. RNS, also called endothelium-derived relaxing factor, activate calcium-dependent potassium channels leading to hyperpolarization of the vascular smooth muscle cell leading to vasodilation. The mechanism of activation involves S-nitrosation of thiols and eventual disulfide formation of vicinal thiols accompanied by the release of NO^- (Bolotina *et al.,* 1994). Hence, RNS regulate vascular tone by activation of calcium-dependent potassium channels, in addition to activation of guanylyl cyclase as discussed above.

PROTEINS INACTIVATED BY NITROSOTHIOL FORMATION

N-Methyl-D-Aspartic Acid Receptor In the central nervous system, glutamate is the main excitatory neurotransmitter. Activation of the *N*-methyl-D-aspartic acid (NMDA) subtype of excitatory amino acid receptors by glutamate results in increased activity of nNOS in the brain. The NO produced acts as a neurotransmitter and is implicated in a variety of functions including synaptic plasticity, cerebral circulation, regulation of circadian rhythm, and production of cerebrospinal fluid. The endogenous NO was found to activate Ras–MAP kinase pathway. However, excessive NMDA receptor activation leads to overproduction of NO, resulting in neuronal damage due to cell death. Paradoxically, the NMDA receptor is inhibited by RNS at a redox modulatory site. Inactivation of NMDA receptor is thought to be due to S-nitrosation at thiol residues and eventual disulfide bond formation (Lipton *et al.,* 1993). RNS provide a neuroprotective signal by inhibiting the NMDA-receptor and hence preventing neural injury caused by high levels of glutamate and RNS (Fig. 7).

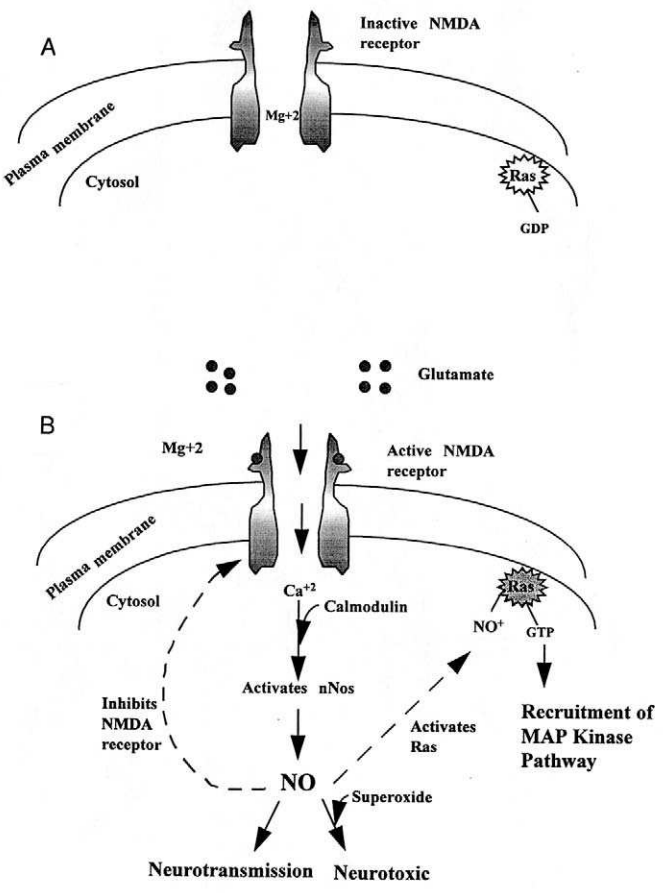


Figure 7 Diverse role of RNS in neurons.

Transcription Factors Transcription factors transduce signals to the transcriptional apparatus by binding to specific DNA sequences of the genes they regulate. Studies suggest that mammalian and bacterial transcription factors can be regulated by S-nitrosation.

NF-κB The classic, inactive cytoplasmic form of NF-κB exists in a trimer of three subunits, p65, p50, and IκBα. On activation, the inhibitory subunit IκBα dissociates, and the dimer of p50/p65 translocates to the nucleus. In addition to NF-κB binding sites in the immunoglobulin κ chain gene, the site has been identified in many other genes including those for cytokines, cytokine receptors, cell adhesion molecules, HIV provirus, and, interestingly, iNOS.

RNS were shown to activate NF-κB in human peripheral blood mononuclear cells. NF-κB activation in human T cells and rat pheochromocytoma cells was found to be dependent on RNS-induced S-nitrosation of Ras at the Cys^{118} residue and subsequent Ras activation (Lander *et al.,* 1995a,b). These studies demonstrate that RNS can trigger an NF-κB response and may provide a mechanistic basis by which RNS trigger NF-κB-dependent gene expression.

In contrast, in nonlymphoid astroglial cells, it was observed that RNS derived from a spermine NONOate inhibits formation of an NF-κB–DNA complex. The direct interac-

tion of RNS with recombinant p50 and p65 subunits was studied, and RNS donors were found to inactivate the DNA-binding activity of the recombinant subunits. The p50 subunit was S-nitrosated at Cys^{62}, and this modification seemed to be responsible for inhibition in DNA binding. The Cys^{62} residue of the p50 subunit is conserved in the NF-κB transcription factor family. Moreover, it is located in the peptide loop that makes specific contacts with the DNA consensus sequence. This residue is redox sensitive, as its oxidation and subsequent intersubunit disulfide linkage also abrogates its DNA binding activity (Matthews *et al.,* 1996). Since the promoter of the iNOS gene contains an NF-κB binding motif, RNS inhibit iNOS gene expression by inhibiting NF-κB activation and thus regulate the enzyme by feedback inhibition.

Hence, like oxidative stress, in some settings nitrosative stress activates NF-κB and may evoke an inflammatory response. In other settings, RNS can downregulate its own synthesis, likely through direct modification of the NF-κB subunits. This dual functionality of RNS highlights the complex signaling behavior of free radicals.

OxyR In a cell, intramolecular disulfide bond formation often results in inactivation of protein activity. However, bacteria utilize this mechanism to activate the transcription factor OxyR, which like SoxR, rescues bacteria from oxidative and nitrosative stress. It induces expression of protective genes such as hydrogen peroxidase I and gluthathione reductase. The conserved residues, Cys^{199} and Cys^{208}, are critical for its activation by oxidative stress. Activation is mediated by intramolecular disulfide bond-induced conformational changes between these two cysteine residues. Nitrosative stress is also known to activate OxyR by reacting with cysteine residues (Zheng *et al.,* 1998). It is worthwhile to note that bacterial transcription factors have evolved sophisticated mechanisms to respond to and survive stressful redox environments.

Interaction with Heme and Thiol

HEMOGLOBIN

When administered systemically, cell-free hemoglobin leads to hypertension. This effect is thought to be due to scavenging of RNS by hemoglobin via its heme iron. Hence, many experimental studies utilize hemoglobin to determine RNS-specific effects. Nevertheless, direct interaction of hemoglobin with RNS was not found to modulate any functional properties of hemoglobin. More recently it was demonstrated that hemoglobin can be S-nitrosated. This discovery highlights the importance of RNS in the respiratory cycle and the dynamic properties of hemoglobin in vasoregulation. In the microcirculation and venous system, RNS reside predominantly on the T-state (deoxy) α-chain heme iron of hemoglobin. The β-chain of hemoglobin possesses a highly reactive thiol group at cysteine-93, which is conserved among mammalian species. When venous blood enters the lungs, oxygen favors an allosteric transition with RNS group exchange from the α-chain heme to the β-chain (Cys^{93}) thiol, and O_2 attaches to the heme. This R state (oxy) structure of hemoglobin then re-enters the circulation and, when faced with an O_2 gradient in resistance vessels, releases both O_2 and RNS. The RNS released may bind to the abundant glutathione present in erythrocytes, and the *S*-nitrosoglutathione formed can then dilate the blood vessels. This augments O_2 delivery to the peripheral tissues (Gow and Stamler, 1998). This highlights how redox-regulated residues are conserved at a strategic site and how the allosteric changes in protein conformation control dynamic functions of the protein.

Cytotoxic Consequences

At high concentrations, RNS are cytotoxic. This action of RNS is utilized as a defense mechanism against pathogens and tumor cells. Some of the cytotoxic signals include the following.

RNS inactivate several mitochondrial iron–sulfur enzymes involved in ATP synthesis. These include NADH:ubiquinone oxidoreductase, NADH:succinate oxidoreductase, and *cis*-acotinase. Inactivation of GAPDH by S-nitrosation inhibits glycolysis. RNS bind the nonheme iron of ribonucleotide reductase, attenuating its activity and inhibiting DNA synthesis. Quenching of tyrosyl radical by RNS may be another mechanism involved in inactivation of this important enzyme. Iron of the iron-storage protein ferritin is also a target of RNS. This interaction leads to the release of free iron, which may cause lipid peroxidation. Like other free radicals, RNS can damage DNA by base deamination, resulting in neurotoxicity (Nathan, 1992). Many of the above cytotoxic events occur at high concentration of RNS and are the causative pathways for cell damage seen in ischemia–reperfusion injury, inflammation, and graft rejection.

The identification of endogenous NO was a hallmark discovery (Furchgott and Zawadzki, 1980; Ignarro *et al.,* 1987). It provided a mechanistic understanding of many physiological processes. Extensive studies in the field of RNS-induced signaling have helped us to appreciate the sophistication as well as simplicity of this protean molecule. In response to environmental cues, RNS can posttranscriptionally modify proteins. This leads to allosteric or catalytic modulation and appropriate signaling. Extensive studies discussed above suggest that S-nitrosation of cysteine is as crucial as phosphorylation of tyrosine, serine, and threonine in signal transduction.

A Shift in Paradigm: Reactive Oxygen Species—From Cytotoxic Agents to Signal Transducers?

Reactive oxygen species (ROS) are commonly associated with a scenario of indiscriminate cellular damage. However, new studies demonstrate that ROS are essential components of redox signaling. At subtoxic concentrations, oxidants

such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals (HO·), and lipid hydroperoxides serve as signaling molecules. Some of the physiological events regulated by ROS include cell growth, cell adhesion, apoptosis, ion transport, neuromodulation, and transcription. Many growth factors, hormones, and cytokines are thought to generate ROS on interacting with their respective receptors. Antioxidants were found to inhibit the signaling induced by platelet-derived growth factor, insulin, angiotensin II, vitamin D_3, parathyroid hormone, transforming growth factor-β1, interleukin 1β, and tumor necrosis factor α. The use of antioxidants has established the role of redox species in the propagation of ligand-stimulated cell signaling (Sundaresan *et al.,* 1995).

How Do ROS Signal?

As discussed previously, two important components of signal transduction are Ca^{2+} signaling and protein phosphorylation. Oxidants were found to utilize these components of the cell machinery to relay signals. Some of the signaling events regulated by ROS include activation of transcription factors AP-1 and NF-κB. ROS were also found to activate the enzyme phospholipase A2, products of which play critical roles in the immune response and inflammation.

Oxidants such as H_2O_2, *tert*-butyl hydroperoxide, and linoleic acid hypoperoxide were found to increase cytosolic Ca^{2+} levels and stimulate Ca^{2+} signaling. Ca^{2+} was found to be effluxed mainly from the sarcoplasmic Ca^{2+}-release channels in muscle cells and inositol phosphate-regulated Ca^{2+} channels on endoplasmic reticulum in endothelial cells. Superoxide induced protooncogene expression in proximal tubular epithelium by mobilizing extracellular Ca^{2+} instead of intracellular stores. Oxidants are even known to recruit Ca^{2+}-binding proteins and the mitochondrial Ca^{2+} store for signaling.

Oxidants also relay signals by modulating protein phosphorylation. They have been reported to activate tyrosine and serine/threonine phosphorylation. Although the mechanism is not yet clear, it may involve direct activation of kinase activity or inhibition of phosphatases. Some of the phosphorylation events modulated by oxidants include activation of insulin receptor thyrosine kinase activity, p56Ick, ZAP-70, p72syk, protein kinase C, and MAP kinase. As discussed in preceding sections, cysteine is a sensor of redox changes. Tyrosine phosphatases and protein phosphatases 1 and 2 have reactive cysteine residues in their active site and hence are highly susceptible to inactivation by oxidant-mediated modification of cysteine residues. However, modification of cysteine in the regulatory domain of protein kinase C triggers its activity (Staal *et al.,* 1994; Suzuki *et al.,* 1997). Redox-induced modification of the CYS^{118} residue of Ras protein is known to activate GDP/GTP exchange on this G protein. Redox modification of cysteine and Fe–S clusters also activates the bacterial transcription factor, OxyR and SoxR, respectively (Lander, 1997). A thiol group of ubiquitin-conjugating enzyme is also reported to be susceptible to redox changes, with S-thiolation inhibiting the enzyme activity (Obin *et al.,* 1998).

Table II Some of the Direct Cellular Targets Utilized by RNS and ROS for Signaling

RNS	ROS
Guanylyl cyclase	Phospholipase A2
Nitric oxide synthase	Insulin receptor
Aconitase	Ras
Ras	Tyrosine phosphatase
Calcium-release channels	Protein phosphotase 1 and 2
Calcium-dependent potassium channels	Protein kinase C
Tissue-type plasminogen activator	Ubiquitin-conjugating enzyme
N-Methyl-D-aspartic acid receptor	SoxR
Hemoglobin	NF-κB
SoxR	OxyR
NF-κB	AP-1
Oxy R	

Cellular homeostasis is a tightly controlled phenomenon with checkpoints and regulation at different levels. The ultiamte outcome of signaling induced by RNS and ROS will depend on various cellular factors. The concentration of reactive species is a very important determinant in signaling. This could be regulated at the level of generation or by the free radical scavengers of the cell. Second, the physicochemical nature of the cellular redox state and targets will also influence signaling. The cellular targets utilized by RNS and ROS for signaling are listed in Table II, and some of the factors that favor RNS and ROS as signaling molecules are summarized in Table III.

In conclusion, RNS and ROS have been identified as second messengers that control crucial physiological events. Although the concept of reactive free radicals as signaling molecules is new and requires one to visualize in a chemical manner, rather than in the traditional steric manner, RNS and ROS should be considered as part of the signal transduction cascade. However, a thin line exists between its role as a cytotoxic or signaling molecule. Studies in redox signaling have highlighted how cells effectively utilize redox chemistry to mediate specific signaling that dictates crucial biolog-

Table III Advantages of RNS and ROS as Cellular Messengers

Cell permeable
High chemical reactivity
Short half-life
Cellular targets located in allosteric or catalytic sites
Modified target is unstable

ical events. Many events in every organ of the body are regulated directly or indirectly by RNS and ROS, and many more significant pathways and cellular outcomes are yet to be deciphered. Hence, in-depth studies in the field of RNS- and ROS-induced signaling and gene expression will provide insights into various physiological processes. Ultimately, elucidating the role of RNS and ROS in cell signaling will yield novel targets for drug development.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994). ''Molecular Biology of the Cell.'' Garland. New York, New York.

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368,** 850–853.

Bredt, D. S., and Synder, S. H. (1994). Nitric oxide: A physiologic messenger molecule. *Annu. Rev. Biochem.* **63,** 175–195.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Synder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles ċytochrome P-450 reductase. *Nature* **351,** 714–718.

Drapier, J. C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kuhn, L. C. (1993). Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *EMBO J.* **12,** 3643–3649.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Gow, A. J., and Stamler, J. S. (1998). Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* **391,** 169–173.

Griscavage, J. M., Fukuto, J. M., Komori, Y., and Ignarro, L. J. (1994). Nitric oxide inhibits neuronal nitric oxide synthase by interacting with their heme prosthetic group: Role of tetrahydrobiopterin in modulating inhibitory action of nitric oxide. *J. Biol. Chem.* **269,** 21644–21649.

Hardie, D. G. (1990). ''Biochemical Messengers: Hormones, Neurotransmitters and Growth Factors.'' Chapman & Hall, London.

Hausladen, A., Gow, A. J., and Stamler, J. S. (1998). Nitrosative stress: Metabolic pathway involving the flavohemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14100–14105.

Hidalgo, E., and Demple, B. (1994). An iron-sulfur center essential for transcriptional activation by the redox-sensing Sox-R protein. *EMBO J.* **13,** 138–146.

Ignarro, L. J. (1991). Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.* **41,** 485–490.

Ignarro, L. J., and Kadowitz, P. J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Ann. Pharmacol. Toxicol.* **25,** 171–191.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9268.

Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E., and Byrns, R. E. (1993). Oxidation of NO in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 8103–8107.

Jaffrey, S. R., Cohen, N. A., Rouault, T. A., Klausner, R. D., and Synder, S. H. (1994). The iron-responsive element binding protein: A target for synaptic actions of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12994–12998.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6348–6352.

Lander, H. M. (1997). An essential role for free radicals and derived species in signal transduction. *FASEB J.* **11,** 118–124.

Lander, H. M., Ogiste, J. S., Pearce, S. F. A., Levi, R., and Novogrodsky, A. (1995a). Nitric oxide-stimulated guanine nucleotide exchange on $p21^{ras}$. *J. Biol. Chem.* **270,** 7017–7020.

Lander, H. M., Ogiste, J. S., Teng, K. K., and Novogrodsky, A. (1995b). $p21^{ras}$ as a common signaling target of reactive free and cellular redox stress. *J. Biol. Chem.* **270,** 21195–21198.

Lander, H. M., Milbank, A. J., Tauras, J. M., Hajjar, D. P., Hempstead, B. L., Schwartz, G. D., Kraemer, R. T., Mirza, U. A., Chait, B. T., Campbell-Burk, S., and Quilliam, L. A. (1996). Redox regulation of cell signalling. *Nature* **381,** 380–381.

Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S. V., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Matthews, J. R., Botting, C. H., Panico, M., Morris, H. R., and Hay, R. T. (1996). Inhibition of NF-κB DNA binding by nitric oxide. *Nucleic Acids Res.* **24,** 2236–2242.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6,** 3051–3064.

Nathan, C., and Xie, Q. W. (1994). Nitric oxide synthases: Roles, tolls and controls. *Cell* **78,** 915–918.

Obin, M., Shang, F., Gong, X., Handelman, G., Blumberg, J., and Taylor, A. (1998). Redox regulation of ubiquitin-conjugating enzymes: Mechanistic insights using the thiol-specific oxidant diamide. *FASEB J.* **12,** 561–569.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987). The anti-aggregating properties of vascular endothelium: Interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.* **92,** 639–646.

Staal, F. J. T., Anderson, M. T., Staal, G. E. J., Herzenberg, L. A., Gitler, C., and Herzenberg, L. A. (1994). Redox regulation of signal transduction: Tyrosine phosphorylation and calcium influx. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 3619–3622.

Stamler, J. S. (1994). Reedox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78,** 931–936.

Stamler, J. S., Singel, D., and Loscalzo, J. (1992a). Biochemistry of nitric oxide and its redox-activated forms. *Science* **258,** 1898–1902.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992b). S-nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stamler, J. S., Simon, D. I., Jaraki, O., Osborne, J. A., Francis, S., Mullins, M., Singel, D., and Loscalzo, J. (1992c). S-nitrosylation of tissue-type plasminogen activator confers vasodilatory and antiplatelet properties on enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 8087–8089.

Sundaresan, M., Yu, Z.-X., Ferrans, V. J., Irani, K., and Finkel, T. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* **270,** 296–299.

Suzuki, Y., Forman, H., and Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radicals Biol. Med.* **22,** 269–285.

Xie, Q.-W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256,** 225–228.

Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998). Activation of cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* **279,** 234–237.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279,** 1718–1721.

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CHAPTER 17

Antioxidant Actions of Nitric Oxide

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AN ANTIOXIDANT IS ANY SMALL MOLECULE OR PROTEIN THAT INHIBITS OXIDATION REACTIONS, IRRESPECTIVE OF THE MECHANISM. INHERENT CELLULAR PROPERTIES OR CHANGES IN DIFFERENTIATED CELL FUNCTION THAT LEAD TO INHIBITION OF MOLECULAR OXIDATION REACTIONS CAN ALSO BE PERCEIVED AS ANTIOXIDANT IN NATURE. THIS IS A NECESSARILY BROAD VIEW, BECAUSE THE OXIDANTS FORMED BY BIOLOGICAL SYSTEMS COME IN A BROAD ARRAY OF SHAPES AND SIZES, WITH CORRESPONDING VARIATIONS IN STABILITY, CHEMICAL REACTIVITY, AND SPECIFICITY. SIMILARLY, THE REGULATORY MECHANISMS THAT CONTROL CELLULAR OXIDANT PRODUCTION, INJURIOUS RESPONSES, AND DEFENSE REACTIONS REQUIRE A BROAD VIEW OF ANTIOXIDANT REACTIONS. WHILE EMBRACING THIS EXPANSIVE DEFINITION OF AN ANTIOXIDANT, WE ARE NOW FACED WITH THE CHALLENGE OF UNDERSTANDING THE PROFOUND INFLUENCES THAT NO EXERTS IN REGULATING BOTH PRO-OXIDANT AND ANTIOXIDANT REACTIONS AT THE CELL AND MOLECULAR LEVEL. THIS CHEMICALLY SIMPLE MOLECULE ENLISTS MULTIPLE MOLECULAR MECHANISMS WHEN MEDIATING ANTIOXIDANT ACTIONS AND ACTS AT MULTIPLE LEVELS IN TISSUES, OFTEN SIMULTANEOUSLY. THESE MECHANISMS INCLUDE DIRECT CHEMICAL TERMINATION OF FREE RADICAL REACTIONS, COMPLEXATION WITH REACTIVE METAL CENTERS, REGULATION OF GENE EXPRESSION OF KEY OXIDANT SCAVENGERS, AND ALTERATION OF INFLAMMATORY CELL–TARGET CELL INTERACTIONS. FOLLOWING, WE ADDRESS THE CONCEPT OF ''INACTIVATING'' NO BY REACTION WITH SUPEROXIDE ($O_2^{\cdot-}$) AND PRESENT THE PRINCIPAL MECHANISMS WHEREBY NO CAN MANIFEST OXIDANT-PROTECTIVE PROPERTIES. IT IS IMPORTANT TO NOTE THAT THE FOCUS OF THIS CHAPTER IS TO DEFINE MECHANISMS UNDERLYING DIRECT PRO-OXIDANT AND ANTIOXIDANT ACTIONS OF NO. ALTHOUGH OTHER PROPERTIES OF NO, ALONG WITH CELLULAR LOCALIZATION AND MODULATION OF NITRIC OXIDE SYNTHASES, ALSO PLAY AN IMPORTANT ROLE IN CONTROLLING NO FUNCTION, THESE ARE NOT DISCUSSED IN DETAIL AS THEY ARE COVERED IN DEPTH ELSEWHERE IN THIS VOLUME.

Radical Termination Reaction between Nitric Oxide and Superoxide—Not an Antioxidant Property of Nitric Oxide

After the description of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO), there was early confusion as to whether the reaction of NO with superoxide ($O_2^{\cdot-}$) was pro-oxidative or antioxidant in nature, since the interaction of $O_2^{\cdot-}$ with NO often induced subsequent abrogation of NO-dependent physiological responses (Rubanyi *et al.,* 1991). Although this was termed an inactivation or scavenging reaction, confusion arose from the failure to appreciate at the time that the ''inactivation'' product of the diffusion-limited reaction of NO with $O_2^{\cdot-}$ was actually a potent oxidizing and nitrating species, peroxynitrite ($ONOO^-$)

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(Beckman *et al.,* 1990; Hogg *et al.,* 1992; Ischiropoulos, 1998). We now know that when $ONOO^-$ is formed in the predominant detection system for $O_2^{\cdot-}$, the reduction of ferrous cytochrome *c* (Cyt c^{2+}), the $ONOO^-$ will reoxidize ferric cytochrome *c* (Cyt c^{3+}), in addition to mediating other heme and amino acid modifications of this protein [Eqs. (1) and (2)] (Thomson *et al.,* 1995; Cassina *et al.,* 2000).

$$\text{Cyt } c^{3+} + O_2^{\cdot-} \rightarrow \text{Cyt } c^{2+} + O_2 \quad (1)$$

$$\text{Cyt } c^{2+} + ONOO^- \rightarrow \text{Cyt } c^{3+} + NO_2^-, NO_3^- \quad (2)$$

Thus, when investigators were measuring the influence of endogenously produced or exogenously administered NO on cell $O_2^{\cdot-}$ production, a secondary oxidant was being produced that also impairs $O_2^{\cdot-}$ detection. Knowledge of the oxidative chemistry and cell biology of the reaction of NO with $O_2^{\cdot-}$ has advanced significantly since then and can be broadly summarized by Fig. 1. The reaction of NO with $O_2^{\cdot-}$ to yield $ONOO^-$ generates both oxidized and nitrated products on reacting with a diverse array of biomolecules. Both the oxidation and nitration products of $ONOO^-$ will manifest secondary biological effects; thus, the view that $O_2^{\cdot-}$ inactivates NO is a simplification that does not take into account the diverse biological effects of this reactive nitrogen species.

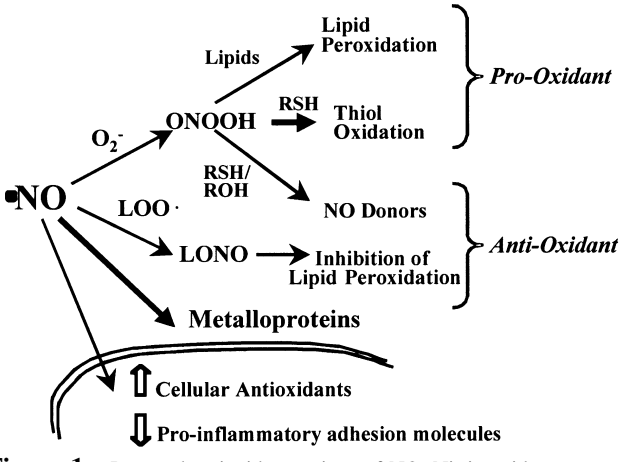


Figure 1 Pro- and antioxidant actions of NO. Nitric oxide can exert both pro-oxidant and antioxidant effects, depending on other reactants and on the nature of the reaction. Pro-oxidant effects associated with NO are generally attributed to formation of $ONOO^-$ and subsequent oxidation of lipids or thiols. For example, lipid peroxidation of low density lipoprotein (LDL) is a key step in the oxidative hypothesis of atherosclerosis, and oxidation of cellular thiols, namely, glutathione, depletes antioxidant levels and thus decreases the capacity of cells to survive an oxidative insult. On the other hand, $ONOO^-$ can exhibit antiatherosclerotic effects via reaction with thiols and hydroxide functional groups on carbohydrates, yielding NO donor compounds. Release of NO from these compounds can then protect the vasculature through a number of mechanisms, including direct reaction with LOO·, which results in a termination of lipid peroxidation. Furthermore, long-term exposure of vascular cells to NO can increase cellular antioxidants and downregulate expression of adhesion molecules during inflammation. Important metalloproteins modulated by NO activity include lipooxygenases and cyclooxygenases. Peroxynitrite also reacts with carbon dioxide in a reaction that promotes tyrosine nitration.

In broad perspective, it is generally viewed that the reaction of NO with $O_2^{\cdot-}$ will lead to pro-oxidative effects, but there are always exceptions in complex biological milieus. For example, NO might divert $O_2^{\cdot-}$ reactivity away from a particularly toxic target molecule or endpoint reaction, and vice versa. At low concentrations, $ONOO^-$ may act as a signaling molecule, explaining cytoprotective effects that have been reported in both platelets and vascular systems (Go *et al.,* 1999; Moro *et al.,* 1994; Brown *et al.,* 1998). In a cellular milieu this could be occurring through the competitive and kinetically favorable ''redirection'' of the reactivities of both NO and $O_2^{\cdot-}$ to the formation of the highly thiol-reactive product $ONOO^-$. The net outcome can then be the rerouting of the individual free radical reactivities of NO and $O_2^{\cdot-}$ via $ONOO^-$ formation and its subsequent reaction with the regenerable antioxidant glutathione (GSH) and modification of thiol-containing proteins in signaling cascades.

Regulation of Nonenzymatic and Enzymatic Lipid Peroxidation

General Aspects of Lipid Oxidation

One of the first indications that NO could inhibit lipid peroxidation came from studies of the heme protein- and metal-initiated lipid peroxidation reactions underlying food rancidification (Kanner, 1979). Indeed, it had long been known that oxidation of meat could be prevented by the addition of nitrite (NO_2^-), resulting in the formation of a nitroso complex with the heme iron of myoglobin. More recently, concepts related to this and other mechanisms of regulation of lipid oxidation by NO have been extended to understanding the pathophysiology of human diseases. Most notably, in atherosclerosis it has been argued that lipid peroxidation plays a central pathogenic role. We now have more insight into the protective roles that NO can play toward oxidizing membrane and lipoprotein lipids (Rubbo *et al.,* 1994; Hogg and Kalyanaraman, 1999; O'Donnell *et al.,* 1997), and we present as background some of the basic elements of lipid peroxidation biochemistry.

Nitric oxide is an ideal molecule to mediate lipid oxidative reactions. First, as a pro-oxidant, it reacts with $O_2^{\cdot-}$ to yield the initiator of lipid oxidation, $ONOO^-$. In contrast, from an antioxidant perspective, the physical properties of NO result in a highly lipid-protective role in the absence of $O_2^{\cdot-}$ levels that would support significant $ONOO^-$ production. The unpaired electron of NO makes it possible for NO to readily react with other radical intermediates formed during lipid oxidation and thus terminate radical reaction sequences. The charge neutrality of NO, its lipophilicity, and its low Stokes radius also permits facile diffusion through as well as concentration in membranes and lipoproteins (Liu *et al.,* 1998). Finally, the direct or indirect reactivity of NO with heme-, iron–sulfur-, and thiol-containing proteins will modulate downstream reactions that can either stimulate or inhibit lipid oxidation.

Controlled lipid peroxidation reactions are essential in normal physiology. For example, the directed and stereospecific insertion of oxygen into unsaturated fatty acids is used by lipoxygenases and cyclooxygenases to generate a family of lipid oxidation products that serve as signaling molecules. Nonenzymatic lipid oxidation mechanisms lack this specificity and, in the absence of sacrificial antioxidants, have the capacity for uncontrolled amplification reactions of increasing intensity, known as propagation reactions.

The different facets of lipid peroxidation reactions start with the initiation event, in which a hydrogen atom is abstracted from an unsaturated fatty acid, yielding L· (Fig. 2). In some cases not only radical species, but also metal complexes and metalloproteins can serve to abstract hydrogen atoms from unsaturated fatty acids (as in the case of lipoxygenases and cyclooxygenase). Then, molecular oxygen rapidly adds to yield an alkyl peroxyl radical (LOO·). The next phase involves propagation reactions, catalyzed by the reaction of peroxyl radical intermediates with vicinal unsaturated fatty acids. At this stage, the lipid peroxidation process is susceptible to termination by lipophilic antioxidants (e.g., tocopherols, carotenoids, lycopenes), via reduction of the peroxyl radical to a hydroperoxide (LOOH). Reduced metals enter into the reaction scheme by reacting with LOOH species and forming the alkoxyl radical (LO·) and epoxyallylic radical species [L(O)·], both of which are capable of stimulating lipid oxidation by catalyzing further propagation reactions. Importantly, NO can act at two key steps in this reaction pathway to inhibit lipid peroxidation. One is the kinetically preferred termination of lipid peroxyl radicals via NO reaction with LOO· intermediates, and the other is by reaction with catalytic metal centers that can then either initiate or propagate lipid peroxidation. As will be described, NO can (a) inhibit controlled enzymatic reactions that generate oxidized lipid signaling molecules and (b) limit membrane and lipoprotein oxidation reactions. Diverse mechanisms will initiate lipid peroxidation when tissues are exposed to inflammatory mediators or redox-active xenobiotics. Before addressing these mechanisms, it is important to convey that secondary products of NO are also capable of initiating lipid oxidation.

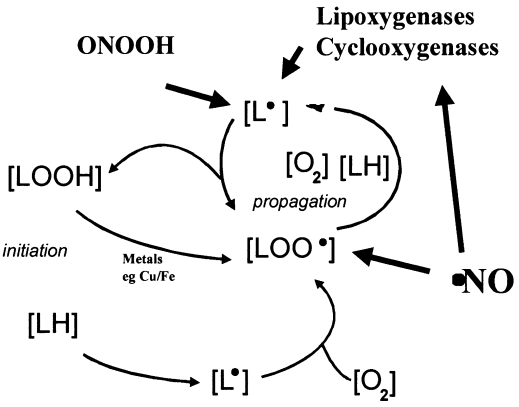


Figure 2 Lipid peroxidation and targets for NO interaction. In this scheme, lipid oxidation is initiated in an unsaturated fatty acid (LH) to form the alkyl radical (L·). The alkyl radical in turn reacts rapidly with oxygen, thus precluding effective scavenging of this species, and forms the peroxyl radical (LOO·). Peroxyl radicals react slowly with vicinal lipids in the propagation phase of peroxidation (dotted lines) and can thus be effectively scavenged.

Initiation of Lipid Peroxidation by NO-Derived Species

A number of NO-derived reactive species can initiate lipid peroxidation, including nitrogen dioxide (NO_2·) and, most notably, $ONOO^-$, which displays unique properties as a mediator of lipid oxidation. On a molar basis, $ONOO^-$ is a more potent lipid oxidant than hydrogen peroxide (H_2O_2), and, unlike H_2O_2, it does not require metal catalysis (Radi *et al.,* 1991). Lipid oxidation mechanisms frequently depend on metal-mediated decomposition of a preexisting lipid peroxide, whereas $ONOO^-$ can attack lipids in microenvironments where metals are not present or are bound in catalytically inactive forms. Typically, initiation of lipid oxidation by free radical or oxidizing species in key targets such as low density lipoprotein (LDL) requires depletion of endogenous antioxidants to a critically low level before significant unsaturated fatty acid oxidation occurs; however, this precept is now not felt to be applicable to atherosclerotic lesions (Niu *et al.,* 1999). Importantly, because of its reactivity, $ONOO^-$ can bypass lipophilic antioxidant defenses to directly oxidize unsaturated fatty acids (Darley-Usmar *et al.,* 1992). Thus, once formed, $ONOO^-$ is capable of promoting a number of reactions that are potentially atherogenic, including the oxidation of LDL (Hogg *et al.,* 1992).

Finally, the inflammatory conditions during which NO and $ONOO^-$ are generated can have relatively greater or lower concentrations of $O_2^{\cdot-}$. This leads to the interesting manifestation of either pro-oxidant or antioxidant actions of NO (Figs. 1 and 3) (Rubbo *et al.,* 1994). When NO does not exceed local $O_2^{\cdot-}$ concentrations, NO will stimulate lipid oxidation via $ONOO^-$ formation and reaction. When NO is replete in a microenvironment and in "excess" of $O_2^{\cdot-}$, it can manifest a predominantly inhibitory role toward lipid peroxidation via termination of LOO·. Increases in rates of tissue NO production may thus restore the balance between NO and $O_2^{\cdot-}$ in favor of NO and suppress both chemical and secondary cellular components of atherosclerosis or other tissue inflammatory processes (Darley-Usmar *et al.,* 1992). This lipid-protective action of NO is even more likely to occur when non-superoxide-dependent mechanisms are initiating lipid oxidation (e.g., reduced transition metals, peroxyl radicals), because $ONOO^-$ formation would not be expected. In support of these concepts, it is noted that NO donor agents protect cells from damage elicited by transition metals, H_2O_2, alkyl hydroperoxides, oxidized LDL, and xanthine oxidase-derived $O_2^{\cdot-}$ and H_2O_2 (Malo-Ranta *et al.,* 1994; Gutierrez *et al.,* 1996; Rubbo *et al.,* 1996; Jessup *et al.,* 1992; Hogg *et al.,* 1993; Yates *et al.,* 1992). The precur-

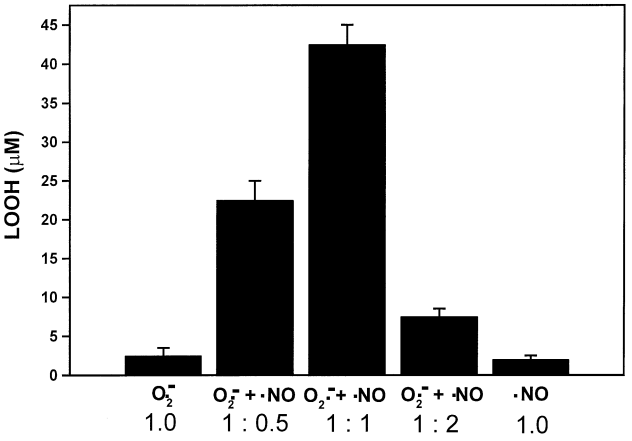


Figure 3 The influence of different rates of $O_2^{\cdot-}$ and ·NO generation on liposome membrane lipid oxidation. Phosphatidylcholine (PC) liposomes (6.6 mg ml^{-1}) were oxidized for 30 min in stirred incubations containing 50 m*M* potassium phosphate, 100 μ*M* EDTA-Fe^{3+}, pH 7.4. Superoxide was generated by xanthine oxidase (XO) plus 10 m*M* acetaldehyde, and GSNO was used as a source of NO. XO and GSNO concentrations ranged up to 5 mU ml^{-1} for XO and 660 μ*M* for GSNO and were calibrated to give the noted proportional rates of $O_2^{\cdot-}$ and ·NO production, with maximal rates of radical production being 1 μ*M* min^{-1} for each at a ratio of 1:1 for $O_2^{\cdot-}$ and ·NO (adapted from Rubbo *et al.,* 1994).

sor of NO synthesis, L-arginine, may also work through a similar mechanism (Drexler *et al.,* 1991; Cooke *et al.,* 1992).

Radical Termination Reactions between Nitric Oxide and Peroxyl Radicals

It is evident from the previous sections that more than one mechanism for initiation of lipid oxidation may be important in atherosclerosis and other inflammatory diseases, lending support to the strategy of targeting protective interventions toward the second stage of the peroxidation process, LOO·-mediated propagation reactions, rather than by limiting initiation events. The importance of LOO· in membrane and lipoprotein oxidation lies in the fact that it allows lipid peroxidation to be self-sustained through regeneration and recruitment of more unsaturated fatty acid into the cycle (Fig. 2). In this milieu of autocatalytic propagation reactions, NO can intervene by avidly reacting with lipid epoxyallylic radical [L(O·)], alkoxyl radical (LO·), and LOO· species with rate constants of $\sim 2 \times 10^9\ M^{-1}\ s^{-1}$, yielding both nitrogen oxide- and hydroperoxide-containing products (Wallington *et al.,* 1992; O'Donnell *et al.,* 1999a; Maricq and Szente, 1996; Frost and Smith, 1990; Padmaja and Huie, 1993). The reaction of NO with LOO· species would thus readily predominate over the much slower initiation of secondary peroxidation propagation reactions by LOO· with vicinal unsaturated lipids ($k = 1.3 \times 10^3\ M^{-1}\ s^{-1}$) (Leibler, 1994).

When inhibiting lipid peroxidation propagation reactions, NO undergoes an initial termination reaction with organic peroxyl radicals to form organic peroxynitrites (LOONO). During this overall reaction, two molecules of NO are consumed, because the original LOONO that is formed rapidly decomposes ($t_{1/2} = 0.2$–0.6 s) to species that react with additional NO (O'Donnell *et al.,* 1997). Alternatively, the LOONO can hydrolyze to yield LOOH and NO_2^- (O'Donnell *et al.,* 1999b).

Nitric Oxide–α-Tocopherol Interactions in Lipid Oxidation

The body possesses endogenous protection mechanisms designed to prevent membrane and lipoprotein lipid oxidation, with the most widely recognized defensive molecule being vitamin E, and in particular the vitamin E isomer α-tocopherol. However, the often restricted location of α-tocopherol, especially in LDL, and poor bioavailability can constrain its effectiveness as a lipid antioxidant (Kayden and Traber, 1993; Dieber-Rotheneder *et al.,* 1991). Phenolic antioxidants such as α-tocopherol function through the donation of the hydrogen atom of a phenolic group to the peroxyl radical, to form a stable hydroperoxide [Eq. (3)]:

$$\text{LOO}\cdot + \text{ArOH} \rightarrow \text{LOOH} + \text{ArO}\cdot \qquad (3)$$

The kinetic efficiency of an antioxidant compound must be high, because propagation of lipid peroxidation, albeit slow ($k = 1.3 \times 10^3\ M^{-1}\ s^{-1}$), is a self-sustaining reaction that may quickly overwhelm chain-breaking antioxidants. The first requirement of an effective lipid antioxidant is that the compound must react with LOO· faster than this propagating species can react with adjacent unsaturated fatty acids. It is important to note that this is a necessary but not sufficient condition for suppression of lipid peroxidation. Second, the reactions in which the newly formed antioxidant radical initiates further peroxidation or reconverts the LOOH to LOO· must be slow. By these criteria, α-tocopherol gives mixed results. First, the reaction of α-tocopherol with LOO· to yield the α-tocopheroxyl radical is not fast, occurring at a rate constant of $\sim 5 \times 10^5\ M^{-1}\ s^{-1}$, with this rate affected by the alkyl chain length, degree of unsaturation, and esterified nature of the fatty acid undergoing peroxidation (Serbinova and Packer, 1994). Because of resonance stabilization of the phenoxyl radical and its potential subsequent rereduction by ascorbate, thiols, or enzyme-dependent mechanisms, the α-tocopheroxyl radical product has only limited capability to further propagate radical chain reactions under most biological conditions (Kagan and Packer, 1994).

Interestingly, on the basis of relative rate constants, it is predicted that in most biological conditions the reaction of ·NO with LOO· will predominate over both the reduction of LOO· by α-tocopherol and the initiation of secondary peroxidation propagation reactions by LOO· with vicinal unsaturated lipids. Thus, in tissues having an ambient concentration of 50 n*M* NO and 10–20 μ*M* α-tocopherol, the efficiency of the two molecules in reacting with LOO· is approximately equal. When it is also considered that (a) NO is replenished by an enzymatic mechanism, (b) NO concentrates 20- to 30-fold in a lipophilic milieu, and (c) NO is freely diffusible and thus can terminate lipid and possibly

protein radical species with little or no regard to the spatial orientation of the radical intermediate within membrane or lipoprotein microenvironments, NO appears to be a more versatile antioxidant (O'Donnell *et al.,* 1997; Rubbo *et al.,* 2000).

Because the scavenging of LOO· results in the formation of an α-tocopheroxyl radical, its fate must also be considered. This is crucial, as the scavenging of propagating peroxyl radicals is limited by the available number of electrons that can be channeled to the termination reaction. Physiologically, this problem is overcome through a series of redox shuttles that ultimately utilize cellular metabolism as the source of reducing equivalents (Buettner, 1993). An example of this type of mechanism is the recycling of the α-tocopheroxyl radical by ascorbate (Sato *et al.,* 1990). Under conditions where the lipid peroxidation reaction occurs in a compartment to which the ascorbate radical has restricted access, such as the lipid rich core of a lipoprotein, inhibition of peroxidation will be synergistic (Kalyanaraman *et al.,* 1992). In the case of NO, its activity as an antioxidant is coupled to the reducing equivalents in the cell (e.g., NADPH) that are required for NO synthesis by nitric oxide synthases (NOS). Thus, the level of antioxidant protection could be controlled in inflammatory sites by the availability and diffusion characteristics of NO.

It has been observed in more complex and multiple-antioxidant-containing oxidizing lipid systems that NO preferentially reacts with lipid radical species, with α-tocopherol consumption not occurring until ·NO concentrations fell below a critical level (Rubbo *et al.,* 2000; Goss *et al.,* 1995). In addition, α-tocopherol and ·NO cooperatively inhibited lipid peroxidation, exhibiting greater antioxidant capacity than the pair α-tocopherol/ascorbate. Pulse radiolysis analysis showed no direct reaction between ·NO and α-tocopheroxyl radical, inferring that peroxyl radical termination reactions were the principal lipid-protective mechanism mediated by ·NO. These observations reaffirm the concept that ·NO is a potent chain-breaking antioxidant toward peroxidizing lipids, via termination reactions with lipid radical species, thus preventing α-tocopherol loss. In comparison, the rereduction of α-tocopheroxyl radical by ascorbate was a comparatively less efficient mechanism for preserving α-tocopherol than ·NO-mediated termination of peroxyl radicals, owing to slower ascorbate–α-tocopherol and ascorbate–LOO· reaction kinetics and limited transfer of reducing equivalents from ascorbate in the aqueous phase.

Nitric Oxide Modulation of Enzymatic Lipid Oxidation Processes

Enzymatic oxidation products of unsaturated lipids activate signal transduction reactions via interaction with G-protein-coupled receptors. The best known oxidized lipid mediators are arachidonate-derived eicosanoids, including prostaglandins, thromboxanes, leukotrienes, and epoxides. These species are generated through oxidation reactions coordinated by prostaglandin endoperoxide H synthase (PGHS), lipoxygenases (LOX), and cytochrome P-450 isoenzymes.

Generally, NO is inhibitory toward the enzymatic generation of oxidized lipid mediators. Because enzyme-catalyzed lipid oxidation products, especially hydroperoxide derivatives, can decompose through pro-oxidant and nonenzymatic propagation reactions, NO-mediated inhibition of lipid-oxidizing enzymes will ultimately result in limiting of both enzymatic and nonenzymatic lipid oxidation reactions. The inhibition of enzymatic lipid oxidation results from either (a) termination of lipid radical intermediates (LOX) or (b) formation of heme–nitrosyl complexes (cytochrome P-450). The exception to this paradigm is PGHS, where inhibition, activation, or no effect has been variously observed, depending on the cellular system examined.

Prostaglandin Endoperoxide H Synthase

Prostaglandins (PG) are generated by PGHS, of which there are both constitutive (PGHS-1) and inducible (PGHS-2) isoforms. As a first step in catalysis, the enzyme oxidizes arachidonic acid to a cyclic endoperoxide, prostaglandin G_2 (PGG_2), by a cyclooxygenase activity, then a peroxidase reduces the peroxide to a hydroxide. This concerted catalytic event ultimately yields the endoperoxide prostaglandin H_2 (PGH_2) (Smith and De Witt, 1996). The peroxidase and cyclooxygenase activities of PGHS are separated by the heme prosthetic group and function independently of one another. Under inflammatory conditions, PGHS-2 expression is upregulated by proinflammatory cytokines, which include interleukin-1 (IL-1) and tumor necrosis factor (Swierkosz *et al.,* 1995; Akarasereenont *et al.,* 1995; Vane *et al.,* 1994; De Witt, 1991; Davidge *et al.,* 1995). As these cytokines also induce inducible NOS (iNOS), high levels of both PG and NO will be produced together *in vivo.*

Reactive nitrogen species have multiple effects on PGHS. In assay systems, including purified PGHS-2, platelets, endothelial cells, and RAW 264.7 cells, and *in vivo,* in rats, NO potently stimulates PG production (Davidge *et al.,* 1995; Salvemini *et al.,* 1993, 1994; Manfield *et al.,* 1996). However, other investigators have found NO either to inhibit PGHS or to have no effect (Stadler *et al.,* 1993; Curtis *et al.,* 1996; Nakatsuka and Osawa, 1994). In contrast, for some cell types (rat microglial cells and peritoneal macrophages), NO suppresses LPS-induced PGHS-2 expression, resulting in apparent enzyme inhibition (Minghetti *et al.,* 1996; Habib *et al.,* 1997). Probable mitigating factors in these opposing outcomes, in addition to the manifestation of unique differentiated cell responses to NO, can include variations in NO concentration and underlying rates of oxidant production by cells.

Nitric oxide can interact with PGHS during turnover in several ways. These include formation of an iron–nitrosyl complex (K_d = 0.92 m*M*) (Tsai *et al.,* 1994), termination of the catalytic tyrosyl radical, reduction of compounds 1 and 2 of the peroxidase cycle, and possible termination of enzyme-bound lipid alkyl or peroxyl radicals (Curtis *et al.,* 1996; Goodwin *et al.,* 1998; Gunther *et al.,* 1997). Following

reaction of NO with the heme, catalytic tyrosyl radical, or lipid radical intermediates, enzyme inhibition would be expected. However, NO reaction with compounds 1 and 2 would likely lead to enzyme activation. Intriguingly, a balance between inhibitory and activating effects of NO may also take place, as NO has little effect on the activity of purified PGHS (Curtis *et al.,* 1996). It has also been shown that $ONOO^-$ is a peroxidase substrate for both PGHS isoforms (Landino, 1996). This suggests that $ONOO^-$ could promote PG synthesis during inflammatory events, where accelerated rates of production of NO and $O_2^{\cdot-}$ can be occurring.

LIPOXYGENASES

Lipoxygenases are non-heme, iron-containing enzymes that oxidize polyenoic fatty acids, forming the corresponding lipid hydroperoxides. In mammalian systems, at lease three isoforms are known. The best characterized, 5-LOX, is found in leukocytic cells (Ford-Hutchinson, 1994). 5-LOX generates precursors for the synthesis of leukotrienes, lipid products that stimulate a number of inflammatory and immune cell functions, including chemotaxis and aggregation, as well as playing central roles in inflammation and allergies. 12-LOX is found predominantly in platelets and monocytes. The *in vivo* activities of its product, 12(S)HPETE (12(S)hydroperoxyeicosatetraenoic acid), are unknown, but *in vitro* studies show that it inhibits platelet aggregation (Brune *et al.,* 1991; Nyby, 1996). 15-LOX is highly expressed in reticulocytes during maturation, where it degrades intracellular membranes. Expression of 15-LOX can be induced in monocytes by IL-4 and IL-13 (Nassar, 1996; Conrad, 1992). The precise role of 15-LOX products are unknown, but *in vitro* effects include stimulation of epidermal growth factor receptor signaling (Glasgow, 1997). A central role for 15-LOX in initiation and progression of atherosclerosis has been suggested by the observation that its products are found in atherosclerotic lesions (Kuhn, 1994, 1997; Folcik, 1995).

Lipoxygenases contain a single nonheme iron that cycles between Fe^{2+} and Fe^{3+} during turnover. Resting LOX is predominantly reduced and requires oxidation by hydroperoxides before dioxygenation can take place. Oxidation of arachidonate or linoleate is then catalyzed by the ferric enzyme as shown in Fig. 4.

Inhibition of several LOX isoforms (soybean, rabbit, and human 15-LOX, human platelet 12-LOX) by NO has been reported (Holzhutter, 1997; Weisner, 1996; Kanner, 1992; O'Donnell, 1996, Natasuka, 1994) and was originally suggested to be due to formation of an iron–nitrosyl complex with the ferrous enzyme. However, this species has been shown to be formed only at high NO concentrations, and thus its involvement in enzyme inhibition under biological conditions is unlikely (Rubbo, 1995; Nelson, 1987; Salerno, 1979; Galpin, 1978). More recent studies have shown that NO is consumed by LOX in a turnover-dependent manner. Also, LOX is inhibited only while NO is present. Mechanistic studies indicate that LOX inhibition is a result of a termination reaction between NO and the enzyme-bound lipid peroxyl radical (O'Donnell *et al.,* 1999b). Following this, dissociation and hydrolysis of the organic peroxynitrite (LOONO) forms LOOH and NO_2^- as products. To complete the catalytic cycle, reoxidation of the enzyme-bound iron is required. Since this is a slow process that proceeds at 20% of the rate of dioxygenation for the soybean 15-LOX, suppression of activity is observed.

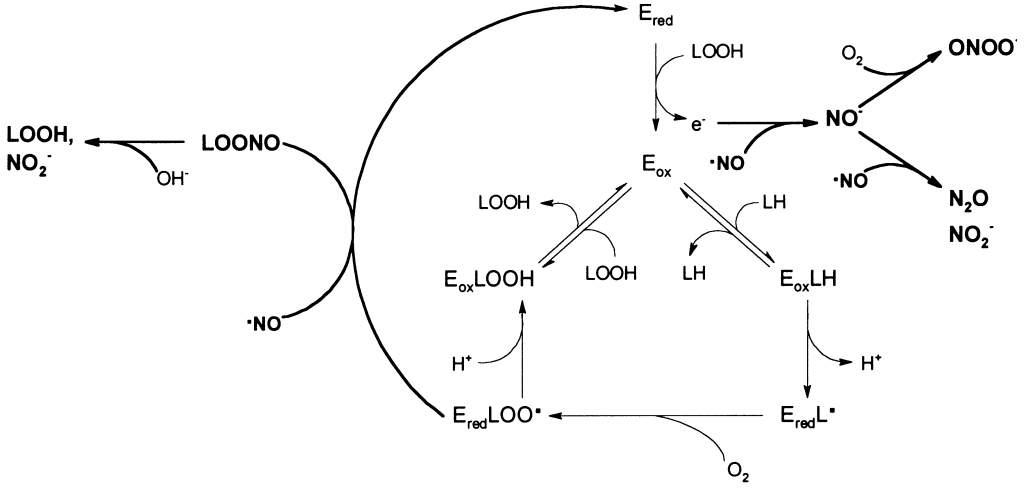


Figure 4 Lipoxygenase-mediated fatty acid oxidation and the influence of NO. Three sites of potential NO reaction are shown: (i) During peroxide (LOOH) activation of LOX, 2 mol ·NO are consumed, via reaction with an electron (e^-) released from the ferrous enzyme (E_{red}) to form nitroxyl anion (NO^-). Secondary reactions of NO^- will consume further ·NO. For example, reaction of NO^- with O_2 or with ·NO can occur as shown. (ii) During dioxygenase turnover, ·NO is consumed through reaction with $E_{red}LOO\cdot$ to form reduced inactive enzyme (E_{red}) and an organic peroxynitrite (LOONO) that can be hydrolyzed to the hydroperoxide (LOOH) and nitrite (NO_2^-). (iii) Finally, at higher ·NO concentrations, a ferrous nitrosyl complex may form ($E\text{-}Fe^{2+}\text{-}\cdot NO$) that slowly decomposes, yielding active enzyme (E*) (adapted from O'Donnell *et al.,* 1999b).

CYTOCHROME P-450

Cytochrome P-450 isozymes are a family of heme-containing enzymes that play central roles in xenobiotic metabolism and lipid oxidation reactions. Arachidonic acid oxidation by cytochrome P-450 occurs through three specific pathways, yielding a variety of oxygenated metabolites, including epoxides and fatty acid alcohols (Capdevila, 1992).

Nonhepatic cytochrome P-450-derived arachidonate metabolites act as intracellular signaling molecules in vascular tissue. The cytochrome P-4504A product 20-hydroxyeicosatetraenoic acid (20-HETE) is a potent vasoconstrictor, produced in vascular smooth muscle cells (Harder, 1997). A second product, 11,12-epoxyeicosatetraenoic acid (11,12-EET), is produced by endothelial cells and becomes avidly esterified into endothelial phospholipid pools (Roslowski, 1996; Van Rollins, 1993). In several studies, EET species were observed to relax blood vessels, leading to the suggestion that EETs may account for (the so far unidentified) endothelial-derived hyperpolarizing factor (EDHF) (Roslowski, 1996). Finally, both thromboxane synthase and prostacyclin synthase, which metabolize the PGHS product PGH_2 to thromboxane A_2 (TXA_2) and prostacyclin (PGI_2), respectively, are cytochrome P-450 enzymes.

Several cytochrome P-450 isoenzymes are inactivated by NO. Activities that are inhibited include arachidonate hydroxylation to 20-HETE, testosterone hydroxylation, oxidation of *p*-nitrophenol and dimethylnitrosamine, generation of TXA_2 and PGI_2, and O-ethylation of 7-ethoxyresorufin (Alonso-Garcia, 1997; Donato, 1997; Wink, 1993; Gergel, 1997; Minamiyama, 1997; Muller, 1996; Stadler, 1994). However, under some conditions generation of TXA_2 is actually stimulated by NO (Rosolowski and Campbell, 1996). The influence of NO on epoxide production by cytochrome P-450 has not been studied. However, ·NO inhibits production of the hydroxylated arachidonate product, 20-HETE, by cytochrome P-4504A in vascular smooth muscle cells. Since 20-HETE is a potent vasoconstrictor, this effect may contribute to vasodilatory effects of NO (Bolz *et al.,* 2000).

The Effect of Nitric Oxide on Oxidative Reactions Mediated by Heme Proteins

Peroxidase reactions are commonplace in biology, and both ''professional'' peroxidases and heme proteins that have ''pseudoperoxidase'' activity [e.g., myoglobin (Mb) or hemoglobin (Hb)] capable of oxidizing lipids *in vitro* are known (Gutteridge, 1987; Galaris *et al.,* 1990; Hogg *et al.,* 1994; Rogers *et al.,* 1995; Sarti *et al.,* 1994; Miller *et al.,* 1996; Paganga *et al.,* 1992; Ziouzenkova *et al.,* 1999; Kalyanaraman *et al.,* 1995). Their contribution to lipoprotein oxidation and atherosclerosis is unclear at present, but in support of a potential role, chlorination of tyrosine has been detected in atherosclerotic lesions. Also, the peroxidase capable of generating hypochlorite, myeloperoxidase, was also found (Daughterty *et al.,* 1994). The mechanism of heme protein-dependent lipid peroxidation involves both initiation and propagation events. Initiation, by abstraction of a hydrogen atom from a polyunsaturated fatty acid, can be promoted by higher oxidation states of heme proteins such as the ferryl oxidation states of Hb or Mb, similar to complexes I and II of peroxidases (Galaris *et al.,* 1990; Miller *et al.,* 1996; Newman *et al.,* 1991; Dee *et al.,* 1991). Specific binding sites for fatty acids on the protein, as has been demonstrated for myoglobin, may facilitate these processes (Rao *et al.,* 1994). Similar to free transition metal ions, heme proteins can also catalytically decompose LOOH via a one-electron redox cycle that cycles between the ferric and ferryl oxidation states of Mb and Hb (Rogers *et al.,* 1995; Sarti *et al.,* 1994; Miller *et al.,* 1996).

Nitric oxide has been shown to inhibit heme protein-dependent oxidation of lipids via reduction of higher heme oxidation states. For example, NO rapidly reduces ferryl Mb to ferric Mb and can also terminate protein radicals associated with ferryl heme formation in complex I (Dee *et al.,* 1991; Kanner *et al.,* 1992; Gorbunov *et al.,* 1995). The precise role of such protein radicals in lipid peroxidation is not known, although initiation events have been suggested (Newman *et al.,* 1991). Finally, binding of NO to ferrous heme, a relatively rapid process, prevents redox cycling and formation of the higher oxidation states of heme required for heme-dependent lipid oxidation reactions to occur (Kanner *et al.,* 1991).

Nitric Oxide-Dependent Regulation of Antioxidant Enzymes

The direct reaction of NO with oxygen radicals can be a cytoprotective mechanism that will be most effective in situations that have a component of propagation in the reaction mechanism, such as lipid peroxidation. The cell signaling roles of NO are also critical in the context of maintaining a healthy vasculature and inhibiting the progression of inflammatory diseases such as atherosclerosis. The classic cell signaling pathway of NO, namely, the activation of soluble guanylate cyclase and formation of cGMP, occurs in vascular smooth muscle and platelets, representing important vasorelaxant and antithrombotic mechanisms (Moncada *et al.,* 1991). Inflammation, although a normal response to injury, also represents a key component of tissue damage that leads to the development of vascular disease. Modulation of cell signaling pathways in a cGMP-independent manner by NO includes inhibition of expression of cytokine-induced adhesion molecules [vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1)]. Binding of circulating leukocytes/monocytes to these integrins facilitates adherence and entrance of inflammatory cells into the arterial wall and thus represents a critical step in both vascular oxidant injury and atherosclerotic plaque development. The NO-mediated downregulation of inflammatory adhesion molecule expression includes inhibition of the

transcription factor NF-κB (De *et al.,* 1995; Peng *et al.,* 1995) and represents an important indirect antioxidant action of NO.

Recent insights indicate that cGMP-independent NO signaling pathways are also capable of regulating transcriptional events that control the expression and synthesis of antioxidant enzymes (Moellering *et al.,* 1998, 1999; Li *et al.,* 1999; Frank *et al.,* 1999; Foresti *et al.,* 1997). The contrast in our understanding of these novel NO-signaling pathways compared to the cGMP-mediated actions of NO is interesting. In the case of the NO/cGMP pathway, a great deal is known about the upstream elements of the signaling cascade but relatively little about downstream events that regulate gene expression. In contrast, the downstream elements of the NO-mediated, cGMP-independent pathways are understood to some extent, but the upstream events and ''receptor'' for NO remains undefined. In this section these aspects will be discussed with particular reference to control of antioxidant enzyme expression.

One of the most important antioxidants in the cell is the small tripeptide glutathione (GSH), which plays an important role in the detoxification of reactive oxygen and nitrogen species (Deneke and Fanburg, 1989). For example, exposure of cells to exogenous oxidants or the endogenous formation of inorganic peroxides (e.g., $ONOO^-$, H_2O_2), lipid peroxides, and their decomposition products (e.g., aldehydes and epoxides) is an unavoidable consequence of oxygen metabolism. The intracellular concentration of these species, whether playing a role in cell signaling or cytotoxicity, is controlled by an integrated network of metabolic enzymes and antioxidants. Glutathione, in its reduced form, is an enzyme cofactor capable of reducing cell protein thiols, enzymatic reduction and undergoing direct reaction with reactive oxygen and nitrogen species. The intracellular concentration of GSH is typically 1–5 m*M;* its actions and the steps leading to its synthesis (Deneke and Fanburg, 1989) are summarized in Fig. 5.

In situations of acute NO-mediated oxidative stress on activation of iNOS, induction of GSH synthesis occurs to maintain adequate levels of the antioxidant, since inhibition of NOS activity causes GSH levels to fall precipitously (Kuo *et al.,* 1996). This is most likely a mechanism specific to the pleitropic stimulus of cytokine treatment. Under physiological conditions, both endothelial and vascular smooth muscle cells exposed to NO at low fluxes are stimulated to synthesize additional GSH (Moellering *et al.,* 1998, 1999).

A recent addition to the family of antioxidant enzymes is heme oxygenase. Heme is widespread throughout the cell and plays a critical role in electron transfer reactions, cell signaling, and oxygen transport (Foresti and Motterlini, 1999). The capacity of the iron in the heme molecule to promote oxidative reactions also makes heme proteins a potential source of toxicity. One of the most important detoxification pathways of free heme is the enzyme heme oxygenase (HO), which catalyzes the decomposition of heme, in turn yielding carbon monoxide and biliverdin as by-products. The further metabolism of biliverdin forms bilirubin,

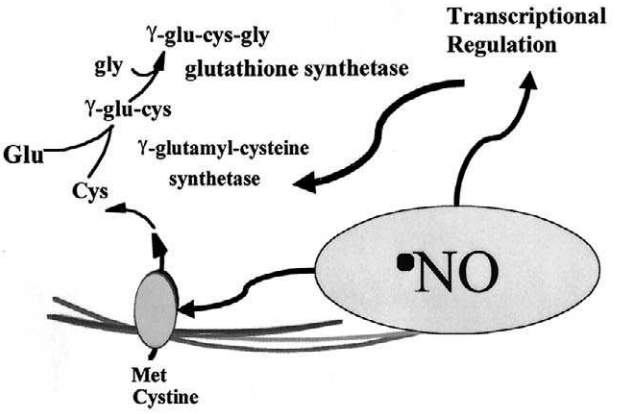


Figure 5 The synthesis of GSH and its interaction with NO. The first step of GSH synthesis, formation of γ-glutamylcysteine by γ-glutamylcysteine synthetase, is rate limiting. The activity of γ-glutamylcysteine synthetase is modulated by pro-oxidants (e.g., H_2O_2) and by nonoxidative mechanisms related to cell density and the availability of glutamate. In the final step of GSH biosynthesis, glycine is incorporated by GSH synthetase. The uptake of the amino acids cystine and methionine are also important in controlling GSH levels.

which also has potent antioxidant properties (Maines, 1988). Carbon monoxide has been associated with cytoprotective effects through mechanisms that have not yet been defined but may include NO-mediated mechanisms (Vercellotti *et al.,* 1994; Thom *et al.,* 2000). One possible explanation is the tight binding of CO to ferrous heme, retarding oxidation to the more pro-oxidant ferrous or ferryl forms of the heme molecule. Of course free iron is also a by-product of this reaction, but relative to heme this is readily sequestered by proteins in the cell and is of limited potential to promote pro-oxidant reactions. Heme oxygenases manifest antioxidant properties through several different mechanisms. The enzyme exists in two isoforms, HO-1 (inducible) and HO-2 (constitutive). Heme oxygenase-1 is induced by a wide variety of toxic stimuli, including free heme. It is not clear whether induction involves oxidant-dependent loss of heme from endogenous proteins or is mediated by an independent signaling pathway. Several studies have convincingly shown that this enzyme is capable of protecting against both oxidative and nitrosative stress (Vercellotti *et al.,* 1994; Applegate *et al.,* 1991).

More recent investigations have revealed a potentially important interaction between heme metabolism and NO, via HO-1 (Juckett *et al.,* 1998). Exposure of diverse cell types to NO, including hepatocytes and aortic endothelial cells, results in induction of HO-1. The concentrations of NO needed to elicit these effects are difficult to estimate from the data presented, but they are certainly higher than levels obtained from endothelial NOS (eNOS) activation and more closely relate to those achieved from iNOS. More detailed studies have shown that the mediator involved could be $ONOO^-$, an observation consistent with the higher levels of NO required to elicit increased HO-1 activity (Foresti *et al.,* 1997). The signal transduction pathway is cGMP-

independent and results in both increased transcription and the stabilization of the mRNA for HO-1.

Summary

Chemical, cell biological, and *in vivo* studies all reveal that NO has the capacity to serve a tissue-protective role in inflammatory diseases that have oxidative injury as a component. In some conditions, for example, a microenvironment rich in $O_2^{\cdot-}$, NO will also amplify oxidant injury via $ONOO^-$ formation. In other instances, when a different milieu of inflammatory mediators is present, NO is capable of terminating radical reactions and limiting overall oxidative tissue injury via overriding competing influences. Because of the diverse reaction pathways and signaling events in which reactive species are capable of participating, it is not practical to ascribe a singular ''antioxidant'' property to NO. Present understanding of the multifaceted tissue-protective actions of NO suggest that (a) NO-mediated stimulation of expression of antioxidant defenses (e.g., GSH biosynthesis), (b) protection of membranes and lipoproteins from oxidation, and (c) inhibition of production of inflammatory mediators (cytokines, eicosanoids, and integrin synthesis/expression) all contribute to the limitation of inflammatory injury to tissues by ·NO.

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References

Akarasereenont, P., Mitchell, J. A., Bakhle, Y. S., Thiemermann, C., and Vane, J. R. (1995). Comparison of the induction of cyclooxygenase and nitric oxide synthase by endotoxin in endothelial cells and macrophages. *Eur. J. Pharmacol.* **273,** 121–128.

Alonso-Galicia, M., Drummond, H. A., Reddy, K. K., Falck, J. R., and Roman, R. J. (1997). Inhibition of 20-HETE production contributes to the vascular responses to nitric oxide. *Hypertension* **29,** 320–325.

Applegate, L. A., Luscher, P., and Tyrrell, R. M. (1991). Induction of heme oxygenase: A general response to oxidant stress in cultured mammalian cells. *Cancer Res.* **51,** 974–978.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Bolz, S.-S., Fisslthaler, B., Pieperhoff, S., De Wit, C., Fleming, I., Busse, R., and Pohl, U. (2000). Antisense oligonucleotides against cytochrome P450 2C8 attenuate EDHF-mediated Ca^{2+} changes and dilation in isolated resistance arteries. *FASEB J.* **14,** 255–260.

Brown, A. S., Moro, M. A., Masse, J. M., Cramer, E. M., Radomsksi, M., and Darley-Usmar, V. (1998). Nitric oxide-dependent and independent effects on human platelets treated with peroxynitrite. *Cardiovasc. Res.* **40,** 380–388.

Brüne, B., and Ullrich, V. (1991). 12-Hydroperoxyeicosatetraenoic acid inhibits main platelet functions by activation of soluble guanylyl cyclase. *Mol. Pharmacol.* **39,** 671–678.

Buettner, G. (1993). The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol and ascorbate. *Arch. Biochem. Biophys.* **320,** 535–543.

Campbell, W. B., and Harder, D. R. (1999). Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circ. Res.* **84,** 484–488.

Campbell, W. B., Gebremedhin, S., Pratt, P. F., and Harder, D. R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ. Res.* **78,** 415–423.

Capdevila, J. H., Falck, J. R., and Estabrook, R. W. (1992). Cytochrome P450 and the arachidonate cascade. *FASEB J.* **6,** 731–736.

Cassina, A. M., Hodara, R., Thomson, L., Souza, J. M., Ischiropoulos, H., Freeman, B. A., and Radi, R. (2000). Cytochrome *c* nitration by peroxynitrite. *J. Biol. Chem.* **275,** (in press).

Conrad, D. J., Kühn, H., Mulkins, M., Highland, E., and Sigal, E. (1992). Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 217–221.

Cooke, J. P., Singer, A. H., Tsao, P., Zera, P., Rowan, R. A., and Billingham, M. E. (1992). Antiatherogenic effects of sml-nmarginine in the hypercholesterolemic rabbit. *J. Clin. Invest.* **90,** 1168–1172.

Curtis, J. F., Reddy, N. G., Mason, R. P., Kalyanaraman, B., and Eling, T. E. (1996). Nitric oxide: A prostaglandin H synthase 1 and 2 reducing cosubstrate that does not stimulate cyclooxygenase activity or prostaglandin H synthase expression in murine macrophages. *Arch. Biochem. Biophys.* **335,** 369–376.

Darley-Usmar, V. M., Hogg, N., O'Leary, V. J., Wilson, M. T., and Moncada, S. (1992). The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radical Res. Commun.* **17,** 9–20.

Darley-Usmar, V. M., Wiseman, H., and Halliwell, B. (1995). Nitric oxide and oxygen radicals: A question of balance. *FEBS Lett.* **369,** 131–135.

Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994). Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J. Clin. Invest.* **94,** 437–444.

Davidge, S. T., Baker, P. N., McLaughlin, M. K., and Roberts, J. M. (1995). Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. *Circ. Res.* **77,** 274–283.

De, C. R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A. J., Shin, W. S., and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* **96,** 60–68.

Dee, G., Rice-Evans, C., Obeyesekera, S., Meraji, S., Jacobs, M., and Bruckdorfer, K. R. (1991). The modulation of ferryl myoglobin formation and its oxidative effects on low density lipoproteins by nitric oxide. *FEBS Lett.* **294,** 38–42.

Deneke, S. M., and Fanburg, B. L. (1989). Regulation of cellular glutathione. *Am. J. Physiol.* **257,** L163–L173.

De Witt, D. L. (1991). Prostaglandin endoperoxide synthase: Regulation of enzyme expression. *Biochim. Biophys. Acta* **1083,** 121–134.

Dieber-Rotheneder, M., Puhl, H., Waeg, G., Striegl, G., and Esterbauer, H. (1991). Effect of oral supplementation with a α-tocopherol on the vitamin E content of human LDL and its oxidation resistance. *J. Lipid Res.* **32,** 1325–1332.

Donato, M. T., Guillen, M. I., Jover, R., Castell, J. V., and Gomez-Lechon, M. J. (1997). Nitric oxide-mediated inhibition of cytochrome P450 by interferon-γ in human hepatocytes. *J. Pharmacol. Exp. Ther.* **281,** 484–490.

Drexler, H., Zeiher, A. M., Meinzer, K., and Just, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by sml-nmarginine. *Lancet* **338,** 1546–1550.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., and Busse, R. (1999). Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* **401,** 493–497.

Folcik, V. A., Nivar-Aristy, R. A., Krajewski, L. P., and Cathcart, M. K. (1995). Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. *J. Clin. Invest.* **96,** 504–510.

Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (0000). 5-lipoxygenase. *Annu. Rev. Biochem.* **63,** 383–417.

Foresti, R., and Motterlini, R. (1999). The heme oxygenase pathway and its interaction with nitric oxide in the control of cellular homeostasis. *Free Radical Res.* **31,** 459–475.

Foresti, R., Clark, J. E., Green, C. J., and Motterlini, R. (1997). Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J. Biol. Chem.* **272,** 18411–18417.

Frank, S., Zacharowski, K., Wray, G. M., Thiemermann, C., and Pfeilschifter, J. (1999). Identification of copper/zinc superoxide dismutase as a novel nitric oxide-regulated gene in rat glomerular mesangial cells and kidneys of endotoxemic rats. *FASEB J.* **13,** 869–882.

Frost, M. J., and Smith, I. W. M. (1990). Rate constants for the reactions of CH_3O and C_2H_5O with NO over a range of temperature and total pressure. *J. Chem. Soc. Faraday Trans.* **86,** 1757–1762,

Galaris, D., Sevanian, A., Cadenas, E., and Hochstein, P. (1990). Ferrylmyoglobin-catalyzed linoleic acid peroxidation. *Arch. Biochem. Biophys.* **281,** 163–169.

Galpin, J. R., Veldink, G. A., Vliegenthart, J. F. G., and Boldingh, J. (1978). The interaction of nitric oxide with soybean lipoxygenase-1. *Biochim. Biophys. Acta* **536,** 356–362.

Gergel, D., Misik, V., Riesz, P., and Cederbaum, A. I. (1997). Inhibition of rat and human cytochrome P4502E1 catalytic activity and reactive oxygen radical formation by nitric oxide. *Arch. Biochem. Biophys.* **337,** 239–250.

Glasgow, W. C., Hui, R., Everhart, A. L., Jayawickreme, S. P., Angerman-Stewart, J., Han, B. B., and Eling, T. E. (1997). The linoleic acid metabolite, (13*S*)-hydroperoxyoctadecadienoic acid, augments the epidermal growth factor receptor signaling pathway by attenuation of receptor dephosphorylation. character.*J. Biol. Chem.* **272,** 19269–19276.

Go, Y. M., Patel, R. P., Maland, M. C., Park, H., Beckman, J. S., Darley-Usmar, V. M., and Jo, H. (1999). Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of cJun N-terminal kinase in endothelial cells. *Am. J. Physiol.* **277,** H1647–H1653.

Goodwin, D. C., Gunther, M. R., Hsi, L. C., Cresw, B. C., Eling, T. E., Mason, R. P., and Marnett, L. J. (1998). Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover: Detection of the radical derivative of tyrosine 385. *J. Biol. Chem.* **273,** 8903–8909.

Gorbunov, N. V., Osipov, A. N., Day, B. W., Zayas-Rivera, B., Kagan, V. E., and Elsayed, N. M. (1995). Reduction of ferrylmyoglobin and ferrylhemoglobin by nitric oxide: A protective mechanism against ferryl hemoprotein-induced oxidations. *Biochemistry* **34,** 6689–6699.

Goss, S. P. A., Hogg, N., and Kalyanaraman, B. (1995). The antioxidant effect of spermine NONOate in human low-density lipoprotein. *Chem. Res. Toxicol.* **8,** 800–806.

Gunther, M. R., Hsi, L. C., Curtis, J. F., Gierse, J. K., Marnett, L. J., Eling, T. E., and Mason, R. P. (1997). Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation. *J. Biol. Chem.* **272,** 17086–17090.

Gutierrez, H. H., Chumley, P., Rivera, A., and Freeman, B. A. (1996). Nitric oxide regulation of superoxide-dependent lung cell injury: Oxidant-protective actions of endogenously produced and exogenously administered nitric oxide. *Free Radicals Biol. Med.* **21,** 43–52.

Gutteridge, J. M. (1987). The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. *Biochim. Biophys. Acta* **917,** 219–223.

Habib, A., Bernard, C., Lebret, M., Creminon, C., Esposito, B., Tedgui, A., and Maclouf, J. (1997). Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J. Immunol.* **158,** 3845–3851.

Harder, D. R., Lange, A. R., Gebremedhin, D., Birks, E. K., and Roman, R. J. (1997). Cytochrome P450 metabolites of arachidonic acid as intracellular signalling molecules in vascular tissue. *J. Vasc. Res.* **34,** 237–243.

Hogg, N., and Kalyanaraman, B. (1999). Nitric oxide and lipid peroxidation. *Biochim. Biophys. Acta* **1411,** 378–384.

Hogg, N., Darley-Usmar, V. M., Wilson, M. T., and Moncada, S. (1992). The production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem. J.* **281,** 419–424.

Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., and Parthasarathy, S. (1993). Inhibition of low-density lipoprotein by nitric oxide *FEBS Lett.* **334,** 170–174.

Hogg, N., Rice-Evans, C., Darley-Usmar, V., Wilson, M. T., Paganga, G., and Bourne, L. (1994). The role of lipid hydroperoxides in the myoglobin-dependent oxidation of LDL. *Arch. Biochem. Biophys.* **314,** 39–44.

Holzhütter, G. G., Wiesner, R., Rathmann, J., Stosser, R., and Kühn, H. (1997). A kinetic model for the interaction of nitric oxide with a mammalian lipoxygenase. *Eur. J. Biochem.* **245,** 608–616.

Ischiropoulos, H. (1998). Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356,** 1–11.

Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T., and Stocker, R. (1992). The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochim. Biophys. Acta* **1180,** 73–82.

Juckett, M., Zheng, Y., Yuan, H., Pastor, T., Antholine, W., Weber, M., and Vercellotti, G. (1998). Heme and the endothelium. Effects of nitric oxide on catalytic iron and heme degradation by heme oxygenase. *J. Biol. Chem.* **273,** 23388–23397.

Kagan, V. E., and Packer, L. (1994). Light-induced generation of vitamin E radicals: Assessing vitamin E regeneration. *Methods Enzymol.* **234,** 316–320.

Kalyanaraman, B., Darley-Usmar, V. M., Wood, J., Joseph, J., and Parathasarathy, S. (1992). Synergistic interaction between the probucol phenoxyl radical and ascorbic acid in inhibiting the oxidation of LDL. *J. Biol. Chem.* **267,** 6789–6795.

Kalyanaraman, B., Darley-Usmar, V., Struck, A., Hogg, N., and Parthasarathy, S. (1995). Role of apolipoprotein B-derived radical and α-tocopheroxyl radical in peroxidase-dependent oxidation of low density lipoprotein. *J. Lipid Res.* **36,** 1037–1045.

Kanner, J. (1979). *S*-Nitrosocysteine, an effective antioxidant in cured meat. *J. Am. Oil Chem. Soc.* **56,** 74–76.

Kanner, J., Harel, S., and Granit, R. (1991). Nitric oxide as an antioxidant. *Arch. Biochem. Biophys.* **289,** 130–136.

Kanner, J., Harel, S., and Granit, R. (1992). Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemoglobin. *Lipids* **27,** 46–49.

Kayden, H. J., and Traber, M. G. (1993). Absorption, lipoprotein transport and regulation of plasma concentrations of vitamin E in humans. *J. Lipid Res.* **34,** 343–358.

Kühn, H., Belkner, J., Zaiss, S., Fährenklemper, T., and Wohlfeil, S. (1994). Involvement of 15-lipoxygenase in early stages of atherogenesis. *J. Exp. Med.* **179,** 1903–1911.

Kühn, H., Heydeck, D. L., Hugou, I., and Gniwotta, C. (1997). In vivo action of 15-lipoxygenase in early stages of human atherogenesis. *J. Clin Invest.* **99,** 888–893.

Kuo, P. C., Abe, K. Y., and Schroeder, R. A. (1996). Interleukin-1-induced nitric oxide production modulates glutathione synthesis in cultured rat hepatocytes. *Am. J. Physiol.* **271,** C851–C862.

Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996). Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15069–15074.

Li, H., Marshall, Z. M., and Whorton, A. R. (1999). Stimulation of cystine uptake by nitric oxide: Regulation of endothelial cell glutathione levels. *Am. J. Physiol.* **276,** C803–C811.

Liebler, D. C. (1994). Tocopherone and epoxytocopherone products of vitamin E oxidation. *Methods Enzymol.* **234,** 310–316.

Liu, X., Miller, M. J. S., Thomas, D. D., and Lancaster, J. R. (1998). Accelerated reaction of nitric oxide with oxygen within the hydrophobic

interior of biological membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2175–2179.

Maines, M. D. (1988). Heme oxygenase: Function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* **2,** 2557–2568.

Malo-Ranta, U., Ylä-Hertualla, S., Metsä-ketelä, T., Jaakkola, O., Moilanes, E., Vourinen, P., and Nikkari, T. (1994). Nitric oxide donor GEA 3162 inhibits endothelial cell-mediated oxidation of low density lipoprotein. *FEBS Lett.* **337,** 179–183.

Manfield, L., Jang, D., and Murrell, G. A. (1996). Nitric oxide enhances cyclooxygenase activity in articular cartilage. *Inflamm. Res.* **45,** 254–258.

Maricq, M. M., and Szente, J. J. (1996). Kinetics of the reaction between ethylperoxy radicals and nitric oxide. *J. Phys. Chem.* **100,** 12374–12379.

Miller, Y. I., Felikman, Y., and Shaklai, N. (1996). Hemoglobin induced apolipoprotein B crosslinking in low-density lipoprotein peroxidation. *Arch. Biochem. Biophys.* **326,** 252–260.

Minamiyama, Y., Takemura, S., Imaoka, S., Funae, Y., Tanimoto, Y., and Inoue, M. (1997). Irreversible inhibition of cytochrome P450 by nitric oxide. *J. Pharmacol. Exp. Ther.* **283,** 1479–1485.

Minghetti, L., Polazzi, E., Nicolini, A., Creminon, C., and Levi, G. (1996). Interferon-γ and nitric oxide down-regulate lipopolysaccharide-induced prostanoid production in cultured rat microglial cells by inhibiting cyclooxygenase-2 expression. *J. Neurochem.* **66,** 1963–1970.

Moellering, D., McAndrew, J., Patel, R. P., Cornwell, T., Lincoln, Cao, X., Messina, J. L., Forman, H. J., Jo, H., and Darley-Usmar, V. M. (1998). Nitric oxide-dependent induction of glutathione synthesis through increased expression of γ-glutamylcysteine synthetase. *Arch. Biochem. Biophys.* **358,** 74–82.

Moellering, D., Mc, A. J., Patel, R. P., Forman, H. J., Mulcahy, R. T., Jo, H., and Darley-Usmar, V. M. (1999). The induction of GSH synthesis by nanomolar concentrations of NO in endothelial cells: A role for γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase. *FEBS Lett.* **448,** 292–296.

Moncada, S., Palmer, R. M., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Moro, M. A., Darley-Usmar, V. M., Goodwin, D. A., Read, N. G., Zamora-Pino, R., Feelisch, M., Radomski, M. W., and Moncada, S. (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6702–6706.

Muller, C. M., Scierka, A., Stiller, R. L., Kim, Y. M., Cook, D. R., Lancaster, J. R., Buffington, C. W., and Watkins, W. D. (1996). Nitric oxide mediates hepatic cytochrome P450 dysfunction induced by endotoxin. *Anesthesiology* **84,** 1435–1442.

Nakatsuka, M., and Osawa, Y. (1994). Selective inhibition of the 12-lipoxygenase pathway of arachidonic acid metabolism by sml-nmarginine or sodium nitroprusside in intact human platelets. *Biochem. Biophys. Res. Commun.* **200,** 1630–1634.

Nassar, G. M., Morrow, J. D., Roberts, L. J., Lakkis, F. G., and Badr, K. F. (1994). Induction of 15-lipoxygenase by interleukin-13 in human blood monocytes. *J. Biol. Chem.* **269,** 27631–27634.

Nelson, M. J. (1987). The nitric oxide complex of ferrous soybean lipoxygenase-1. *J. Biol Chem.* **262,** 12137–12142.

Newman, E. S., Rice-Evans, C. A., and Davies, M. J. (1991). Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochem. Biophys. Res. Commun.* **179,** 1414–1419.

Niu, X., Zammit, V., Upston, J. M., Dean, R. T., and Stocker, R. (1999). Coexistence of oxidized lipids and α-tocopherol in all lipoprotein density fractions isolated from advanced human atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1708–1718.

Nossuli, T. O., Hayward, R., Jensen, D., Scalia, R., and Lefer, A. M. (1998). Mechanisms of cardioprotection by peroxynitrite in myocardial ischemia and reperfusion injury. *Am. J. Physiol.* **275,** H509–H519.

Nyby, M. D., Sakaki, M., Ideguchi, Y., Wynne, H. E., Hori, M. T., Berger, M. E., Golub, M. S., Brickman, A. S., and Tuck, M. L. (1996). Platelet lipoxygenase inhibitors attenuate thrombin- and thromboxane mimetic-induced intracellular calcium mobilization and platelet aggregation. *J. Pharmacol. Exp. Ther.* **278,** 503–509.

O'Donnell, V. B., Chumley, P. H., Hogg, N., Bloodsworth, A., Darley-Usmar, V. M., and Freeman, B. A. (1997). Nitric oxide inhibition of lipid peroxidation: Kinetics of reaction with lipid peroxyl radicals and comparison with α-tocopherol. *Biochemistry* **36,** 15216–15223.

O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999a). Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species: Peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12,** 83–92.

O'Donnell, V. B., Taylor, K. B., Parthasarathy, S., Kuhn, H., Koesling, D. A., Friebe, A., Bloodsworth, A., Darley-Usmar, V. M., and Freeman, B. A. (1999b). 15-Lipoxygenase catalytically consumes nitric oxide and impairs activation of guanylate cyclase. *J. Biol. Chem.* **274,** 20083–20091.

Padmaja, S., and Huie, R. E. (1993). The reaction of nitric oxide with organic peroxyl radicals. *Biochem. Biophys. Res. Commun.* **195,** 539–544.

Paganga, G., Rice-Evans, C., Rule, R., and Leake, D. (1992). The interaction between ruptured erythrocytes and low-density lipoproteins. *FEBS Lett.* **303,** 154–158.

Peng, H. B., Libby, P., and Liao, J. K. (1995). Induction and stabilization of I κ B alpha by nitric oxide mediates inhibition of NF-κ B. *J. Biol. Chem.* **270,** 14214–14219.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288,** 481–487.

Rao, S. I., Wilks, A., Hamberg, M., Ortiz, D., and Montellano, P. R. (1994). The lipoxygenase activity of myoglobin. Oxidation of linoleic acid by the ferryl oxygen rather than protein radical. *J. Biol. Chem.* **269,** 7210–7216.

Rogers, M. S., Patel, R. P., Reeder, B. J., Sarti, P., Wilson, M. T., and Alayash, A. I. (1995). Pro-oxidant effects of cross-linked haemoglobins explored using liposome and cytochrome *c* oxidase vesicle model membranes. *Biochem. J.* **310,** 827–833.

Rosolowski, M., and Campbell, W. B. (1996). Synthesis of hydroxyeicosatetraenoic (HETEs) and eopoxyeicosatrienoic acids (EETs) by cultured bovine artery endothelial cells. *Biochim. Biophys. Acta* **1299,** 267–277.

Rubanyi, G., Ho, E. H., Cantor, E. H., Lumma, W. C., and Botelho, L. H. P. (1991). Cytoprotective function of nitric oxide: Inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* **181,** 1392–1397.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation: Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **265,** 26066–26075.

Rubbo, H., Parthasarathy, S., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1995). Nitric oxide inhibition of lipoxygenase-dependent liposome and low density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324,** 15–25.

Rubbo, H., Darley-Usmar, V., and Freeman, B. A. (1996). Nitric oxide regulation of tissue free radical injury. *Chem. Res. Toxicol.* **9,** 809–820.

Rubbo, H., Radi, R., Anselmi, D., Kirk, M., Barnes, S., Butler, J., Eiserich, J. P., and Freeman, B. A. (2000). Nitric oxide reaction with lipid peroxyl radical spares α-tocopherol during lipid peroxidation: Greater oxidant protection from the pair nitric oxide/α-tocopherol than α-tocopherol/ascorbate. *J. Biol. Chem.* **275,** 10812–10818.

Salerno, J. C., and Siedow, J. N. (1979). The nature of the nitric oxide complexes of lipoxygenase. *Biochim. Biophys. Acta* **579,** 246–251.

Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7240–7244.

Salvemini, D., Seibert, K., Masferrer, J. L., Misko, T. P., Currie, M. G., and Needleman, P. (1994). Endogenous nitric oxide enhances prosta-

glandin production in a model of renal inflammation. *J. Clin. Invest.* **93,** 1940–1947.

Sarti, P., Hogg, N., Darley-Usmar, V. M., Sanna, M. T., and Wilson, M. T. (1994). The oxidation of cytochrome-*c* oxidase vesicles by hemoglobin. *Biochim. Biophys. Acta* **1208,** 38–44.

Sato, K., Niki, E., and Shimasaki, H. (1990). Free radical mediated chain oxidation of LDL and its synergistic inhibition of vitamin E and vitamin C. *Arch. Biochem. Biophys.* **279,** 402–405.

Serbinova, E. A., and Packer, L. (1994). Antioxidant properties of α-tocopherol and α-tocotrienol. *Methods Enzymol.* **234,** 354–366.

Smith, W. L., and De Witt, D. L. (1996). Prostaglandin endoperoxide H synthases-1 and -2. *Adv. Immunol.* **62,** 167–214.

Stadler, J., Harbrecht, B. G., Di Silvio, M., Curran, R. D., Jordan, M. L., Simmons, R. L., and Billiar, T. R. (1993). Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J. Leukocyte Biol.* **53,** 165–172.

Stadler, J., Trockfeld, J., Schmalix, W. A., Brill, T., Siewert, J. R., Greim, H., and Doehmer, J. (1994). Inhibition of cytochromes P4501A by nitric oxide. *Proc. Natl. Acad. Sci. USA* **91,** 3559–3563.

Swierkosz, T. A., Mitchell, J. A., Warner, T. D., Botting, R. M., and Vane, J. R. (1995). Co-induction of nitric oxide synthase and cyclooxygenase: Interactions between nitric oxide and prostanoids. *Br. J. Pharmacol.* **114,** 1335–1342.

Thom, S., Fisher, D., Xu, Y., Notarfrancesco, K., and Ischiropoulos, H. (2000). Adaptive responses and apoptosis in endothelial cells exposed to carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 1305–1310.

Thomson, L., Trujillo, M., Telleri, R., and Radi, R. (1995). Kinetics of cytochrome c^{2+} oxidation by peroxynitrite: Implications for superoxide measurements in nitric oxide-producing biological systems. *Arch. Biochem. Biophys.* **319,** 491–497.

Tsai, A. L., Wei, C., and Kulmacz, R. J. (1994). Interaction between nitric oxide and prostaglandin H synthase. *Arch. Biochem. Biophys.* **313,** 367–372.

Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Croxtall, J., and Willoughby, D. A. (1994). Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2046–2050.

VanRollins, M., Kaduce, T. L., Knapp, H. R., and Spector, A. A. (1993). Epoxyeicosatrienoic acid metabolism in endothelial cells. *Circ. Res.* **34,** 1931–1942.

Vercellotti, G. M., Balla, G., Balla, J., Nath, K., Eaton, J. W., and Jacob, H. S. (1994). Heme and the vasculature: An oxidative hazard that induces antioxidant defenses in the endothelium. *Art. Cells Blood Subs. Immobi. Biotech.* **22,** 207–213.

Wade, M. L., and Fitzpatrick, F. A. (1997). Nitric oxide modulates the activity of the hemoproteins prostaglandin I_2 synthase and thromboxane A_2 synthase. *Arch. Biochem. Biophys.* **347,** 174–180.

Wallington, T. J., Dagaut, P., and Kurylo, M. J. (1992). Ultraviolet absorption cross sections and reaction kinetics and mechanisms for peroxy radicals in the gas phase. *Chem. Rev.* **92,** 667–710.

Wiesner, R., Rathmann, J., Holzhütter, H. G., Stosser, R., Mäder, K., Nolting, H., and Kühn, H. (1996). Nitric oxide oxidizes a ferrous mammalian lipoxygenase to a pre-activated ferric species. *FEBS Lett.* **389,** 229–232.

Wink, D. A., Osawa, Y., Darbyshire, J. F., Jones, C. R., Eshenaur, S. C., and Nims, R. W. (1993). Inhibition of cytochromes P450 by nitric oxide and a nitric oxide-releasing agent. *Arch. Biochem. Biophys.* **300,** 115–123.

Yates, M. T., Lambert, L. E., Whitten, J. P., McDonald, I., Mano, M., Ku, G., and Mao, S. J. T. (1992). A protective role for nitric oxide in the oxidative modification of low density lipoproteins by mouse macrophages. *FEBS Lett.* **309,** 135–138.

Ziouzenkova, O., Asatryan, L., Akmal, M., Tetta, C., Wratten, M. L., Loseto-Wich, G., Jurgens, G., Heinecke, J., and Sevanian, A. (1999). Oxidative cross-linking of ApoB100 and hemoglobin results in low density lipoprotein modification in blood. Relevance to atherogenesis caused by hemodialysis. *J. Biol. Chem.* **274,** 18916–18924.

CHAPTER 18

Mechanisms through Which Reactive Nitrogen and Oxygen Species Interact with Physiological Signaling Systems

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NITRIC OXIDE (NO), REACTIVE OXYGEN SPECIES (ROS), AND REACTIVE NITROGEN SPECIES (RNS) DERIVED FROM NO HAVE MULTIPLE INTERACTIONS WITH CELLULAR CONTROL MECHANISMS THAT RANGE FROM FUNDAMENTAL SIGNALING PROCESSES TO SENSING SYSTEMS FOR THE LEVELS OF INDIVIDUAL ROS AND RNS. SOME OF THE MOST POTENT EFFECTS OF NO ARE ITS INTERACTIONS WITH HEMOPROTEINS IN PROCESSES INCLUDING THE STIMULATION OF SOLUBLE GUANYLATE CYCLASE (SGC), THE INHIBITION OF CYTOCHROME OXIDASE, AND THE INACTIVATION OF NO BY HEMOGLOBIN. THE METABOLISM OF PEROXIDE BY ENZYMES INCLUDING CATALASE, HEME PEROXIDASES, AND GLUTATHIONE PEROXIDASE RESULTS IN THE ACTIVATION OF SENSITIVE SIGNALING MECHANISMS FOR ROS INCLUDING THE STIMULATION OF SGC, PROSTAGLANDIN FORMATION, AND THIOL REDOX-REGULATED PROCESSES, RESPECTIVELY. THE REACTION OF NO WITH SUPEROXIDE ACTIVATES ADDITIONAL SIGNALING MECHANISMS VIA THE FORMATION OF PEROXYNITRITE AND OTHER RNS BY PROCESSES INCLUDING MODIFICATION OF IRON–SULFUR CENTERS AND THIOL NITROSATION, NITRATION, AND OXIDATION. MANY ADDITIONAL REGULATORY PROCESSES SEEM TO RESULT FROM INTERACTIONS BETWEEN NO AND ROS SIGNALING SYSTEMS AND FROM THE PERTURBATION OF ANTIOXIDANT DEFENSE MECHANISMS. THUS, THE INTERACTIONS OF RNS AND ROS WITH SIGNALING SYSTEMS HAVE EVOLVED INTO MULTIPLE REGULATORY ROLES IN PHYSIOLOGICAL SYSTEMS AND PATHOPHYSIOLOGICAL PROCESSES.

Introduction

This chapter focuses on examining how the physiological chemistry and enzymatic interactions of reactive species derived from nitric oxide (NO) and the reduction of oxygen are incorporated into fundamental cellular signaling mechanisms. Emphasis will be placed on processes involving reactive nitrogen species (RNS) and reactive oxygen species (ROS) that do not appear to be associated with conditions which result in irreversible cellular injury and dysfunction.

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Several of the preceding chapters of this book have considered important aspects of the production of NO, the metabolism of NO, and the chemical reactivities and properties of key RNS. The chemical properties of RNS and ROS have been extensively investigated. Components of physiological systems such as antioxidant defense mechanisms have a major role in controlling the species that are present. The interactions of certain RNS and ROS with enzymes and other components result in the formation of additional reactive intermediates that often participate in the sensing of these species and activation of signaling processes. Thus, it is important to consider which RNS and ROS are present in physiological systems in amounts that are linked to the activation of signaling processes discussed in subsequent sections of this chapter and to consider how metabolic interactions of these species could be linked to their detection.

Roles for NO-Derived Species in Physiological NO Detection Mechanisms

In this section, the NO-derived species that potentially form in the absence of an interaction with ROS are discussed. Consideration of the species that form as a result of interactions of NO with ROS is a very important aspect of this topic, which is evaluated later in this chapter.

NO–Heme Species

The binding of NO to the Fe^{2+} or ferrous form of heme groups on proteins is one of the most potent actions of NO. However, many of the hemoproteins in cells have their heme stabilized in a higher oxidation state, such as the ferric or Fe^{3+} form, or have the heme group sequestered by the structure of the protein in a manner that prevents the binding of NO. Some hemoproteins have the iron of their heme groups entering oxidation states higher than Fe^{3+} (such as ferryl or Fe^{4+}, and species termed compound I and II) as transient intermediates in their catalytic reaction. NO can also function as an electron donor to heme groups in higher oxidation states. This can attenuate oxidation reactions catalyzed by the hemoprotein (Wink *et al.,* 1995). NO also seems to have the ability to reduce Fe^{3+} heme to its Fe^{2+} form when present at very high levels (Keilin and Hartree, 1937).

GUANYLATE CYCLASE

Activation of the soluble form of guanylate cyclase (sGC) is known to occur at low nanomolar concentrations of NO as a result of NO binding its Fe^{2+}-heme forming a Fe–NO species (Ignarro, 1989). The binding of NO to the heme results in enzyme activation as a result of NO causing the scission of the histidine bond to the Fe^{2+} of the heme (Deinum *et al.,* 1996; Yu *et al.,* 1994). Although the formation of NO–heme on sGC has been suggested to cause oxidation of the heme to its Fe^{3+} form (Dierks and Burstyn, 1998), other studies indicate that the binding of NO is reversible, and the regeneration of the Fe^{2+} as a result of the dissociation of NO is a process involved in the termination of enzyme stimulation (Kharitonov *et al.,* 1997). Some of the inhibitors of sGC stimulation, such as methylene blue (Dierks and Burstyn, 1998) and ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) (Schrammel *et al.,* 1996), seem to attenuate sGC stimulation by oxidizing the NO–Fe^{2+}-heme complex to its Fe^{3+} form, which appears to be resistant to stimulation by NO. Evidence suggests that an NADPH-dependent oxidoreductase may control the redox state of the heme of sGC and that this may be an important component of the sensitivity of sGC in tissues to activation by NO (Gupte *et al.,* 1998). Aspects of the mechanism of activation of sGC by NO are described in detail in other chapters of this book.

CYTOCHROME OXIDASE

At low concentrations NO binds the heme–copper centers of cytochrome oxidase through an interaction that is thought to involve both of these centers (Torres *et al.,* 1998). As discussed in greater detail later in this chapter, the binding of NO to this site results in a reversible inhibition of mitochondrial electron transport (Brown *et al.,* 1997). An interesting aspect of the binding of NO to cytochrome oxidase is that it appears to have a competitive interaction with molecular oxygen (Brown *et al.,* 1997). A competition of this type suggests that NO could function in a manner which shifts the range of mitochondrial sensing of oxygen from the nanomolar concentrations normally seen in isolated mitochondria up into the micromolar range, which has often been observed in tissue spectroscopy studies (Jobsis, 1972). It has been reported that cytochrome oxidase can metabolize NO to N_2O (Brown *et al.,* 1997). However, there is little evidence suggesting that cytochrome oxidase is an important site for the metabolism of NO in tissues (Stubauer *et al.,* 1998).

CATALASE

Catalase is an important metabolizing system for hydrogen peroxide (H_2O_2) (Chance *et al.,* 1979) and a key participant in the stimulation of sGC by H_2O_2 (Burke and Wolin, 1987; Cherry and Wolin, 1989). Evidence exists for interactions of NO with the heme of catalase that result in the observation of both reversible and irreversible inhibition of this enzyme. The reversible inhibition of catalase appears to be associated with a reversible binding of NO to the heme of the enzyme (Brown, 1995). In contrast, under conditions where an irreversible inhibition of catalase has been observed, a species resembling the inactive compound II of catalase has been detected (Mohazzab-H. *et al.,* 1996). Compound II is thought to be a ferryl heme-containing form of catalase and appears to be a relatively stable, inactivated form of this enzyme. Although little is known regarding how NO functions in tissues as an inhibitor of catalase, it has been observed that levels of NO which cause the formation of peroxynitrite in vascular tissue (a 2-min exposure to 50 n*M* NO) result in a prolonged inhibition of catalase activity (Wo-

lin *et al.,* 1998). Thus, under conditions where NO inhibits catalase, it will function to impair the metabolism of peroxide and prevent the stimulation of sGC by H_2O_2. This action of NO has been observed in vascular tissue (Mohazzab-H. *et al.,* 1996).

CYTOCHROME P-450

NO has been demonstrated to inhibit the metabolic activities of cytochrome P-450 through what appears to be a reversible binding of NO to its heme group and through an irreversible process caused by RNS (Wink and Mitchell, 1998). The metabolism of arachidonic acid by cytochrome P-450 is a pathway for the formation of eicosanoids that have important roles in intracellular and transcellular signaling processes. Evidence suggests that NO appears to function in endothelium as an inhibitor of the formation of mediators by cytochrome P-450, where the loss of NO production results in the generation of cytochrome P-450-derived vasoactive eicosanoids (Sun *et al.,* 1998).

CYCLOOXYGENASE AND PROSTAGLANDIN METABOLISM

Since NO has been observed to have both stimulatory and inhibitory actions on production of prostaglandins in intact cells and tissues, there has been much interest in how NO influences the activity of cyclooxygenase (COX) and prostaglandin-metabolizing enzymes. It has been shown that NO reacts with a tyrosyl radical formed during the cyclooxygenase reaction, causing an irreversible inhibition of this enzyme as a result of nitrotyrosine formation (Goodwin *et al.,* 1998). Oxidized products of NO may also influence prostaglandin metabolism. The regulation of COX and prostaglandin metabolism by NO mechanisms is further discussed later in this chapter.

HEMOGLOBIN AND MYOGLOBIN

NO has an extremely potent interaction with the oxygen-bound heme of proteins including hemoglobin and myoglobin. NO reacts with Fe^{2+}-O_2 forms of these proteins at what appear to be diffusion controlled reaction rates, producing the Fe^{3+} forms of the hemoproteins and nitrate (Doyle and Hoekstra, 1981). While this process is clearly very important in signaling as a result of its terminating the biological actions of NO *in vivo,* formation of the methemoproteins and nitrate is not known to be associated with activation of additional signaling processes. It is difficult to detect the oxidation of hemoglobin by NO in erythrocytes because of the extremely efficient actions of methemoglobin reductase activity present in these cells (Hultquist, 1978). Hemoglobin-like proteins have the potential to promote pathophysiological responses as a result of their ability the cooxidize biological constituents such as lipids through peroxidase reactions. NO may function as an antioxidant by attenuating these processes as a result of its ability to reduce the higher oxidation states of iron (e.g., ferryl or Fe^{4+}) that catalyze these reactions (Wink *et al.,* 1995).

NO–Iron Species

The formation of $(RS)_2Fe(NO)_2$ complexes have been observed in tissues at what appears to be high nanomolar to low micromolar levels of NO (Hibbs *et al.,* 1990; Mulsch *et al.,* 1993). These NO complexes are potentially linked to several different signaling mechanisms. First, one needs to consider where the iron came from and what the consequence of its removal might be. NO has been reported to release iron by disrupting iron–sulfur centers on proteins such as aconitase and complexes I and II in the mitochondrial electron transport chain (Hibbs *et al.,* 1990). While damage to the iron–sulfur centers is generally considered an irreversible processes which would be beyond the scope of this chapter, evidence exists that there may be iron-dependent mechanisms of repair for iron–sulfur centers that are damaged by NO (Hibbs *et al.,* 1990). As is considered later in this chapter, the removal of iron from iron–sulfur centers would cause a prolonged inhibition of mitochondrial electron transport. NO also causes a loss of iron from the cytosolic aconitase, converting this enzyme into a form of the iron responsive binding protein (Drapier and Bouton, 1996). Under conditions where peroxynitrite forms, the iron responsive binding protein lacks its normal ability to bind and activate the iron responsive element system, and this prevents activation of the expression of the transferin receptor and of downregulation of ferritin production, which would normally promote an increase in cellular iron uptake. This action of NO on cellular iron metabolism may be more of a protective antioxidant effect than a signaling mechanism. The availability of iron could be a process that indirectly affects signaling as a result of its influencing the release of iron and the formation of $(RS)_2Fe(NO)_2$ complexes. These Fe–NO complexes also are likely to serve both as tissue storage forms of NO and as a chelated form of iron that is potentially resistant to particiation in oxidant stress-induced cellular injury.

NO–Thiol Species

There is substantial evidence that the nitrosation of thiols occurs in tissues (Gaston *et al.,* 1994; Stamler *et al.,* 1992). However, the actual process involved in nitrosation seems rather poorly understood. Iron–NO species related to $(RS)_2Fe(NO)_2$ complexes have been demonstrated to generate nitrosated thiols or thionitrites (RSNO) in the presence of physiological levels of thiols (Boese *et al.,* 1995). It has also been suggested that oxidation reactions of species formed from the reversible binding of NO to thiols result in the formation of RSNO (Gow *et al.,* 1997). Although these mechanisms appear to form RSNO species, there may be some more sensitive mechanisms for the formation of RSNO species under physiological conditions. As discussed later in this chapter, oxidation products of NO are potentially a physiologically relevant source of $RSNO_x$ species (Davidson *et al.,* 1997). Once RSNO species are formed, they may

function in transnitrosation reactions, which would transfer the NO group to thiols that have enhanced chemical reactivities, and this could be a component of a regulatory mechanism (Arnelle and Stamler, 1995). Some of the systems potentially regulated by thiol nitrosation-related processes are considered later in this chapter.

Mechanisms of Detection of ROS by Physiological Signaling Systems

Signaling mechanisms through which ROS can be detected are discussed here in a manner that permits consideration, later in this chapter of how NO and its metabolites interact with the individual ROS and with the systems they appear to regulate. Signaling mechanisms for ROS have been the subject of several reviews (Wolin, 1996; Wolin *et al.,* 1996, 1999a; Lander, 1997; Suzuki *et al.,* 1997; Wolin and Mohazzab-H., 1997; Abe and Berk, 1998).

Control of the Production and Levels of ROS

Cells appear to have basal sources of ROS production and signaling mechanisms. Pathophysiological states often activate ROS production by additional systems. Tissues contain oxidase enzymes that transfer electrons to oxygen resulting in the production of ROS. Most of the enzymes that reduce oxygen donate one electron to molecular oxygen, producing superoxide anion. Although superoxide readily reacts with itself at physiological pH, the enzyme superoxide dismutase (SOD) markedly accelerates the conversion of superoxide to H_2O_2 and molecular oxygen. In animal tissues there are three forms of SOD. SOD-1 is a Cu,Zn-containing form of SOD that is found in the cytosol of most cells. SOD-2 is a Mn form of SOD present in the mitochondrial matrix. SOD-3 is an extracellular Cu,Zn-SOD that is bound to the matrix of certain tissues (Oury *et al.,* 1994). It is important to note that the level of SOD activity is a major regulator of the reaction of NO with superoxide, an interaction that appears to result in the activation of signaling mechanisms considered later in this chapter. Most of the H_2O_2 present in tissues that appears to participate in signaling mechanisms seems to originate from systems that produce superoxide anion and from the metabolism of superoxide by SOD (Wolin and Mohazzab-H., 1997). Catalase, glutathione (GSH) peroxidase, and heme peroxidase enzymes are the major cellular metabolizing systems for H_2O_2 in most tissues (Chance *et al.,* 1979). Many of the signaling mechanisms linked to peroxide appear to originate from its metabolism by these enzymes. The reaction of peroxide with hemoproteins or certain chelated forms of Fe^{2+} result in the generation of species that have chemical reactivities similar to the extremely reactive hydroxyl radical. Although these hydroxyl radical-like species are likely to be participants in tissue injury, some of these species may have the potential to initiate signaling processes though oxidation reactions that they promote.

ROS Generation by Mitochondria

Mitochondria normally produce superoxide anion in amounts that appear to be relatively small compared to the rates of oxygen reduction to H_2O by the cytochrome oxidase reaction. Under normal circumstances it seems that mitochondria release only a small amount of the ROS that they produce, and due to the high levels of MnSOD present, the primary species that they appear to release is H_2O_2. Two major sites of superoxide production have been located in the complex I (NADH dehydrogenase) and coenzyme Q_{10} segments of the electron transport chain. These sites produce superoxide in a manner that seems to be primarily dependent on the availability of electrons or degree of reduction of these sites, suggesting that superoxide anion production might be increased in resting states. Although little is known regarding the regulatory role of the basal production of ROS by mitochondria, studies have provided evidence that mitochondrial superoxide could have an important influence on the interaction of NO with mitochondrial respiration and perhaps apoptosis (Poderoso *et al.,* 1998; Wallace, 1999).

ROS Generation by NAD(P)H Oxidases

Tissues contain several different NADPH and NADH oxidases that are potentially linked to signaling processes.

NADPH Oxidases Phagocytic cells contain a NADPH oxidase whose activity is controlled by signaling pathways associated with activation of these cells (Thelen *et al.,* 1993). Although substantial evidence is accumulating suggesting that the components of the phagocytic NADPH oxidase are present in other cell types (Cross and Jones, 1991; Griendling and Ushio-Fukai, 1997), only minimal information is available on the function of this system in these other cell types. Cytochrome P-450 and cytochrome P-450-like enzymes such as NO synthase (NOS) appear to be able to generate superoxide from NADPH at their heme site in a manner that may be of relevance to physiological signaling processes. For example, the depletion of tetrahydrobiopterin has been shown to change the endothelium-derived relaxant mediator generated by porcine coronary endothelium from NO to H_2O_2 (Cosentino and Katusic, 1995), through a process that appears to originate from the role this cofactor has in controlling electron transfer in the NOS reaction (Rusche *et al.,* 1998). Substrates for cytochrome P-450 enzymes also seem to have a major influence on promoting and inhibiting superoxide generation by this system. The flavin sites of cytochrome P-450 reductase may also contribute to the generation of oxidant species when redox cycling agents or metals such as iron are available as electron acceptors (Cenas *et al.,* 1994). It appears that the various types of NADPH oxidases discussed here will have important roles in signaling when these systems are producing oxidant species.

NADH Oxidases Although early investigations of the sources of ROS production in tissues detected a prominent role for NADH oxidase activity (Chance *et al.,* 1979), this activity was only relatively recently recognized as a poten-

tially important system that could be linked to physiological control mechanisms (Griendling and Ushio-Fukai, 1997; Wolin *et al.,* 1999b). It was initially observed that cellular growth factors regulated NADH oxidase activity (Morre and Brightman, 1991), and evidence supporting the significance of this system in the control of this process is accumulating (Griendling and Ushio-Fukai, 1997). Observations on the effects of lactate and pyruvate on vascular tissue resulted in the hypothesis that the redox status of cytosolic NAD(H) could have a major influence on the production of vasoactive ROS by NADH oxidase (Omar *et al.,* 1993; Wolin *et al.,* 1996; Wolin *et al.,* 1999b). Evidence has continued to accumulate supporting the concept that cytosolic NAD(H) redox and NADH oxidase-derived ROS have important roles in mechanisms that control vascular function (Wolin *et al.,* 1999b). It appears that the behaviors of the glycolytic enzymes that produce and metabolize NADH and of the mitochondrial shuttle mechanisms that import NADH into the mitochondria are basic cellular processes designed to keep the redox status of cytosolic NAD(H) in a highly oxidized state (Rabinowitz *et al.,* 1998). Thus, the availability of cytosolic NADH is potentially an important process that controls oxidant signaling mechanisms. As the production of superoxide by NADH oxidase appears to increase over the range of oxygen levels that typically exist in physiological systems (Mohazzab-H. and Wolin, 1994), the availability of oxygen as a substrate for NADH oxidases is potentially an additional important aspect of the way this system functions. Evidence appears to be rapidly accumulating in multiple cell types suggesting that NAD(P)H oxidases are important sources of basal oxidant production linked to signaling processes controlling cellular function. The dependence of the activity of NADH oxidase on the availability of cytosolic NADH and oxygen may enable this system to function as a key cellular metabolic and oxygen sensor, in addition to its potential role in receptor regulated oxidant signaling processes.

Other Sources of ROS Production

Cyclooxygenase and xanthine oxidase are other sources of production of ROS, and they appear to generate these species in amounts that have been observed to alter signaling processes. Cyclooxygenase is the initial enzyme in the pathway that produces prostaglandins, and it seems to be found in a wide variety of cell types. The catalytic mechanism of COX appears to produce ROS by causing a one-electron oxidation of cofactors such as NAD(P)H. This results in the generation of a free radical intermediate form of NAD(P) that reduces oxygen to superoxide anion (Kukreja *et al.,* 1986). Cyclooxygenase has been demonstrated to be a significant source of vasoactive ROS in the cerebral microcirculation (Marshall and Kontos, 1991). It appears that bradykinin receptor-mediated stimulation of prostaglandin production in endothelium results in the simultaneous generation of superoxide anion (Holland *et al.,* 1990). Xanthine dehydrogenase/oxidase is an enzyme localized primarily in the vascular endothelium. It appears that the oxidase activity of xanthine dehydrogenase is increased by thiol oxidation or proteolysis of the enzyme under multiple pathophysiological conditions such as ischemia–reperfusion (Granger, 1988). Although other sources of ROS production have been observed, including H_2O_2-generating enzymes in peroxisomes (Chance *et al.,* 1979), these other sources have not been linked to the production of ROS in tissues in amounts that appear to regulate signaling mechanisms.

Control of Superoxide Anion Levels and H_2O_2 Production by SOD

The activity of each of the three major types of SOD appears to have a considerable role in controlling the local levels of superoxide anion and the interactions between superoxide and signaling mechanisms. This is because the local activity of SOD influences the distribution of ROS that are present, and each oxygen-derived species has its own interactions with signaling systems. Superoxide anion normally reacts with itself to form oxygen and H_2O_2. SOD functions as a catalyst to markedly accelerate this process. The rate constants indicate that SOD has a reaction rate with superoxide that is $2 \times 10^9\ M^{-1}\ s^{-1}$ compared to the rate of $8 \times 10^4\ M^{-1}\ s^{-1}$ for the reaction of superoxide with itself (Fridovich, 1985). It is important to keep in mind that the efficiency of SOD is also directly dependent on the amount of SOD activity present. Thus, SOD activity is typically low in the extracellular environment, unless appreciable levels of SOD-3 are present. In contrast, SOD-1 and SOD-2 levels are generally quite high in the intracellular environment of cells, and this results in the presence of intracellular levels of superoxide in the picomolar range (Fridovich, 1985), which are likely to be substantially below the concentrations that appear to influence known signaling processes. However, since the rate of reaction of superoxide with NO of $7 \times 10^9\ M^{-1}\ s^{-1}$ is three times its reaction rate with SOD, elevated levels of NO can compete with SOD for the scavenging of superoxide, and this enables superoxide to influence signaling processes (see later) through the generation of peroxynitrite. Thus, the local levels of SOD directly control the extent to which superoxide interacts with NO and other systems that metabolize superoxide.

Peroxide Metabolizing Systems and the Control of H_2O_2

Peroxide metabolizing enzymes including catalase, GSH peroxidase, and heme peroxidases, appear to have two major roles in oxidant signaling mechanisms. They control the cellular levels of peroxides, and the metabolism of peroxide by these enzymes is linked to several of the most sensitive signaling processes. Under normal circumstances, these peroxide-metabolizing systems are thought to keep the intracellular levels of H_2O_2 in the high picomolar to low nanomolar concentration range (Chance *et al.,* 1979).

Catalase Catalase is a hemoprotein that metabolizes H_2O_2 to water and oxygen through the formation of an intermediate of catalase termed compound I, in a manner that does not require the presence of electron donors or acceptors.

Although catalase activity is markedly enriched in peroxisomes, there is substantial evidence that its activity is present in the cytosol and possibly other cellular organelles. Catalase has the ability to cometabolize other substances, typically resulting in a two-electron oxidation of the substance by the compound I intermediate of catalase. Although little is known regarding which cellular constituents are cometabolized by catalase, it is likely that tissues contain substances which serve as electron donors to catalase. When compound I of catalase reacts with a one-electron donor, it forms a compound II intermediate, which is a rather stable inactivated form of catalase. It is important to note that both superoxide and perhaps NO appear to serve as one-electron donors to catalase (Brown, 1995; Kono and Fridovich, 1982). As previously mentioned, the metabolism of peroxide by catalase has been demonstrated to promote activation of the production of cGMP by sGC. Both superoxide and NO have been demonstrated to inhibit the H_2O_2-elicited cGMP-associated relaxation responses in vascular preparations (Cherry *et al.,* 1990; Mohazzab-H. *et al.,* 1996). Thus, catalase is important both in controlling tissue peroxide levels and in promoting the activation of sGC when it is metabolizing H_2O_2, and species such as NO and superoxide appear to be important, physiologically relevant inhibitors of these actions of catalase.

GSH Peroxidase One of the most important systems for the metabolism of H_2O_2 are GSH peroxidase enzymes. This enzymatic activity is often the most prominent peroxide metabolizing system present in cells. GSH peroxidase activity is a primary metabolic pathway for peroxide consumption in both the cytosol and mitochondria (Chance *et al.,* 1979). The function of GSH peroxidases appears to be directly dependent on the concentration of GSH, and GSH is used to reduce H_2O_2 to water. Tissue GSH levels are typically in the low millimolar concentration range, and GSH is maintained in its reduced state by the high levels of NADPH-dependent GSH reductase activity present in tissues. The consumption of peroxide by GSH peroxidase is linked to metabolism through the regeneration of NADPH by systems such as glucose metabolism through the pentose phosphate pathway. An elevation in the amount of peroxide in tissues causes increases in the levels of oxidized GSH (GSSG) under conditions where the rate of production of GSSG exceeds its rate of removal. The increase in GSSG levels appears to promote a stimulation of signaling as a result of enzyme-catalyzed formation of disulfides of GSH with protein thiols [S-thiolation (RSSG)]. As superoxide (Blum and Fridovich, 1985) and peroxynitrite (Asahi *et al.,* 1995) appear to inhibit GSH peroxidase activity, high levels of production of these species are likely to promote increases in peroxide levels as a consequence of the inactivation of GSH peroxidase activity. Thus, GSH peroxidase is important both in controlling tissue peroxide levels and in promoting signaling by providing GSSG for S-thiolation-type modifications of proteins.

Heme Peroxidases The heme peroxidases detected in mammalian tissues seem to be present in amounts that are significantly less than the other peroxide metabolizing systems, suggesting that they are designed more to function in signaling mechanisms controlled by peroxide levels than to have a significant role in the control of cellular peroxide levels. However, the heme peroxidases found in phagocytic cells (such as myeloperoxidase) are present in amounts sufficient to be a significant source of cellular peroxide consumption. The purpose of these peroxidases appears to be the generation of ROS (such as hypochlorous acid), which are more reactive than H_2O_2, for use in phagocytic cell defense mechanisms. Whereas several hemoproteins in cells have been demonstrated to have heme peroxidase activity, COX appears to be the only enzyme other than the phagocytic cell peroxidases that has been documented to express this activity under physiological conditions.

Interactions of Superoxide Anion with Signaling Systems

Superoxide anion appears to have several interactions with cellular signaling systems. The most sensitive mechanism seems to originate from the ability of elevated levels of NO to function as a scavenger of superoxide in competition with the SOD reaction. The signaling aspects of this process that generate peroxynitrite are considered later in this chapter. As superoxide production increases and/or the activity of SOD becomes impaired, superoxide will begin to scavenge NO in both the extracellular and intracellular environment in tissues. If NO levels are relatively low, the amount of peroxynitrite that forms may not be sufficient to interact with other signaling systems. An elevation of tissue superoxide levels has been demonstrated to inhibit vascular relaxation to H_2O_2, which appears to be a result of sGC stimulation by catalase-mediated peroxide metabolism, suggesting that catalase activity in tissues can be inhibited by elevated levels of superoxide (Cherry *et al.,* 1990). The iron–sulfur center of aconitase appears to be a target for superoxide, and an inactivation of this enzyme is known to result in an impairment of mitochondrial function (Gardner *et al.,* 1994). It is well documented that superoxide can inactivate aconitase in intact tissues, and observation of an inactivation of aconitase has been used as a method of detection of elevated levels of intracellular superoxide anion. Elevated levels of superoxide are thought to reductively release iron from iron–sulfur centers and from ferritin. Reactions of the iron that is released with other ROS or RNS may result in the modulation of other signaling processes or the initiation of tissue injury (Wink and Mitchell, 1998). The regulatory actions of both NO and catecholamines such as epinephrine and norepinephrine are very sensitive to disruption by superoxide in the extracellular environment, where there is often low SOD activity; as a result, these regulatory substances have extremely high rates of reaction wit superoxide (Abrahamsson *et al.,* 1992; Wolin and Belloni, 1985).

Interactions of H_2O_2 with Signaling Systems

In this section the processes through which H_2O_2 interacts with signaling systems are discussed with a focus on provid-

ing background for considering how NO and its metabolites interact with these systems. It is important to keep in mind that the activity of peroxide metabolizing systems, which have a significant influence on H_2O_2 concentrations, will modulate the expression of all of the other peroxide signaling systems as a result of controlling the levels of H_2O_2.

Systems Linked to the Metabolism of Peroxide by Catalase—Regulation of sGC Activity

The activation of sGC by H_2O_2 metabolism by catalase has been examined both with purified enzymes and in intact vascular tissue (Burke and Wolin, 1987; Cherry and Wolin, 1989), demonstrating the potential physiological significance of this process. The stimulation of sGC appears to require a continuous rate of H_2O_2 metabolism by catalase and levels of peroxide in the high picomolar to low nanomolar range, which cause the formation of the compound I species of catalase. The function of this mechanism appears to be extremely dependent on the presence of tissue SOD activity, because it is very susceptible to inhibition by superoxide (Cherry *et al.,* 1990) and the interaction of superoxide with NO (Wolin *et al.,* 1998; see later). The activity of GSH peroxidase has been demonstrated in vascular tissue to have a major influence on the ability of H_2O_2 to elicit responses mediated by the stimulation of sGC as a result of controlling the amounts of peroxide metabolized by catalase (Mohazzab-H. *et al.,* 1999). Catalase may cooxidize other substances present in tissues (Chance *et al.,* 1979). This could function to inhibit the stimulation of sGC (Burke-Wolin and Wolin, 1990), or the products of these cooxidation reactions might interact with additional signaling processes. However, only minimal information is available on the metabolites present in tissues that are cooxidized by catalase or on signaling systems that could be influenced by products of cooxidation reactions. The redox state of the heme on sGC could be altered by processes related to oxidant stress (Iesaki *et al.,* 1998), and this could be an additional mechanism that influences the sensitivity of this system to activation. Thus, multiple processes are likely to control the expression of the ability of H_2O_2 to influence the activity of sGC as a result of its metabolism by catalase.

Systems Linked to the Metabolism of Peroxide by GSH Peroxidase–Thiol Redox as a Regulatory Mechanism

The generation of GSSG as a result of the metabolism of peroxides by GSH peroxidase seems to be a key pathway for linking H_2O_2 to signaling processes. Normal cellular metabolism is thought to maintain very low GSSG levels. As GSH oxidation begins to occur, it appears that S-thiolation of proteins is a major pathway for the GSSG produced (Brigelius, 1985; Gilbert, 1984; Thomas *et al.,* 1995). If the rate of production of GSSG exceeds its rate of removal and GSSG accumulates, it is likely that the NADP(H) pool will be highly oxidized and that the mechanisms controlling the redox status of thiols on proteins will become impaired. This metabolic condition is likely to promote oxidation of certain protein thiols in the proximity of each other to form protein disulfides and oxidation of the maintenance thiols that have formed disulfides with GSH in their S-thiolated form. Table I lists some of the proteins known to possess redox-sensitive thiol groups that have the potential to participate in cellular regulatory processes. It appears that a variety of important cellular regulatory systems are potentially regulated by thiol redox processes, including key metabolic enzymes (Brigelius, 1985; Gilbert, 1984), protein kinase C (Gopalakrishna *et al.,* 1999), potassium channels (Bolotina *et al.,* 1994; Duprat *et al.,* 1995; Weir and Archer, 1995), calcium channels (Elliott and Koliwad, 1995; Jones *et al.,* 1983), sGC (Brandwein *et al.,* 1981), and tyrosine phosphatases (Hecht and Zick, 1992). The extent to which GSH redox is altered probably determines which components of cellular regulatory processes are modified and what the resulting consequences of these alterations might be.

Systems Linked to the Metabolism of Peroxide by Heme Peroxidases—Cyclooxygenase and Prostaglandins

Heme peroxidases could be hypothesized to be ideal sensors of peroxide because these enzymes have the potential to generate signaling molecules by peroxide-dependent cooxidation reactions. Cyclooxygenase is a hemoprotein with heme peroxidase activity which is one of the most sensitive tissue sensors of H_2O_2 formation (Wolin and Mohazzab-H., 1997). The reaction of COX with H_2O_2 appears to place the heme of this enzyme in the oxidation state required for the conversion of arachidonic acid to prostaglandin (PG) G_2 (Wei *et al.,* 1995). Prostaglandin G_2 is a peroxide that as a result of its conversion to PGH_2 replaces the need for H_2O_2 to sustain the COX reaction. Several of the PGH_2-metabolizing enzymes are hemoproteins, and one of these enzymes,

Table I Proteins Containing Redox-Sensitive Thiols with Potential for Roles in Cellular Regulation Due to Their Extreme Sensitivity to Oxidation, S-Thiolation, or Nitrosation[a]

Metabolic enzymes	Ion transport processes
Carbonic anhydrase	Calcium channels
Creatine kinase	Calcium-regulated potassium channels
Glyceraldehyde-3-phosphate dehydrogenase	Cyclic nucleotide-gated cation channels
Glycogen phosphorylase	
Signaling systems	
Caspases	
Janus kinases	
NMDA receptors	
$p21^{ras}$	
Protein kinase C	
Soluble guanylate cyclase	
Tyrosine phosphatases	

[a] See text for discussion of the mechanisms that regulate these proteins and for references containing information on the reactivities or regulatory roles of the thiols on these proteins.

PGI_2 synthase, appears to be sensitive to inactivation of peroxide-associated oxidant stress (Moncada *et al.,* 1976). Although phagocytic cells have high heme peroxidase activity (e.g., myeloperoxidase), these enzymes appear to be more involved in the production of cytotoxic species such as hypochlorous acid for use in defense mechanisms, and little evidence exists for a role of these systems in signaling processes. Thus, COX appears to be a heme peroxidase that is very sensitive to activation by peroxide, and the profile of prostaglandins that are formed may also be altered by oxidant stress.

Interactions of Other ROS with Signaling Systems

A variety of additional ROS appear to originate from the chemistry and metabolism of superoxide and H_2O_2, and some of these may interact with signaling systems in a manner that promotes physiological responses. The reaction of H_2O_2 with various chelated forms of ferrous (Fe^{2+}) iron including heme produces very reactive species, which include hydroxyl radical and oxidized iron species with hydroxyl radical-like chemical reactivity. The formation of hydroxyl radical-like species appears to promote physiological responses (Marshall and Kontos, 1991; Wei *et al.,* 1996). However, the manner through which these species interact with signaling pathways seems to be very poorly understood. The metabolism of peroxide by myeloperoxidase forms hypochlorous acid (HOCl), and HOCl readily reacts with amines (e.g., RNH_2) to form chloramines (RNHCl). Chloramines appear to elicit physiological responses (Tamai *et al.,* 1993); however, the mechanisms involved in the interactions of these species with signaling pathways are generally not known. Certain ROS-associated chemical and enzymatic reactions seem to cause the formation of singlet O_2, a reactive species that has the two unpaired electrons normally present in molecular O_2 ($\cdot O-O\cdot$) in a paired molecular structure (O=O). Although the manner through which a species of this type interacts with signaling pathways is essentially not understood, all of the reactive species described in this section have the potential to cause depletion of components of antioxidant systems, which may produce similar forms of oxidant stress and modulation of signaling systems. For example, many of the species discussed in this section have the potential to oxidize cellular GSH and to cause the formation of oxidized lipids, which often have significant biological activity.

Mechanisms Detecting Interactions between NO and ROS by Physiological Signaling Systems

Signaling mechanisms resulting from interactions between NO- and ROS-regulated systems and processes associated with the detection of RNS are discussed in this section. Aspects of the signaling mechanisms of the type considered here have been the subject of reviews (Wink and Mitchell, 1998; Wolin *et al.,* 1998).

Control of the Production and Levels of RNS

The generation rates of ROS and NO, and the activities of the various antioxidant or metabolizing systems present for ROS and RNS, are likely to function in a manner that markedly controls the amounts of the individual species present and their interactions with signaling processes. The most important interaction appears to be the reaction of NO with superoxide because of its extremely efficient rate constant, and because it involves the primary species produced by NOS and oxidase reactions that produce ROS. The product of this reaction is peroxynitrite, a substance with a half-life of around 1 s. Peroxynitrite has nitrating activity and a hydroxyl radical-like reactivity at physiological pH (Ischiropoulos, 1998; Pryor and Squadrito, 1995; Squadrito and Pryor, 1998). Under physiological conditions, several constituents are present in biological systems at concentrations that significantly compete with the spontaneous decomposition of peroxynitrite. Peroxynitrite reacts with the physiological carbon dioxide–bicarbonate buffering system to produce a nitrating species thought to be nitrosoperoxycarbonate ($ONO_2CO_2^-$, Squadrito and Pryor, 1998). The formation of this species appears to enhance tyrosine nitration, and it decomposes to generate nitrogen dioxide (NO_2). The major thiol in physiological systems with which ROS and RNS react is GSH. Peroxynitrite appears to directly react with GSH to form *S*-nitroglutathione ($GSNO_2$), a substance which spontaneously decomposes to NO (Balazy *et al.,* 1998). Under conditions of the reaction of NO with superoxide, the species present may also form *S*-nitrosoglutathione (GSNO). This is likely to occur as a result of the simultaneous presence of NO and nitrogen dioxide, which react to form N_2O_3, a species that directly produces nitrosated thiols (Goldstein and Czapski, 1996). There may be additional substances that have significant rates of reaction with peroxynitrite, or there may be biologically active species that are derived from peroxynitrite. However, the effects of peroxynitrite on signaling mechanisms discussed in this section support the importance of thiol modification and tyrosine nitration as potentially key aspects of the manner through which these species interact with biological systems. Antioxidants are likely to limit the actions of peroxynitrite and its derived RNS. Peroxynitrite appears to readily oxidize thiols (Radi *et al.,* 1991), and cellular GSH may have a major role in preventing many of the additional chemical reactions with cellular constituents that peroxynitrite has been shown (Pryor and Squadrito, 1995) to catalyze. Urate may also be an important physiological scavenger of peroxynitrite (Kooy *et al.,* 1994; Xie and Wolin, 1996) in species that maintain high levels of urate, such as primates and humans (Ames *et al.,* 1981). Peroxynitrite also seems to interact with heme peroxidases (Floris *et al.,* 1993); however, the significance of this process in controlling peroxynitrite levels and signaling is not well understood. Thus, the biological chemistry of

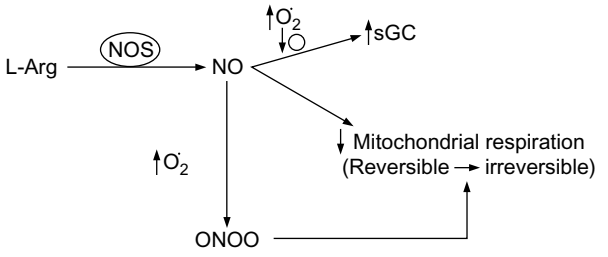


Figure 1 Effects of increases in the levels of superoxide anion (O_2^-) on signaling systems activated by low levels of NO and the consequences of peroxynitrite formation ($ONOO^-$).

the products of the reaction of NO with superoxide may have a major impact of the manner through which RNS interact with cellular signaling systems.

Interactions of ROS with Signaling Systems Regulated by NO

In this section the effects of increasing ROS on some of the better understood signaling systems mediated by NO are considered. Figure 1 contains a model showing some of the important effects superoxide has on NO signaling.

Effects of ROS on the Production of NO

ROS have several interactions that could influence the production of NO. It has been demonstrated that the various forms of SOD in tissues function to minimize the inactivation of NO by superoxide and that Cu,Zn-SOD activity in the endothelium seems to be essential for the release but not the production of NO (Mugge *et al.,* 1991; Omar *et al.,* 1991; Oury *et al.,* 1994). Although the conditions typically used for the assay of NOS activity often promote an interaction of NO with superoxide produced by the NADPH oxidase activity of this enzyme, the levels of SOD in cells that synthesize NO are probably high enough under most circumstances to minimize the effects of superoxide generation by NOS on signaling responses mediated by unmetabolized NO. However, pathophysiological conditions such as exposure of endothelium to elevated levels of low density lipoprotein have been demonstrated to promote detectable levels of NOS-derived superoxide production by these cells, and this is likely to influence NO signaling (Pritchard *et al.,* 1995). It has been demonstrated that a deficiency of the tetrahydrobiopterin cofactor for NOS seems to convert this enzyme in porcine coronary arteries from a source of endothelium-derived NO to a generator of vasoactive levels of H_2O_2 (Cosentino and Katusic, 1995). Whereas cellular antioxidant systems probably prevent ROS from directly influencing the NOS reaction, ROS may influence signaling systems that control the activity of NOS. For example, H_2O_2 has been shown to promote the release of NO from endothelium (Furchgott, 1991), and this appears to be caused by its activating the release of calcium in these cells (Elliott and Koliwad, 1995). ROS have also been shown to influence the expression of NOS activity (Ramasamy *et al.,* 1998). Thus, ROS can control the production of NO by influencing the signaling mechanisms that control NOS activity and expression. SOD activity appears to be essential for he stabilization of NO by preventing its inactivation by superoxide, and pathophysiological conditions can promote an uncoupling of NADPH consumption from NO production by the NOS reaction. A consequence of uncoupling is probably a lowering in NO production and a shift in signaling controlled by NOS to processes involving ROS and RNS.

Effects of ROS on the Stimulation of sGC by NO

The activation of sGC by NO is markedly influenced by the ROS. As previously mentioned, superoxide reacts with NO, and this attenuates the activation of sGC. Evidence is emerging that this effect of superoxide is an important contributor to many pathophysiological conditions. The effects of NO on the stimulation of sGC by H_2O_2 are discussed later in this chapter. As extracellular concentrations of peroxide approach the millimolar concentration range, H_2O_2 is also an inhibitor of NO-mediated responses associated with the stimulation of sGC (Fayngersh *et al.,* 1993). Thus, it appears that this action of peroxide might be associated with a high level of oxidant stress.

Effects of ROS on the Inhibition of Mitochondrial Respiration by NO

The potent reversible inhibition of respiration by NO is converted to an irreversible effect under conditions where a significant reaction between NO and superoxide is occurring (Xie and Wolin, 1996). Under these conditions, superoxide generation does not markedly alter the potency of NO as an inhibitor of respiration, suggesting that mitochondrial respiration by tissues is likely to be readily inhibited by peroxynitrite formed through the reaction of NO with superoxide. The irreversible inhibition of respiration by peroxynitrite appears to be primarily mediated by damage to iron–sulfur centers of enzymes such as aconitase in the Krebs cycle and complex I and II in the mitochondrial electron transport chain (Hibbs *et al.,* 1990). Studies examining the properties of macrophage cytotoxicity (Hibbs *et al.,* 1990) have detected evidence that availability of Fe^{2+} may help reverse the damage caused to iron–sulfur centers under conditions where peroxynitrite seems to be forming.

Potential Effects of ROS on Other NO-Mediated Signaling Processes

Most of the other actions of NO have not been subjected to studies critically examining the effects of superoxide generation. The interaction of species derived from the reaction of NO with superoxide is considered later in this chapter. It could be hypothesized that responses mediated by unmetabolized NO would be inhibited by a subsequent exposure to increased levels of superoxide. However, responses thought to be mediated by NO that are dependent on the formation of peroxynitrite and not mediated by a subsequent regeneration of NO might be enhanced by increased levels of superoxide.

Interactions of NO with Signaling Systems Regulated by ROS

In this section the effects of increasing NO on some of the better understood signaling systems mediated by ROS are considered. Figure 2 contains a model showing some of the fundamental interactions of NO with ROS signaling mechanisms.

EFFECTS OF NO ON THE PRODUCTION OF ROS

Systems that control the production of ROS are likely to be influenced by NO and its RNS metabolites. The availability of electron donor substrates, the enzymatic activity of some of the oxidases that produce ROS, and the antioxidant systems that metabolize or interconvert ROS are systems that can potentially be influenced by NO and its RNS metabolites. Although it is not possible to consider all of the potential mechanisms that could be involved in these interactions, some of the better documented processes will be mentioned. It is likely that the stimulation of cGMP production by NO will influence cellular activation mechanisms that control ROS production by systems such as the NADPH oxidase of phagocytic cells (Kurose *et al.,* 1995). The manner through which NO inhibits mitochondrial respiration seems to affect the electron transport chain in a fashion that should increase the production of ROS (Poderoso *et al.,* 1998). As mitochondrial function becomes more impaired by RNS, it is likely that this will attenuate the removal of cytosolic NADH by the mitochondrial shuttle systems, potentially resulting in an increase in the production of ROS by the NADH oxidase that utilizes cytosolic NADH (Rabinowitz *et al.,* 1998; Wolin *et al.,* 1996). Elevated levels of RNS are associated with the inhibition of mitochondrial SOD (Ischiropoulos *et al.,* 1992), catalase (Mohazzab-H. *et al.,* 1996), and GSH peroxidase activity (Asahi *et al.,* 1995) and with the oxidation of GSH (Radi *et al.,* 1991). Thus, NO and RNS are likely to influence the production of ROS through multiple processes. At lower levels of NO production, several of its effects are likely to contribute to physiological signaling processes, and some of these processes will be discussed in this section and later in this chapter.

EFFECTS OF NO ON THE STIMULATION OF sGC BY H_2O_2

Exposure of vascular tissue to 50 n*M* NO for 2 min causes a marked attenuation of subsequent relaxation responses to H_2O_2 that are thought to result from a stimulation of sGC activity by increased peroxide metabolism by catalase (Mohazzab-H. *et al.,* 1996). This inhibitory effect of NO appears to depend on the formation of peroxynitrite (Wolin *et al.,* 1998). However, it is not known if peroxynitrite is inhibiting catalase or if it is producing NO donors that subsequently release over time levels of NO sufficient to inhibit catalase activity. A direct exposure of catalase in buffer to NO under conditions similar to the studies on vascular tissue suggest that the heme of catalase is converted to its compound II form, which is a rather stable, inactive species of catalase. High levels of peroxide are known to reconvert catalase back to its active ferric form (Lardinois, 1995), and this seems to occur when NO-treated preparations are assayed for catalase activity in the presence of high levels of H_2O_2 (Mohazzab-H. *et al.,* 1996). Thus, NO appears to have a potent inhibitory effect on the stimulation of sGC by H_2O_2, which seems to result from the formation of peroxynitrite and an attenuation of the function of catalase.

POTENTIAL EFFECTS OF NO ON THE STIMULATION OF PROSTAGLANDIN PRODUCTION BY ROS

Since the metabolism of peroxide by cyclooxygenase appears to be a fundamental component of the initiation and sustainment of the cyclooxygenase reaction, many of the previously considered effects of NO on ROS production and peroxide metabolism could influence the activity of this enzyme in either a stimulatory or inhibitory manner. It appears that peroxynitrite can mimic the effects of the metabolism of peroxide on initiating the cyclooxygenase reaction (Landino *et al.,* 1996). The literature contains multiple observations indicating that NO stimulates and inhibits the production of prostaglandins by tissues. In addition, PGI_2 synthase appears to be readily inhibited by peroxynitrite (Zou and Ullrich, 1996). Thus, NO has multiple potential sites of interaction with the systems that control the production of prostaglandins by tissues, which are currently rather poorly understood.

EFFECTS OF NO ON THE INHIBITION OF MITOCHONDRIAL RESPIRATION BY SUPEROXIDE

Evidence for the inhibition of mitochondrial function by superoxide evolved from studies on the mechanisms of oxygen toxicity. Mitochondrial aconitase, which is inhibited as a result of damage to its iron–sulfur center, is considered to be a cellular site of the pathophysiological actions of superoxide (Gardner *et al.,* 1994). Studies suggest that the presence of NO has the potential to enhance by approximately 10-fold the potency of the irreversible inhibitory actions of superoxide on mitochondrial respiration (Xie and Wolin, 1996). The mechanism involved in this enhancement appears to originate from the formation of peroxynitrite, a species whose actions were discussed previously in this chapter. The significance of this hypothesized potentially important role of NO in the toxicity of oxygen is likely to be better defined by future studies in this field.

Signaling Mechanisms Originating from RNS Derived from ROS

This section focuses on how species derived from interactions between ROS and NO or RNS could be potentially linked to the control of signaling systems. Properties of the interactions between ROS and RNS that seem to be of the most significance in physiological systems were considered previously in this chapter. It appears that peroxynitrite may be one of the most important species involved in the interactions of ROS-derived RNS with signaling systems. Some potential targets for the initiation of signaling mechanisms by peroxynitrite include key thiols, tyrosine, iron–sulfur

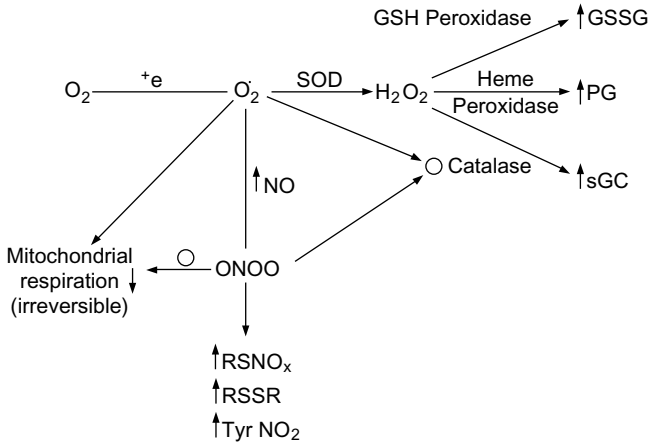


Figure 2 Effects of increases in the levels of NO on regulatory systems activated by low levels of ROS and additional signaling interactions that occur as the levels of peroxynitrite ($ONOO^-$) increase. Potential systems regulated by oxidized GSH (GSSG), protein S-thiolation or thiol oxidation (RSSR), and protein nitrosation ($RSNO_x$) are listed in Table I.

centers, and unsaturated fatty acids. Figure 2 contains a model showing how changes in the levels of NO and superoxide are linked to signaling processes as a result of the production of peroxynitrite.

Signaling Mechanisms Potentially Originating from Interactions of RNS with Thiols

Peroxynitrite can modify thiols by reactions that cause nitrosation (RSNO, Goldstein and Czapski 1996), nitration ($RSNO_2$, Balazy *et al.,* 1998), and oxidation reactions (Radi *et al.,* 1991), which result in disulfide formation (RSSR'), S-thiolation (RSSG), and thiol oxidation products (RSO_x). Most of these interactions of peroxynitrite with thiols should be given serious consideration as processes that can be involved in signaling because they are usually readily reversible or have the potential to produce tissue storage forms of NO ($RSNO_x$). It is likely that many of the thiol-containing signaling systems previously considered in this chapter as targets for regulation by ROS-elicited alterations in thiol redox are potential sites of action of peroxynitrite. Table I lists some of the proteins known to possess redox-sensitive thiol groups that have the potential to participate in cellular regulatory processes. Some of the thiol-containing cellular sites that have been demonstrated to be regulated by NO-mediated mechanisms include the key metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase (Mohr *et al.,* 1994) and creatine kinase (Gross *et al.,* 1996), calcium-regulated potassium channels (Bolotina *et al.,* 1994), cyclic nucleotide-gated cation channels (Broillet and Firestein, 1996), Janus kinases (Duhe *et al.,* 1998), tyrosine phosphatases (Caselli *et al.,* 1995), and *N*-methyl-D-aspartate (NMDA) receptors (Lipton *et al.,* 1993).

Signaling Mechanisms Potentially Originating From Interactions of RNS with Other Systems

There are additional signaling related processes that are potentially regulated by RNS. It has been suggested that the nitration of protein tyrosine groups could influence signaling mechanisms regulated by tyrosine phosphorylation (Ischiropoulos, 1998). Modifications of this type could potentially alter the function of a protein in a manner similar to tyrosine phosphorylation, or, if the nitration reaction was a very efficient process on a tyrosine group normally regulated by phosphorylation, it could function as an inhibitor of the phosphorylation-linked signaling. Cells that have significant levels of heme peroxidase activity might also be able to nitrate protein tyrosine groups for regulatory purposes through a more recently documented peroxide-catalyzed metabolism by the peroxidase of the NO decomposition product nitrite (Sampson *et al.,* 1998). However, it is likely that this tyrosine nitration reaction will not be of importance in the majority of cell types present in mammalian organisms *in vivo* because most cells typically contain low heme peroxidase activity. In addition, most of the peroxide needed for this process is likely to be derived from superoxide (which should function to promote peroxynitrite formation), and it is not clear that adequate levels of nitrite are available. Modification of iron–sulfur centers by peroxynitrite is a well-documented process, which was previously discussed in several sections of this chapter related to its role in the control of mitochondrial function and iron metabolism. Peroxynitrite appears to also modify unsaturated fatty acids to form oxidized lipids, with some of the lipids possessing NO_x functional groups (Rubbo *et al.,* 1995). Although these lipids were initially considered for their role in the pathological actions of peroxynitrite, it is possible that certain of the key products of these reactions might be biologically active participants in signaling processes. It is also conceivable that peroxynitrite or other RNS could have interactions with signaling processes through chemical or metabolic reactions in addition to those considered in this chapter.

Signaling Mechanisms of Physiological Processes That Appear to Be Regulated by Systems That Detect RNS and ROS

In this section some of the better documented roles for the ROS and RNS signaling mechanisms discussed in this chapter in physiological and pathophysiological regulatory processes are highlighted. Several of the systems mentioned, but not discussed in detail, are considered in greater depth in other chapters of this book.

The Importance of the Regulation of sGC by ROS and RNS

There appear to be multiple mechanisms through which ROS and RNS can alter the activity of sGC. The most potent interactions with sGC include the stimulation of sGC by NO and H_2O_2 metabolism by catalase. Although the stimulation of sGC by NO has been studied in multiple systems, evidence for the regulation of sGC through peroxide metabolism by catalase has only been described in a few vascular

preparations. Multiple redox systems, including GSH peroxidase, NO, superoxide, and the presence of electron donors for catalase, may function in tissues normally to inhibit expression of the stimulation of sGC by the catalase-dependent mechanism. Because the production of H_2O_2 is typically regulated by oxygen tension, tissue redox, and certain receptor regulated oxidases, the control of sGC activity by the catalase mechanism is likely to have a role in responses involved in the sensing of changes in oxygen tension, lactate produced by tissue hypoxia, and receptor-regulated oxidant signaling. The peroxide-dependent regulation of sGC appears to participate in responses of pulmonary arteries to hypoxia (Burke-Wolin and Wolin, 1990; Wolin *et al.,* 1996), of coronary arteries to posthypoxic reoxygenation (Mohazzab-H. *et al.,* 1999), and of the relaxation of multiple vascular preparations to levels of lactate that are produced during exercise (Wolin *et al.,* 1999b).

Attenuation of the regulation of sGC by an inactivation of NO by superoxide appears to be one of the most potent biological actions of superoxide that is observed in physiological systems. The activities of the various forms of SOD present in tissues seem to have a major role in controlling the expression of this interaction. Because it appears that a NO-regulated form of sGC is present in a large number of cell types, processes including the attenuation of NO stimulation of sGC by superoxide and perhaps the formation of NO donors in tissues as a result of the production of significant levels of peroxynitrite at elevated levels of NO should be considered as fundamental cellular signaling processes. It is already well documented that the attenuation of NO-mediated stimulation of sGC by superoxide can antagonize the actions of NO that are involved in promoting smooth muscle relaxation, the inhibition of platelet aggregation, the inhibition of neutrophil adhesion, and neurotransmission in a variety of experimental systems.

The Importance of the Regulation of Arachidonic Acid Metabolism by ROS and RNS

The potent effects of peroxide on stimulating prostaglandin formation by cyclooxygenase appears to be a basic component of certain physiological regulatory processes. Thus, the role of additional effects of NO, RNS, and ROS on arachidonic acid metabolism in biological processes remains to be better defined. For example, certain oxygen-elicited vascular responses, such as the acute increase in blood flow (reactive hyperemia) following a brief arterial occlusion (Wolin *et al.,* 1990) and the promotion of the production of prostaglandin formation by inflammatory processes, appear to be mediated in part by the production of H_2O_2 (Gurtner and Burke-Wolin, 1991). Although it is well documented that the activity of prostacyclin or PGI_2 synthase is inhibited by peroxynitrite and elevated levels of peroxide (Moncada *et al.,* 1976; Zou and Ullrich, 1996), and that several diseases associated with oxidant stress alter the production of prostaglandins (Gurtner and Burke-Wolin, 1991), the role of ROS and RNS in these changes remains to be better defined.

The Importance of the Regulation of Aspects of Tissue Metabolism and Respiration by ROS and RNS

NO controls tissue oxygen consumption in conscious dogs in a manner that appears to be independent of hemodynamic changes and increases in respiration promoted by exercise and cardiac pacing (Shen *et al.,* 1994, 1995; Bernstein *et al.,* 1996). It is also well documented that conditions associated with increased oxidant stress, such as hyperoxia (Gardner *et al.,* 1994) and tissue ischemia/reperfusion (Granger, 1988), and increased peroxynitrite production during macrophage cell killing (Hibbs *et al.,* 1990) are associated with irreversible effects mitochondrial function. NO appears to regulate respiration in a manner that improves the efficiency of tissue oxygen utilization (Bernstein *et al.,* 1996; Wolin *et al.,* 1999a), whereas the formation of peroxynitrite seems to cause a loss of efficiency of oxygen utilization (Schulz *et al.,* 1997). Thus, NO, superoxide, peroxynitrite, and other species are likely to have important roles in controlling mitochondrial function under physiological and pathophysiological conditions. The function of certain other thiol-containing metabolic enzymes, including glyceraldehyde-3-phosphate dehydrogenase (Mohr *et al.,* 1994) and creatine kinase (Gross *et al.,* 1996), also appears to be readily inactivated by tissue injury processes associated with oxidant stress and/or the formation of RNS. Many of the consequences of these metabolic effects of NO, peroxynitrite, and ROS on these systems remain to be elucidated.

The Importance of the Regulation of Thiol Redox Processes by ROS and RNS

Multiple interactions of ROS and RNS with systems that are influenced by thiol redox have been discussed throughout this chapter. These interactions include modification of thiols by several different forms of oxidation including S-thiolation, nitrosation, and nitration. It is likely that many different forms of signaling are mediated by the various ways thiols can be modified, and each pathway will have subtle ways through which thiol redox processes control the systems being regulated. The susceptibility of each of the thiol groups on proteins to modification is influenced by the reactivity and accessibility to the process it is being modified by. Thus, aspects of the processes that control thiol redox may permit a diversity of systems to be regulated by the behavior of individual thiol groups that are components of signaling mechanisms for each regulatory system. Many key components of cellular regulatory systems have thiol groups whose redox status appears to influence the function of the system. Table I lists some of the enzymes influenced by thiol redox that are potential contributors to cellular control mechanisms. Thus, thiol redox processes can participate in the signaling mechanisms that control metabolism (Gilbert, 1984; Brigelius, 1985; Mohr *et al.,* 1994; Gross *et al.,* 1996; Ji *et al.,* 1999), potassium channels (Bolotina *et al.,* 1994; Weir and Archer, 1995), calcium channels (Elliott and Koliwad, 1995; Jones *et al.,* 1983), cyclic nucleotide-gated

cation channels (Broillet and Firestein, 1996), protein kinase C (Gopalakrishna *et al.,* 1999), sGC (Brandwein *et al.,* 1981), tyrosine phosphatases (Hecht and Zick, 1992; Caselli *et al.,* 1995), and NMDA receptor function (Lipton *et al.,* 1993). These mechanisms are likely to be involved in the control of important physiological responses, and many of the signaling systems are activated by ROS and RNS through mechanisms that are not well understood. Some of these mchanisms and physiological processes are mentioned next.

Other Physiological Regulatory Systems that Appear to Be Controlled by ROS and RNS

The nature of many of the processes discussed in this chapter suggests they have important fundamental roles in signaling mechanisms involving ROS and RNS. Multiple additional actions of ROS and RNS on signaling systems involved in physiological responses have been observed, but the actual manner through which the ROS or RNS interact with the signaling system have often not been identified. Some examples include the activation of several phospholipases, protein kinase C, extracellular signal regulated kinases, and mitogen-activated protein kinases and the regulation of multiple signaling and gene expression processes through redox-sensitive transcription factors including nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (Lander, 1997; Suzuki *et al.,* 1997; Abe and Berk, 1998). These processes have important roles in the control of smooth muscle contractile behavior, in the function of antioxidant systems, in the expression of adhesion proteins, and in cell proliferation, apoptosis, and many other basic physiological processes.

Concluding Remarks

Evidence is accumulating that ROS and RNS interact with cellular control mechanisms in a manner that is consistent with the many of the processes being components of fundamental signaling mechanisms. Most of the individual ROS and RNS have unique ways of interacting with or being sensed by signaling systems. There appear to be second messenger-like regulatory processes that are controlled by rapid changes in the levels of specific ROS and perhaps RNS that are formed during normal physiological responses such as the sensing of oxygen tension and stimulation of receptors. Slightly higher levels or a more prolonged exposure to increased lower levels of ROS and RNS appear to participate in the control of additional regulatory processes important to physiological or pathophysiological function such as controlling the expression of adhesion proteins, the activation of cellular proliferation, and the expression of multiple genes. Levels of ROS and RNS that strongly perturb antioxidant defense systems also seem to activate signaling responses. However, it appears that these mechanisms may be more involved in pathophysiological processes, including responses of tissues to injury, such as apoptosis, and in the activation of mechanisms of repair. Thus, ROS and RNS appear to participate in a diversity of important signaling mechanisms, and much remains to be learned regarding how these systems are designed to function.

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References

Abe, J.-I., and Berk, B. C. (1998). Reactive oxygen species as mediators of signal transduction in cardiovascular disease. *Trends Cardiovasc. Med.* **8,** 59–64.

Abrahamsson, T., Brandt, U., Marklund, S. L., and Sjoqvist, P.-O. (1992). Vascular bound recombinant extracellular superoxide dismutase type C protects against the detrimental effects of superoxide radicals on endothelium-dependent relaxation. *Circ. Res.* **70,** 264–271.

Ames, B. N., Cathcart, R., Schwiers, E., and Hochstein, P. (1981). Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6858–6862.

Arnelle, D. R., and Stamler, J. S. (1995). NO^+, NO· and NO^- donation by *S*-nitrosothiols: Implications for regulation and physiological function by S-nitrosylation and acceleration of disulfide formation. *Arch. Biochem. Biophys.* **318,** 279–285.

Asahi, M., Fujii, J., Suzuki, K., Seo, H. G., Kuzuya, T., Hori, M., Tada, M., Fuji, S., and Taniguchi, N. (1995). Inactivation of glutathione peroxidase by nitric oxide. *J. Biol. Chem.* **270,** 21035–21039.

Balazy, M., Kaminski, P. M., Mao, K., Tan, J., and Wolin, M. S. (1998). *S*-Nitroglutathione: A product of the reaction between peroxynitrite and glutathione that generates nitric oxide. *J. Biol. Chem.* **273,** 32009–32015.

Bernstein, R. D., Ochoa, F. Y., Xu, X., Forfia, P., Shen, W., Thompson, C. I., and Hintze, T. H. (1996). Function and production of nitric oxide in the coronary circulation of conscious dog during exercise. *Circ. Res.* **79,** 840–848.

Blum, J., and Fridovich, I. (1985). Inactivation of glutathione peroxidase by superoxide radical. *Arch. Biochem. Biophys.* **240,** 500–508.

Boese, M., Mordvintcev, P. I., Vanin, A. F., Busse, R., and Mulsch, A. (1995). S-Nitrosation of serum albumin by dinitrosyl–iron complexes. *J. Biol. Chem.* **270,** 29244–29249.

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368,** 850–853.

Brandwein, H. J., Lewicki, J. A., and Murad, F. (1981). Reversible inactivation of guanylate cyclase by mixed disulfide formation. *J. Biol. Chem.* **256,** 2958–2962.

Brigelius, R. (1985). Mixed disulfides: Biological functions and oxidative stress. *In* ''Oxidative Stress'' (H. Sies, ed.), pp. 243–272. Academic Press, London.

Broillet, M.-C., and Firestein, S. (1996). Direct activation of the olfactory cyclic nucleotide-gated channel through modification of sulfhydryl groups by NO compounds. *Neuron* **16,** 377–385.

Brown, G. C. (1995). Reversible binding and inhibition of catalase by nitric oxide. *Eur. J. Biochem.* **232,** 188–191, 1995.

Brown, G. C., McBride, A. G., Fox, E. J., McNaught, K. St. P., and Borutaite, V. (1997). Nitric oxide and oxygen metabolism. *Biochem. Soc. Trans.* **25,** 901–904.

Burke, T. M., and Wolin, M. S. (1987). Hydrogen peroxide elicits pulmonary arterial relaxation and guanylate cyclase activation. *Am. J. Physiol.* **252,** H721–H732.

Burke-Wolin, T. M., and Wolin, M. S. (1990). Inhibition of cGMP-associated pulmonary arterial relaxation to H_2O_2 and O_2 by ethanol. *Am. J. Physiol.* **258,** H1267–H1273.

Caselli, A., Chiarugi, P., Camici, G., Manao, G., and Ramponi, G. (1995). In vivo activation of phosphotyrosine protein phosphatases by nitric oxide. *FEBS Lett.* **374,** 249–252.

Cenas, N., Anusevicius, Z., Bironaite, D., Bachmanova, G. I., Archakov, A., and Ollinger, K. (1994). The electron transfer reactions of NADPH: cytochrome P450 reductase with nonphysiological oxidants. *Arch. Biochem. Biophys.* **315,** 400–406.

Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59,** 527–605.

Cherry, P. D., and Wolin, M. S. (1989). Ascorbate activates soluble guanylate cyclase via H_2O_2-metabolism by catalase. *Free Radicals Biol. Med.* **7,** 485–490.

Cherry, P. D., Omar, H. A., Farrell, K. A., Stuart, J. S., and Wolin, M. S. (1990). Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. *Am. J. Physiol.* **259,** H1056–H1062.

Cosentino, F., and Katusic, Z. S. (1995). Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. *Circulation* **91,** 139–144.

Cross, A. R., and Jones, O. T. G. (1991). Enzymatic mechanisms of superoxide production. *Biochim. Biophys. Acta* **1057,** 291–298.

Davidson, C. A., Kaminski, P. M., and Wolin, M. S. (1997). Nitric oxide elicits prolonged relaxation of bovine pulmonary arteries via endogenous peroxynitrite generation. *Am. J. Physiol.* **273,** L437–L444.

Deinum, G., Stone, J. R., Babcock, G. T., and Marletta, M. A. (1996). Binding of nitric oxide and carbon monoxide to soluble guanylate cyclase as observed with resonance raman spectroscopy. *Biochemistry* **35,** 1540–1547.

Dierks, E. A., and Burstyn, J. N. (1998). The deactivation of soluble guanylate cyclase by redox-active agents. *Arch. Biochem. Biophys.* **351,** 1–7.

Doyle, M. P., and Hoekstra, J. W. (1981). Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Biochem.* **14,** 351–358.

Drapier, J., and Bouton, C. (1996). Modulation by nitric oxide of metaloprotein regulatory activities. *BioEssays* **18,** 1–8.

Duhe, R. J., Evans, G. A., Erwin, R. A., Kirken, R. A., Cox, G. W., and Farrar, W. L. (1998). Nitric oxide and thiol redox regulation of Janus kinase activity. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 126–131.

Duprat, F., Guillemare, E., Romey, G., Fink, M., Lesage, F., and Lazdunski, M. (1995). Susceptibility of cloned K^+ channels to reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11796–11800.

Elliott, S., and Koliwad, S. K. (1995). Oxidant stress and endothelial membrane transport. *Free Radicals Biol. Med.* **19,** 649–658.

Fayngersh, R., Burke-Wolin, T., and Wolin, M. S. (1993). Selective inhibitory actions of hydrogen peroxide on relaxing mechanisms in the calf pulmonary artery. *Am. Rev. Resp. Dis.* **147,** A16.

Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993). Interaction of myeloperoxidase with peroxynitrite: A comparison with lactoperoxidase, horseradish peroxidase and catalase. *Eur. J. Biochem.* **215,** 767–775.

Fridovich, I. (1985). Superoxide dismutase, regularities and irregularities. *Harvey Lect.* **79,** 51–75.

Furchgott, R. F. (1991). Interactions of H_2O_2 and NO in modifying tone in vascular smooth muscle: The SOD paradox. *In* ''Resistance Arteries, Structure and Function'' (M. J. Mulvany, ed.), pp. 216–220. Elsevier, New York.

Gardner, P. R., Nguyen, D. H., and White, C. W. (1994). Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12248–12252.

Gaston, B., Drazen, J. M., Loscalzo, J., and Stamler, J. S. (1994). The biology of nitrogen oxides in airways. *Am. J. Respir. Crit. Care Med.* **149,** 538–551.

Gilbert, H. F. (1984). Redox control of enzyme activities by thiol/disulfide exchange. *Methods Enzymol.* **107,** 330–351.

Goldstein, S., and Czapski, G. (1996). Mechanism of the nitrosation of thiols and amines by oxygenated ·NO solutions: The nature of the nitrosating intermediates. *J. Am. Chem. Soc.* **118,** 3419–3425.

Goodwin, D. C., Gunther, M. R., Hsi, L. C., Crews, B. C., Eling, T. E., Mason, R. P., and Marnett, L. J. (1998). Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. *J. Biol. Chem.* **273,** 8903–8909.

Gopalakrishna, R., Gundimeda, U., Anderson, W. B., Colburn, N. H., and Slaga, T. J. (1999). Tumor promotor benzoyl peroxide induces sulfhydryl oxidation in protein kinase C: Its reversibility is related to cellular resistance to peroxide-induced cytotoxicity. *Arch. Biochem. Biophys.* **363,** 246–258.

Gow, A. J., Buerk, D. G., and Ischiropoulos, H. (1997). A novel reaction mechanism for the formation of *S*-nitrosothiol in vivo. *J. Biol. Chem.* **272,** 2841–2845.

Granger, D. N. (1988). Role of xanthine oxidase and granulocytes in ischemia–reperfusion injury. *Am. J. Physiol.* **255,** H1269–H1275.

Griendling, K. K., and Ushio-Fukai, M. (1997). NADH/NADPH oxidase and vascular function. *Trends Cardiovasc. Med.* **7,** 301–307.

Gross, W. L., Bak, M. I., Ingwall, J. S., Arstall, M. A., Smith, T. W., Balligand, J.-L., and Kelly, R. A. (1996). Nitric oxide inhibits creatine kinase and regulates rat heart contractile reserve. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5604–5609.

Gupte, S., Rupawalla, B. A., Phillibert, D., and Wolin, M. S. (1998). Potential influence of heme redox on nitric oxide-elicited pulmonary artery relaxation and guanylate cyclase activation. *Circulation* **98,** I-342–I-343.

Gurtner, G. H., and Burke-Wolin, T. (1991). Interactions of oxidant stress and vascular reactivity. *Am. J. Physiol.* **260,** L207–L211.

Hecht, D., and Zick, Y. (1992). Selective inhibition of protein tyrosine phosphatase activities by H_2O_2 and vanadate in vitro. *Biochem. Biophys. Res. Commun.* **188,** 773–779.

Hibbs, J. B., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J.-C., Amber, I. J., and Lancaster, J. R. (1990). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of sml-nmarginine: A molecular mechanism regulating cellular proliferation that targets intracellular iron. *In* ''Nitric Oxide from sml-nmArginine: A Cellular Bioregulatory System'' (S. Moncada and J. B. Hibbs, eds.), pp. 189–223. Elsevier, Amsterdam.

Holland, J. A., Pappolla, M. A., Wolin, M. S., Pritchard, K. A., Rogers, N. J., and Stemerman, M. B. (1990). Bradykinin induces superoxide anion release from human endothelial cells. *J. Cell. Physiol.* **143,** 21–25.

Hultquist, D. E. (1978). Methemoglobin reduction system of erythrocytes. *Methods Enzymol.* **52,** 463–475.

Iesaki, T., Gupte, S., and Wolin, M. S. (1998). A flavoprotein mechanism appears to prevent an oxygen-dependent inhibition of cGMP-associated nitric oxide-elicited relaxation of bovine coronary arteries. *Circulation* **98,** I-666.

Ignarro, L. J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ. Res.* **65,** 1–21.

Ischiropoulos, H. (1998). Biological tryosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356,** 1–11.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D., and Beckman, J. S. (1992). Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298,** 431–437.

Ji, Y., Akerbloom, T. P. M., Sies, H., and Thomas, J. A. (1999). S-nitrosylation and S-glutathiolation of protein sulfhydryls by *S*-nitroso glutathione. *Arch. Biochem. Biophys.* **362,** 67–78.

Jobsis, F. F. (1972). Oxidative metabolism at low PO_2. *FASEB J.* **31,** 1404–1413.

Jones, D. P., Thor, H., Smith, M. T., Jewell, S. A., and Orrenius, S. (1983). Inhibition of ATP-dependent microsomal Ca^{2+} sequestration during oxidative stress and its prevention by glutathione. *J. Biol. Chem.* **258,** 6390–6393.

Keilin, D., and Hartree, E. F. (1937). Reaction of nitric oxide with heamoglobin and metheamoglobin. *Nature* **139,** 548.

Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997). Kinetics of nitric oxide dissociation from five- and six-coordinate nitrosyl hemes and heme proteins, including soluble guanylate cyclase. *Biochemistry* **36,** 6814–6818.

Kono, Y., and Fridovich, I. (1982). Superoxide radical inhibits catalase. *J. Biol. Chem.* **257,** 5751–5754.

Kooy, N. W., Royall, J. A., Ischiropoulos, H., and Beckman, J. S. (1994). Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radicals Biol. Med.* **16,** 149–156.

Kukreja, R. C., Kontos, H. A., Hess, M. L., and Ellis, E. F. (1986). PGH synthetase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circ. Res.* **59,** 612–619.

Kurose, I., Wolf, R., Grisham, M. B., Aw, T. Y., Specian, R. D., and Granger, D. N. (1995). Microvascular responses to inhibition of nitric oxide production. Role of active oxidants. *Circ. Res.* **76,** 30–30.

Lander, H. M. (1997). An essential role of free radicals and derived species in signal transduction. *FASEB J.* **11,** 118–124.

Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996). Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15069–15074.

Lardinois, O. M. (1995). Reactions of bovine liver catalase with superoxide radicals and hydrogen peroxide. *Free Radical Res.* **22,** 251–274.

Lipton, S. A., Choi, Y.-B., Pan, Z.-H., Lei, S. Z., Chen, H.-S. V., Scher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and nitroso-compounds. *Nature* **364,** 626–632.

Marshall, J. J., and Kontos, H. A. (1991). Endothelium and cerebral vascular diseases. *In* ''Cardiovascular Significance of Endothelium-Derived Vasoactive Factors'' (G. M. Rubanyi, ed.), pp. 125–145. Futura, Mount Kisco, New York.

Mohazzab-H., K. M., and Wolin, M. S. (1994). Properties of a superoxide anion generating microsomal NADH-oxidoreductase, a potential pulmonary artery PO_2 sensor. *Am. J. Physiol.* **267,** L823–L831.

Mohazzab-H., K. M., Fayngersh, R. P., and Wolin, M. S. (1996). Nitric oxide inhibits pulmonary artery catalase and H_2O_2-associated relaxation. *Am. J. Physiol.* **271,** H1900–H1906.

Mohazzab-H., K. M., Agarwal, R., and Wolin, M. S. (1999). Influence of glutathione peroxidase on coronary artery responses to alterations in PO_2 and H_2O_2. *Am. J. Physiol.* **276,** H235–H241.

Mohr, S., Stamler, J. S., and, Brune, B. (1994). Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite and related nitrosating agents. *FEBS Lett.* **348,** 223–227.

Moncada, S., Gryglewski, R. J., Bunting, S., and Vane, J. R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides into an unstable substance which inhibits platelet aggregation. *Nature* **263,** 663–665.

Morre, D. J., and Brightman, A. O. (1991). NADH oxidase of plasma membranes. *J. Bioenerget. Biomemb.* **23,** 469–489.

Mugge, A., Elwell, J. H., Peterson, T. E., and Harrison, D. G. (1991). Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. *Am. J. Physiol.* **260,** C219–C225.

Mulsch, A., Mordvinteev, P. I., Vanin, A. F., and Busse, R. (1993). Formation and release of dinitrosyl iron complexes by endothelial cells. *Biochem. Biophys. Res. Commun.* **196,** 1303–1308.

Omar, H. A., Cherry, P. D., Mortelliti, M. P., Burke-Wolin, T., and Wolin, M. S. (1991). Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent & independent nitrovasodilator relaxation. *Circ. Res.* **69,** 601–608.

Omar, H. A., Mohazzab-H., K. M., Mortelliti, M. P., and Wolin, M. S. (1993). O_2 dependent modulation of calf pulmonary artery tone by lactate: Role of H_2O_2 and cGMP. *Am. J. Physiol.* **264,** L141–L145.

Oury, T. D., Chang, L.-Y., Marklund, S. L., Day, B. J., and Crapo, J. D. (1994). Immunocytochemical localization of extracellular superoxide dismutase in human lung. *Lab. Invest.* **70,** 889–898.

Poderoso, J. J., Peralta, J. G., Lisdero, C. L., Carreras, M. C., Radisic, M., Schopfer, F., Cadenas, E., and Boveris, A. (1998). Nitric oxide regulates oxygen uptake and hydrogen peroxide release by isolated beating rat heart. *Am. J. Physiol.* **274,** C112–C119.

Pritchard, K. A., Groszek, L., Smalley, D. M., Sessa, W. C., Wu, M., Villalon, P., Wolin, M. S., and Stemerman, M. B. (1995). Native low density lipoprotein enhances endothelial cell nitric oxide synthase generation of superoxide anion. *Circ. Res.* **77,** 510–518.

Pryor, W. A., and Squadrito, G. L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.* **268,** L699–L722.

Rabinowitz, J. D., Vacchino, J. F., Beeson, C., and McConnell, H. M. (1998). Potentiometric measurement of intracellular redox activity. *J. Am. Chem. Soc.* **120,** 2464–2473.

Radi, R., Beckman, J. S., and Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of endothelial-derived superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Ramasamy, S., Parthasarathy, S., and Harrison, D. G. (1998). Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. *J. Lipid Res.* **39,** 268–276.

Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaraman, B., and Freeman, B. A. (1995). A nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324,** 15–25.

Rusche, K. M., Spiering, M. M., and Marletta, M. A. (1998). Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry* **37,** 15503–15512.

Sampson, J. B., Ye, Y., Rosen, H., and Beckman, J. S. (1998). Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Biophys.* **356,** 207–213.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., and Mayer, B. (1996). Characterization of 1H-[1,2,4]oxadiozolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanyl cyclase. *Mol. Pharmacol.* **50,** 1–5.

Schulz, R., Dodge, K. L., Lopaschuk, G. D., and Clanachan, A. S. (1997). Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am. J. Physiol.* **272,** H1212–H1219.

Shen, W., Xu, X., Ochoa, M., Zhao, G., Wolin, M. S., and Hintze, T. H. (1994). Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. *Circ. Res.* **75,** 1086–1095.

Shen, W., Hintze, T. H., and Wolin, M. S. (1995). Nitric oxide: An important signalling mechanism between the vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation* **92,** 3505–3512.

Squadrito, G. L., and Pryor, W. A. (1998). Oxidative chemistry of nitric oxide: The roles of superoxide, peroxynitrite and carbon dioxide. *Free Radicals Biol. Med.* **25,** 392–403.

Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox-active forms. *Science* **258,** 1898–1902.

Stubauer, G., Giuffre, A., Brunori, M., and Sarti, P. (1998). Cytochrome c oxidase does not catalyze the anaerobic reduction of NO. *Biochem. Biophys. Res. Commum.* **245,** 459–464.

Sun, C.-W., Alonso-Galicia, M., Teheria, M. R., Falck, J. R., Harder, D. R., and Roman, R. J. (1998). Nitric oxide-20-hydroxyeicosatetraenoic acid interaction in the regulation of K^+ channel activity and vascular tone in renal arterioles. *Circ. Res.* **83,** 1069–1079.

Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radicals Biol. Med.* **22,** 269–285.

Tamai, H., Kachur, J. F., Grisham, M. B., Musch, M. W., Chang, E. B., and Gaginella, T. S. (1993). Effect of the thiol oxidizing agent diamide on NH_2Cl-induced rat colonic electrolyte secretion. *Am. J. Physiol.* **265,** C166–C170.

Thelen, M., Dewald, B., and Baggiolini, M. (1993). Neutrophil signal transduction activation and the respiratory burst. *Physiol. Rev.* **73,** 797–821.

Thomas, J. A., Poland, B., and Honzatko, R. (1995). Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Arch. Biochem. Biophys.* **319,** 1–9.

Torres, J., Cooper, C. E., and Wilson, M. T. (1998). A common mechanism for the interaction of nitric oxide with the oxidized binuclear centre and oxygen intermediates of cytochrome *c* oxidase. *J. Biol. Chem.* **273,** 8756–8766.

Wallace, D. C. (1999). Mitochondrial disease in man and mouse. *Science* **283,** 1482–1488.

Wei, C., Kulmacz, R. J., and Tsai, A.-L. (1995). Comparison of branched-chain and tightly controlled reaction mechanisms for prostaglandin H synthesis. *Biochemistry* **34,** 8499–8512.

Wei, E. P., Kontos, H. A., and Beckman, J. S. (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *Am. J. Physiol.* **271,** H1262–H1266.

Weir, E. K., and Archer, S. L. (1995). The mechanism of acute hypoxic pulmonary vasoconstriction: The tale of two channels. *FASEB J.* **9,** 183–189.

Wink, D. A., and Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radicals Biol. Med.* **25,** 434–456.

Wink, D. A., Cook, J. A., Pacelli, R., Liebmann, J., Krishna, M. C., and Mitchell, J. B. (1995). Nitric oxide (NO) protects against cellular damage by reactive oxygen species. *Toxicol. Lett.* **82/83,** 221–226.

Wolin, M. S. (1996). Reactive oxygen species and vascular signal transduction mechanisms. *Microcirculation* **3,** 1–17.

Wolin, M. S., and Belloni, F. L. (1985). Superoxide anion selectively attenuates catecholamine-induced contractile tension in isolated rabbit aorta. *Am. J. Physiol.* **249,** H1127–H1133.

Wolin, M. S., and Mohazzab-H., K. M. (1997). Mediation of signal transduction by oxidants. *In* ''Oxidative Stress and the Molecular Biology of Antioxidant Defenses'' (J. G. Scandalios, ed.), pp. 21–48. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Wolin, M. S., Rodenburg, J. M., Messina, E. J., and Kaley, G. (1990). Similarities in the pharmacological modulation of reactive hyperemia and vasodilation to H_2O_2 in rat skeletal muscle arterioles: Effects of probes for endothelium-derived mediators. *J. Pharmacol. Exp. Ther.* **253,** 508–512.

Wolin, M. S., Burke-Wolin, T. M., Kaminski, P. M., and Mohazzab-H., K. M. (1996). Reactive oxygen species and vascular oxygen sensors. *In* ''Nitric Oxide and Radicals in the Pulmonary Vasculature'' (E. K. Weir, S. L. Archer, and J. T. Reeves, eds.), pp. 245–263. Futura, Armonk, New York.

Wolin, M. S., Davidson, C. A., Kaminski, P. M., Fayngersh, R. P., and Mohazzab-H., K. M. (1998). Oxidant-nitric oxide signaling mechanisms in vascular tissue. *Biochemistry* (*Moscow*) **63,** 810–816.

Wolin, M. S., Xie, Y.-W., and Hintze, T. H. (1999a). NO as a regulator of tissue oxygen consumption. *Curr. Opin. Nephrol. Hypertens.* **8,** 97–103.

Wolin, M. S., Burke-Wolin, T. M., and Mohazzab-H., K. M. (1999b). Roles for NAD(P)H oxidases and reactive oxygen species in vascular oxygen sensing mechanisms. *Respir. Physiol.* **115,** 229–238.

Xie, Y.-W., and Wolin, M. S. (1996). Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration: Involvement in response to hypoxia/reoxygenation. *Circulation* **94,** 2580–2586.

Yu, A. E., Hu, S., Spiro, T. G., and Burstyn, J. N. (1994). Raman resonance spectroscopy of soluble guanyl cyclase reveals displacement of distal and proximal heme ligands by NO. *J. Am. Chem. Soc.* **116,** 4117–4118.

Zou, M.-H., and Ullrich, V. (1996). Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin syntase. *FEBS Lett.* **382,** 101–104.

CHAPTER 19

Nitric Oxide, Oxygen Radicals, and Iron Metabolism

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THE BIOLOGY OF IRON IS INTIMATELY CONNECTED WITH NITRIC OXIDE (NO) AND OXYGEN RADICALS. HERE WE ILLUSTRATE HOW NO AND OXYGEN METABOLITES BIOCHEMICALLY INTERACT WITH IRON AND, IN ADDITION, HOW THESE REACTIVE MOLECULES FUNCTION AS SIGNALS TO REGULATE CELLULAR IRON METABOLISM. WE FIRST PROVIDE AN OVERVIEW OF THE IMPORTANCE OF IRON IN BIOLOGY AND DESCRIBE THE DIFFERENT STRATEGIES THAT CELLS AND ORGANISMS, FROM BACTERIA TO HUMANS, HAVE ADAPTED TO ACQUIRE NUTRITIONAL IRON. EMPHASIS IS GIVEN TO THE POSTTRANSCRIPTIONAL REGULATORY CIRCUIT THAT CONTROLS CELLULAR IRON METABOLISM IN HIGHER EUKARYOTES: THE EXPRESSION OF PROTEINS WITH KEY FUNCTIONS IN IRON UPTAKE, STORAGE, AND UTILIZATION IS COORDINATELY REGULATED AT THE LEVELS OF mRNA TRANSLATION AND STABILITY. THE MECHANISM INVOLVES BINDING OF TWO HOMOLOGOUS CYTOPLASMIC IRON REGULATORY PROTEINS, IRP1 AND IRP2, TO IRON RESPONSIVE ELEMENTS (IREs), CONSERVED STEM–LOOP STRUCTURES IN THE UNTRANSLATED REGIONS OF mRNAs. IRON REGULATORY PROTEINS RESPOND TO IRON BUT ALSO TO IRON-INDEPENDENT SIGNALS, INCLUDING NO AND OXIDATIVE STRESS. MECHANISTIC AND PHYSIOLOGICAL ASPECTS OF THE REGULATION OF IRON METABOLISM BY NO AND OXIDATIVE STRESS ARE DISCUSSED AT THE END OF THE CHAPTER.

The Biology of Iron: Role of Oxygen Radicals and NO

Iron is the second most abundant transition metal on the earth's crust after aluminum. In chemical terms, iron is characterized by its capacity to form a variety of coordination complexes with organic molecules and by its electrochemical redox potential between the two basal ferrous Fe(II) and ferric Fe(III) states. These features form the basis of a provocative evolutionary hypothesis: iron minerals on the earth's crust may have provided a substrate for crucial redox reactions to generate elementary energy sources for primitive forms of life (Wächtershäuser, 1992). Contemporary living cells have exploited the advantageous chemical properties of iron and have utilized iron in macromolecules to fulfill a wide range of fundamental structural and metabolic functions. These include oxygen transport and a plethora of electron transfer and catalytic reactions (Aisen, 1994).

The broad spectrum of iron-dependent biochemical activities implies a dynamic and flexible mode for iron coordination within proteins. Iron-binding proteins can be classified into two major groups, namely, those containing heme or nonheme iron (Frausto da Silva and Williams, 1991). Heme, a very common prosthetic group, is composed of protoporphyrin IX and Fe(II), which can be oxidized to Fe(III) to form hemin. Only Fe(II) can be utilized for enzymatic heme biosynthesis. The insertion of the ferrous iron ion into the tetrapyrrole ring of protoporphyrin IX is catalyzed by ferrochelatase, and this represents the terminal step of the heme biosynthetic pathway. Heme serves as the oxygen carrier in hemoglobin and myoglobin, and it participates in electron

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transfer reactions in cytochromes. Heme also constitutes part of the active site of many enzymes, including nitric oxide synthetases and catalase.

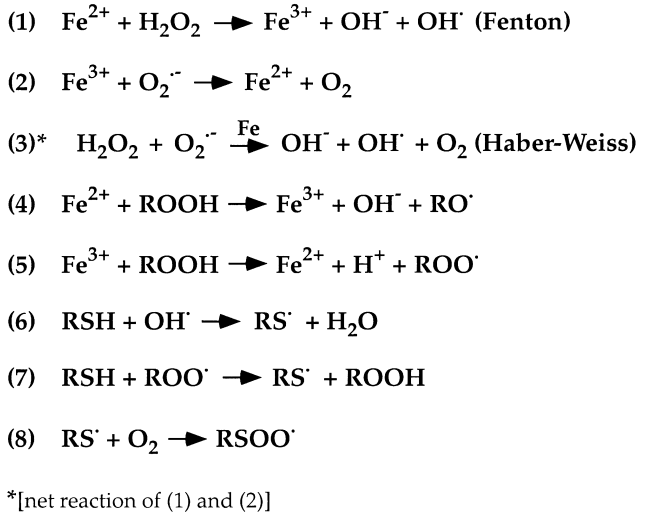
The most prevalent forms of nonheme iron in proteins are the iron–sulfur clusters (Frausto da Silva and Williams, 1991; Beinert *et al.,* 1997). Different types of iron–sulfur clusters have been identified, including 2Fe–2S, 3Fe–4S, and 4Fe–4S. Iron–sulfur clusters play diverse functional roles in electron transfer reactions (Rieske proteins and ferredoxins), transcriptional regulation (bacterial transcription factors SoxR and FNR), structure stabilization (bacterial endonuclease III), and catalysis (dehydratases, including aconitase). Some bacterial enzymes contain more complex iron–sulfur centers together with an additional metal, such as Fe–Mo–S clusters in nitrogenase (to catalyze nitrogen fixation), or Ni–Fe–S clusters in hydrogenase (to catalyze the generation of molecular hydrogen). Mammalian oxidases, such as cyclooxygenase and lipoxygenase, contain a mononuclear iron center instead of heme or iron–sulfur iron. In these enzymes, iron is involved in the activation of the substrate (e.g., arachidonic acid) to be oxidized. The remarkable variety of ways by which iron can coordinate to metalloproteins is also reflected in the unusual diiron–oxo bridge Fe–O–Fe of ribonucleotide reductase, which catalyzes the reduction of ribo- to deoxyribonucleotides, a critical step in DNA synthesis.

Considering the importance of iron as a metal cofactor, one would predict that prokaryotic as well as eukaryotic cells require sufficient supplies of iron for growth, and that iron deficiency in higher organisms, particularly in humans, is associated with various disorders (Andrews, 1999). The vast majority (70%) of iron in the adult human body is utilized in erythroid cells for the synthesis of hemoglobin (Ponka, 1997). The depletion of body iron stores thus results in impaired erythropoiesis, leading to anemia. In light of the constraints and relative inefficiency of dietary iron absorption (see below), it is not surprising that iron deficiency anemia constitutes one of the most common nutritional pathological conditions, affecting ~20% of the world's population (DeMaeyer and Adiels-Tegman, 1985).

Too little iron is bad, but too much iron is even worse. This is evident from the severe disorders of primary and secondary iron overload (Andrews, 1999). Hereditary hemochromatosis is a highly frequent autosomal recessive disease which affects 1:400 individuals in Western populations (Andrews and Levy, 1998; Cox and Kelly, 1998). The defect in hereditary hemochromatosis causes an increase in iron absorption that ultimately leads to iron deposition in tissue parenchymal cells. Repeated blood transfusion, as may be required in the treatment of various anemias, leads to secondary iron overload, mainly in the reticuloendothelial system (Pippard, 1994). Another interesting example of a disorder associated with disturbed iron homeostasis is Friedreich's ataxia, an autosomal recessive disease where iron accumulates in the mitochondria, particularly of the heart and the nervous system (Koenig and Mandel, 1997). In general, excess iron can lead to tissue degeneration and the development of cirrhosis, hepatoma, or heart failure. Interestingly, epidemiological studies suggest that even in healthy individuals, increased body iron stores constitute a risk factor for ischemic heart disease and cancer (Stevens *et al.,* 1988).

The toxicity of iron is based on Fenton/Haber–Weiss chemistry; the iron (or copper)-catalyzed generation of hydroxyl radical (OH·) by derivatives of dioxygen, such as superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2) (Fig. 1). Iron (and copper) also contribute to the generation of organic reactive species, such as peroxyl (ROO·), alkoxyl (RO·), thiyl (RS·), and thiyl–peroxyl (RSOO·) radicals (Fig. 1). Oxygen and organic free radicals are highly aggressive compounds that attack virtually all cellular macromolecules. The result is damage to membranes, proteins, and nucleic acids, which can ultimately lead to cell death or tissue degeneration (Halliwell and Gutteridge, 1990). Cells produce spontaneously ''reactive oxygen intermediates'' (ROIs) by incomplete reduction of dioxygen during aerobic respiration in mitochondria. The superoxide dismutases, catalase, and glutathione peroxidase metabolize superoxide and hydrogen peroxide, and they serve as guardians to protect cells from oxygen-dependent iron toxicity (Cadenas, 1989; Sies, 1997). An increase in levels of ROIs, referred to as oxidative stress, is a hallmark of numerous pathological conditions (Ferrari *et al.,* 1998; Beal, 1998; Sun and Chen, 1998), including inflammation, ischemia–reperfusion injury, and neurodegenerative disorders, such as amylotrophic lateral sclerosis, Alzheimer's, and Parkinson's disease. Oxidative stress has also received attention in the context of aging (Ames *et al.,* 1993; Shigenaga *et al.,* 1994) and cancer (Dreosti, 1998; Poulsen *et al.,* 1998). Thus, it is not surprising that oxidative stress leads to activation of complex genetic defence mechanisms (Baeuerle *et al.,* 1996; Kyriakis and Avruch, 1996).

Apart from being toxic by-products, oxygen radicals are utilized by specialized cells of the immune system to combat bacterial infection (Baggiolini and Thelan, 1991; Hampton



*[net reaction of (1) and (2)]

Figure 1 Iron-catalyzed generation of oxygen and organic free radicals.

et al., 1998). For this purpose, macrophages and neutrophils are stimulated during the ''oxidative burst'' to release high levels of superoxide, generated enzymatically by a plasma membrane-bound NADPH oxidase, that immediately undergoes dismutation to hydrogen peroxide. The effectiveness of this immune response very likely depends on the presence of iron and the recycling of oxygen radicals by Fenton/Haber–Weiss chemistry. In addition, the bactericidal (and cytotoxic) capacity of the oxidative burst is enhanced by nitric oxide (NO) and other reactive oxygen species such as peroxynitrite ($ONOO^-$) and hypochlorite (OCl^-). The inducible nitric oxide synthase (iNOS or NOS2) in macrophages produces NO to inactivate crucial iron-containing proteins in bacteria (or target cells), including aconitase, complexes I and II of the respiratory chain, and ribonucleotide reductase. Peroxynitrite and hypochlorite are potent oxidants; the former is generated spontaneously by superoxide and nitric oxide, whereas the latter is synthesized by the heme-containing enzyme myeloperoxidase from hydrogen peroxide and chloride.

On a chemical basis, it becomes evident that iron, oxygen radicals, and NO are partners that possess an enormous toxic potential. Therefore, their concentrations should be tightly controlled. Oxygen radicals and NO are short-lived intermediates, and their activities are antagonized by degrading enzymes or low-molecular-weight antioxidants. On the other hand, higher organisms have a limited capacity to remove or eliminate iron. Iron stores are instead controlled at the level of absorption, which is, under normal conditions, very limited. Thus, the regulation of iron homeostasis poses a challenge to satisfy the metabolic needs of cells and organisms for iron and at the same time to minimize the risk of iron toxicity in an aerobic environment.

Iron Metabolism

The major problem of iron assimilation by living cells is its limited bioavailability. Soluble Fe(II) is readily oxidized in aqueous solutions to the essentially insoluble (at neutral pH) ferric hydroxide. Many bacteria cope with this problem by synthesizing and secreting ferric iron chelators, the siderophores (Wooldridge and Williams, 1993; Braun, 1997). Similarly, some plants secrete phytosiderophores. These soluble, low-molecular-weight carriers transport iron across cell membranes by binding to specific receptors. In the reducing intracellular milieu, Fe(III) is liberated and converted to Fe(II).

The budding yeast *Saccharomyces cerevisiae* utilizes an elegant mechanism to acquire environmental iron (Klausner and Dancis, 1994; Askwith *et al.,* 1996; Kaplan and O'Halloran, 1996; Dancis, 1998; Radisky and Kaplan, 1999). The first step involves reduction of ferric to ferrous iron in the vicinity of the cell. Subsequently, Fe(II) is either directly internalized by a low-affinity transporter ($K_m = 40$ μM), or reoxidized to Fe(III) and internalized by a distinct high-affinity transporter ($K_m = 0.15$ μM). The identification, molecular cloning, and functional characterization of components of these pathways provided valuable insights into the underlying molecular mechanisms. The first step of Fe(III) reduction is catalyzed by two plasma membrane-bound ferric (and cupric) reductases, encoded by FRE1 (Dancis *et al.,* 1992) and FRE2 (Georgatsou and Alexandraki, 1994). A good candidate for the low-affinity ferrous transporter is encoded by FET4 (Dix *et al.,* 1994), which has six potential transmembrane domains. The high-affinity transporting system comprises two activities: (1) a plasma membrane-bound multicopper ferroxidase, encoded by FET3 (Askwith *et al.,* 1994), that is homologous to mammalian ceruloplasmin, a soluble ferroxidase in the plasma, and (2) a permease, apparently encoded by FTR1 (Stearman *et al.,* 1996). Fet3p, with a single transmembrane domain, associates with Ftr1p, which potentially spans the plasma membrane at six sites. It appears that oxidation of Fe(II) by Fet3p drives Fe(III) to Ftr1p for internalization. The assembly of Ftr1p–Fet3p complexes is required for cell surface expression and for iron transport. Interestingly, related mechanisms appear to operate in some plants. However, these are far less well defined. A gene from *Arabidopsis thaliana,* IRT1, encoding a putative membrane-bound Fe(II) transporter protein has been cloned (Eide *et al.,* 1996) an awaits further functional characterization.

Thus, unicellular organisms and plants have adopted two strategies to assimilate environmental iron for their nutritional needs. These involve either (a) capture of soluble Fe(III) chelates from the extracellular milieu with siderophores, with the iron-loaded siderophores, or even host iron-binding proteins, then being transported by binding to specific cell surface receptors, or (b) reduction of Fe(III) to Fe(II) catalyzed by the extracellular domain of membrane-spanning ferric reductases, and internalization via low- or high-affinity iron transporters.

These principles have been conserved in higher organisms. In mammals, dietary iron is absorbed in the upper part of the small intestine (mainly in the duodenum) (Skikne and Baynes, 1994). Iron is then transported through the mucosal cells to the portal circulation and, finally, delivered to tissues and the erythroid bone marrow. Duodenal iron absorption involves solubilization of Fe(III) in the acidic pH of the intestinal lumen, reduction to Fe(II) (Raja *et al.,* 1992; Riedel *et al.,* 1995) by still ill-characterized reductase(s) and ascorbate, and internalization by a ferrous transporter. In the bloodstream, iron is scavenged by transferrin, a protein that functions like a siderophore and delivers iron to the cells by binding to a specific cell surface receptor (see below). Interestingly, the distinct strategies for intestinal iron absorption and cellular iron uptake are strongly reminiscent of the modes of iron assimilation by yeast and bacteria, respectively.

Early studies employing radiolabeled iron have identified the duodenum as the major site for dietary iron absorption (Skikne and Baynes, 1994). Iron from the intestinal lumen is taken up across the brush border of mature enterocytes. These specialized cells are localized at the microvillar tips

of the duodenal epithelium and derive from precursor mucosal cells in the crypts. The maturation process is associated with migration of the enterocytes from the crypts to the villi. Newly absorbed iron is transported across the basolateral membrane into the circulation and binds to transferrin (Fig. 2). The mature enterocytes are the critical cells for the regulation of dietary iron absorption. In hereditary hemochromatosis the enterocytes absorb pathologically high levels of iron. At the other end of the spectrum, the causative defect in some forms of microcytic anemia lies in the failure of enterocytes to transfer sufficient quantities of iron to the plasma.

Despite the well-established role of enterocytes in the regulation of iron absorption, relatively little progress has been made toward identifying the molecules involved in these processes. However, the more recent identification of genes involved in iron acquisition and its control have greatly stimulated the field. One of these genes encodes a divalent metal transporter, DMT1, first reported in the literature as Nramp2 or DCT1 (Fleming *et al.,* 1997; Gunshin *et al.,* 1997). Genetic studies have implicated DCT1/Nramp2, a member of the Nramp (natural resistance-associated macrophage protein) family of proteins, as a candidate for an intestinal ferrous iron transporter. In microcytic anemia (*mk*) mice, the G185R mutation affects a predicted transmembrane domain of the DCT1/Nramp2 protein. This mutation appears to be responsible for diminished iron absorption that renders the animals anemic due to severe iron deficiency (Fleming *et al.,* 1997). Furthermore, mammalian cells transfected with a DCT1/Nramp2 cDNA (Su *et al.,* 1998), or *Xenopus* oocytes microinjected with mRNA fractions enriched in DCT1/Nramp2 mRNA (Gunshin *et al.,* 1997), display enhanced iron uptake. In addition to Fe(II), DCT1/Nramp2 is also capable of transporting a variety of divalent cations, including Cu(II), Zn(II), Cd(II), Mn(II), and Co(II) (Gunshin *et al.,* 1997).

The pathway of iron transfer across the basolateral membrane of enterocytes is incompletely understood. Genetic approaches may prove to be informative, as a relevant animal model is already available, namely, the sex-linked anemia (*sla*) mouse. Hemizygous male *sla* mice suffer from microcytic anemia. However, in contrast to the *mk* mice that have impaired capacity for iron absorption, the *sla* mice have a defect in iron delivery to the circulation. The gene defective in the *sla* mouse, *Heph,* has been cloned (Vulpe *et al.,* 1999). It encodes hephaestin, a transmembrane-bound protein, that shares homology with ceruloplasmin. The recent identification and cloning of a strong candidate for the long-sought basolateral iron transporter, named IREG1 (McKie *et al.,* 2000) or ferroportin 1 (Donovan *et al.,* 2000) is expected to provide new insights into the mechanisms of iron transport.

Serum transferrin (Tf), a monomeric glycoprotein of 80 kDa, is the carrier of iron in the blood and binds two Fe(III) ions with high affinity ($K_d = 10^{-23}$ *M* at neutral pH) (Ponka *et al.,* 1998). Only ~30% of Tf is saturated with iron under physiological conditions. Tf possesses intrinsic ferroxidase activity and belongs to a family of related proteins found in other biological fluids, such as lactoferrin in milk and ovotransferrin in egg white. Delivery of iron to Tf may involve the ferroxidase activity of ceruloplasmin. In contrast to yeast Fet3p and to intestinal hephaestin, which are both transmembrane proteins, ceruloplasmin is soluble. Its precise biological function is still unclear, but it has always been considered as a factor involved in iron egress from tissues (de Silva *et al.,* 1996). This is also strongly supported by the phenotype of aceruloplasminemic patients, who suffer from impaired ceruloplasmin synthesis and develop tissue iron overload (Harris *et al.,* 1995; Gitlin, 1998). Studies have also implicated ceruloplasmin in hepatocyte iron uptake (Mukhopadhyay *et al.,* 1998), but the physiological significance of this finding is not clear yet. The identification of potential ceruloplasmin-interacting proteins, by analogy to the yeast example, may shed more light on these questions.

The major route for iron uptake into erythroid and nonerythroid cells utilizes the binding of diferric Tf to the cell surface transferrin receptor (TfR) (Fig. 3). TfR is a homo-

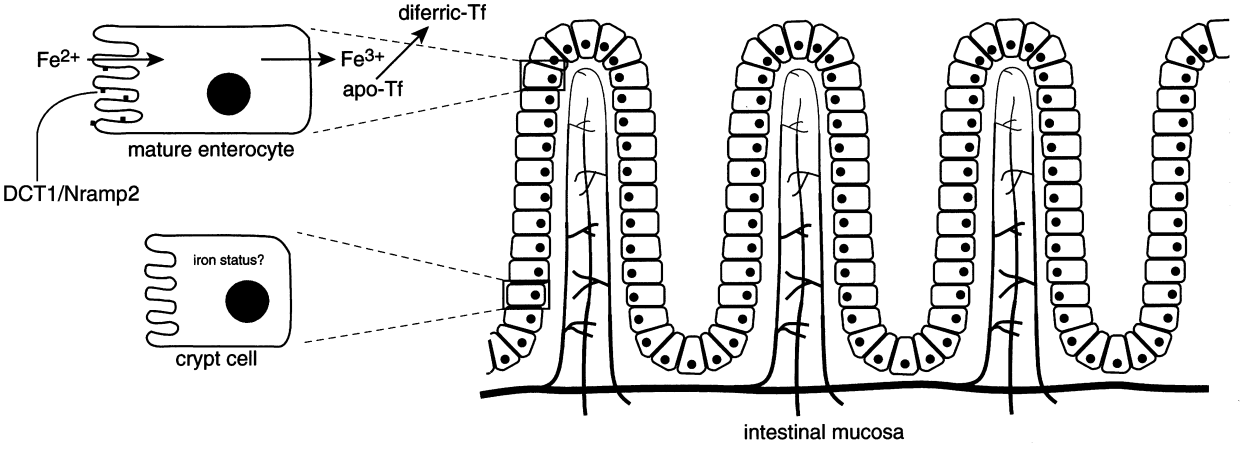


Figure 2 Dietary iron absorption in the duodenum. Ferrous iron is transported from the intestinal lumen across the brush border of mature enterocytes. The apical transport very likely involves the divalent metal transporter DCT1/Nramp2. Following transport across the basolateral membrane of the mature enterocyte, iron is delivered to the circulation and binds to apotransferrin (apo-Tf) to form diferric Tf. The iron status of the precursor cells in the crypts appears to reflect the iron absorption capacity of mature enterocytes.

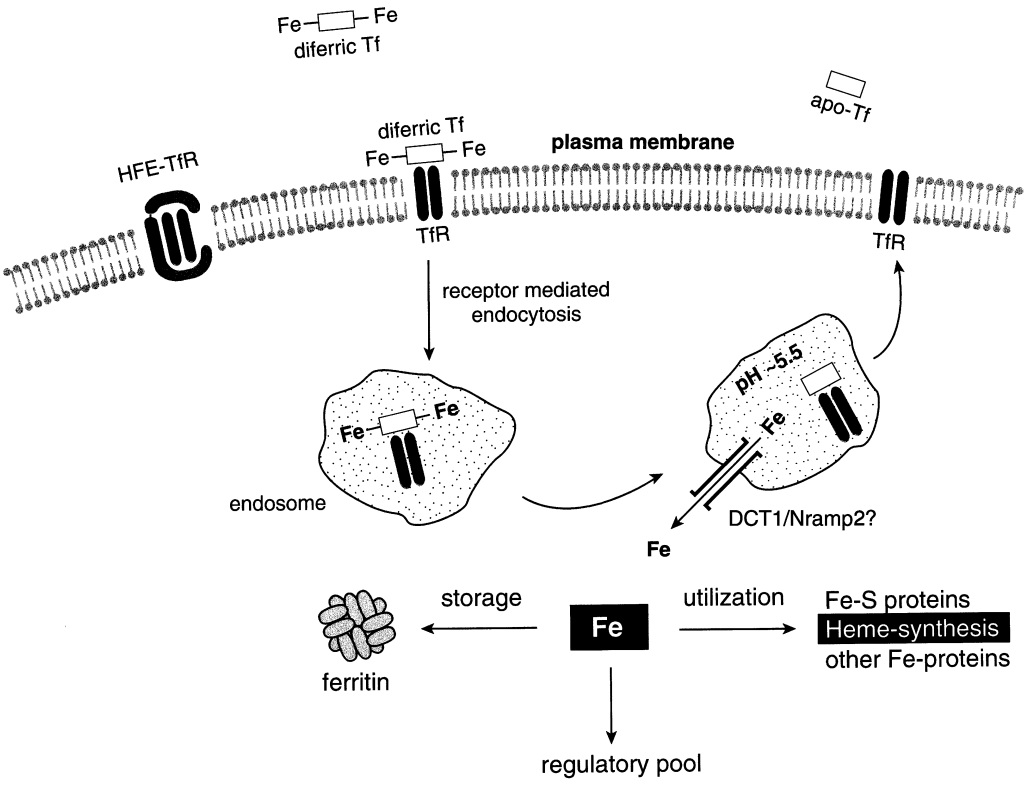


Figure 3 The Tf–TfR cycle in mammalian cells. Extracellular diferric Tf binds to cell surface TfR, and the Tf–TfR complex is internalized by endocytosis. Iron is released, following acidification of the endosome, by a pathway that may involve the activity of the divalent metal transporter DCT1/Nramp2. In the cytoplasm, iron is utilized for the synthesis of iron-containing proteins, and excess is stored in ferritin. ApoTf–TfR complex dissociates at the cell surface. Association of TfR with HFE may prevent binding of extracellular diferric Tf to TfR.

dimeric glycoprotein of 180 kDa that can bind two molecules of Tf. At neutral pH, diferric Tf has a ~500-fold higher binding affinity to the TfR than apoTf (Ponka *et al.,* 1998). The internalization of TfR occurs by classic receptor-mediated endocytosis. In the acidic milieu of the endosome (pH ~5.5) the affinity of ferric iron to TfR-bound Tf drops dramatically, resulting in its release. The apoTf–TfR complex recycles on the cell surface, while ferric iron is reduced to ferrous and delivered from the endosome into the cytosol for utilization by an as yet poorly defined mechanism. A fraction of released iron remains bound to low-molecular-weight chelates, presumably including citrate, ATP, pyrophosphates, or ascorbic acid, and determines the iron status of the cell. This ''regulatory iron pool'' can be monitored by fluorescence techniques (Epsztejn *et al.,* 1997). Not much is known about iron metabolism in intracellular organelles. Genetic and biochemical data suggest that frataxin, a newly characterized gene which is mutated in patients with Friedreich's ataxia (Koenig and Mandel, 1997), has an important role for the maintenance of iron homeostasis in the mitochondria by encoding a protein that stimulates iron efflux (Babcock *et al.,* 1997; Radisky *et al.,* 1999).

Excess iron is sequestered in ferritin, the main intracellular iron storage protein, which is composed of 24 subunits of H- and L-chains (21- and 19-kDa, respectively) (Aisen and Listowski, 1980; Theil, 1987; Ponka *et al.,* 1998). These assemble to a symmetric shell-like structure with a potential to store up to 4500 ferric iron ions. Functional ferritin molecules are composed of different proportions of L- and H-subunits in different tissues. For example, L-subunits predominate in liver and H-subunits in heart ''isoferritins.'' Iron incorporation into ferritin requires a ferroxidase activity associated with H-subunits and a nucleation center associated with L-subunits. Iron mobilization from ferritin, to cover cellular metabolic needs, is incompletely understood and may involve structural changes in ferritin subunits (Takagi *et al.,* 1998). It is also conceivable that utilization of ferritin-bound iron may be coupled with ferritin turnover (Radisky and Kaplan, 1998). Overall, ferritin constitutes a versatile device to sequester and detoxify considerable quantities of iron and, moreover, maintains iron in a soluble, bioavailable form.

The mechanism of intracellular iron transport from the endosome into the cytoplasm is not clear. Genetic data arising from the *mk* mouse and Belgrade (*b*) rat models have implicated DCT1/Nramp2 in this process. DCT1/Nramp2 in the *b* rat carries the same G185R mutation, as in the *mk* mouse, and displays a similar phenotype: a severe iron deficiency anemia (Fleming *et al.,* 1998). Previous reports had suggested that the defect in the *b* rat lies in the failure to deliver transferrin-bound iron into the cytoplasm (Garrick *et*

al., 1993). Taken together, the experimental data obtained with the *mk* mouse and the *b* rat imply that the function of DCT1/Nramp2 may extend from dietary iron absorption in enterocytes to intracellular iron transport across the endosomal membrane in different cell types. Clearly, these attractive speculations await more direct experimental support. The identification of DCT1/Nramp2 is more recent and its characterization thus still preliminary.

Although understanding the function of DCT1/Nramp2 is definitely challenging, the product of another recently identified gene poses no less of a puzzle: HFE is an atypical MHC class I molecule that carries mutations in the vast majority of hereditary hemochromatosis patients (Feder *et al.,* 1996). The most prevalent (>80%) of these is a single C282Y point mutation that abrogates the physical interaction of HFE with β2-microglobulin and thus prevents HFE processing in the Golgi apparatus and HFE expression on the cell surface. Unlike typical MHC class I molecules, HFE does not present peptide antigens (Lebrón *et al.,* 1998). Genetic and clinical evidence suggests that HFE negatively regulates iron absorption in the intestine and, possibly, iron release from macrophages. An unequivocal function of HFE in the control of iron metabolism has been established by the targeted disruption of the HFE gene in mouse: HFE knockout mice develop iron overload in tissue parenchymal cells (Beutler, 1998; Zhou *et al.,* 1998). Interestingly, the same phenotype was earlier observed in β2-microglobulin knockout mice (de Sousa *et al.,* 1994; Rothenberg and Voland, 1996), suggesting that processing of HFE is critical for its function. However, the mechanism for the exact role of HFE in iron metabolism remains as yet elusive. The finding that HFE has the potential to interact with the TfR and decrease the affinity of Tf for the TfR (Parkkila *et al.,* 1997; Gross *et al.,* 1998; Lebrón *et al.,* 1998) has raised the possibility that HFE may have an inhibitory effect on iron uptake by TfR. On the other hand, iron uptake studies in crypt and villar enterocytes, together with the intracellular localization of HFE in crypt cells, have led to an alternative hypothesis (Waheed *et al.,* 1999). According to this, HFE may positively regulate iron delivery from plasma to immature crypt enterocytes by the Tf–TfR route. The iron status of these precursor cells appears to reflect the capacity of mature enterocytes for dietary iron absorption. A signaling function of HFE may be critical for programming differentiating mucosal cells to reduced dietary iron absorption on maturation. This hypothesis is supported by the finding that HFE knockout mice express inappropriately high levels of DCT1/Nramp2 mRNA (Fleming *et al.,* 1999). Moreover, DCT1/Nramp2 mRNA levels are increased in duodenal biopsy samples of hemochromatosis patients carrying the C282Y mutation in HFE (Zoller *et al.,* 1999).

The functional characterization of DCT1/Nramp2 and HFE has generated new questions about the role of these proteins in iron metabolism. The assessment of the biological implications associated with the function of DCT1/Nramp2 and HFE has created immense excitement. However, at this point functional hypotheses have to be viewed as such and require further experimental evaluation.

Posttranscriptional Regulation of Iron Metabolism

The expression of many genes involved in mammalian iron metabolism is controlled at the posttranscriptional level in response to iron concentration (Klausner *et al.,* 1993; Kühn, 1994; Hentze and Kühn, 1996; Rouault *et al.,* 1996; Richardson and Ponka, 1997; Pantopoulos and Hentze, 1999). Thus, in iron-starved mammalian cells, which do not need to store but rather take up iron, ferritin mRNA translation is inhibited, and the otherwise unstable TfR mRNA is stabilized. However, in iron-loaded cells, which have to store excess iron and reduce additional uptake, ferritin mRNA is readily translated, and TfR mRNA is prone to degradation. The common denominator in these homeostatic responses is the presence of so-called iron responsive elements (IREs) in the untranslated regions (UTRs) of these mRNAs. The IRE is a phylogenetically conserved hairpin structure (Theil, 1994), originally identified in the 5' UTR of the mRNAs encoding ferritin H- and L-chains (Hentze *et al.,* 1987). Soon thereafter, multiple IREs were found in the 3' UTR of TfR mRNAs (Müllner and Kühn, 1988; Casey *et al.,* 1989). Typical IREs consist of about 30 nucleotides that form a structured loop with the sequence 5'-CAGUGN-3' and a stem with moderate stability ($\Delta G \approx -7$ kcal/mol), interrupted by a bulge that contains an unpaired 5' C residue (Fig. 4).

The IRE constitutes a binding site for either of two iron regulatory proteins, IRP1 and IRP2, which are activated for

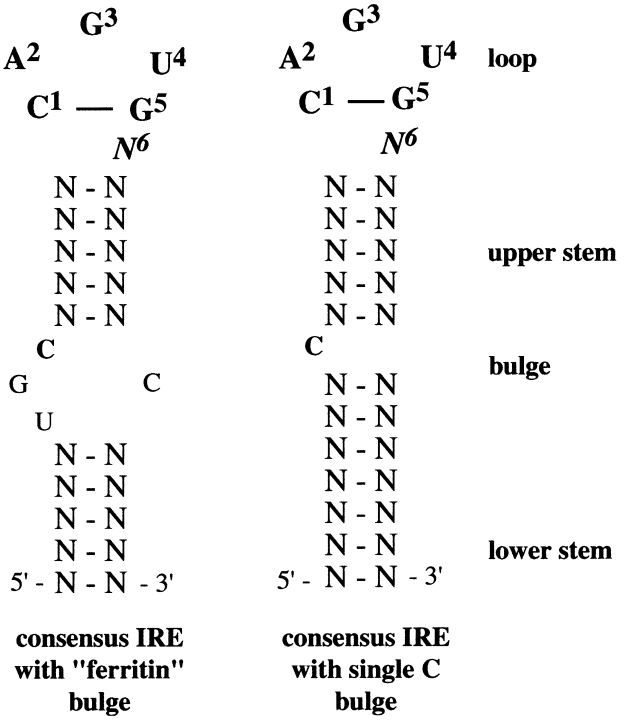


Figure 4 The IRE consensus motif consists of a hexanucleotide loop (5'-CAGUGN-3') and a stem, interrupted by a bulge with an unpaired C residue. Base pairing between C^1 and G^5 is functionally important. N^6 could be any nucleotide but not G, which would potentially disrupt C^1–G^5 interaction by C^1–G^6 pairing. The bulge may consist of an asymmetric tetranucleotide, as in many ferritin mRNAs (left), or of a single C residue (right).

high-affinity IRE binding by iron starvation. The IRE–IRP binding results in specific translational arrest of ferritin mRNAs (Rouault *et al.,* 1988) and stabilization of TfR mRNA (Koeller *et al.,* 1989). Conversely, in iron-replete cells, IRE binding activity decreases dramatically, permitting ferritin H- and L-chain mRNA translation and TfR mRNA degradation (Fig. 5). Taken together, these IRE–IRP interactions, in the context of their respective mRNAs, are necessary

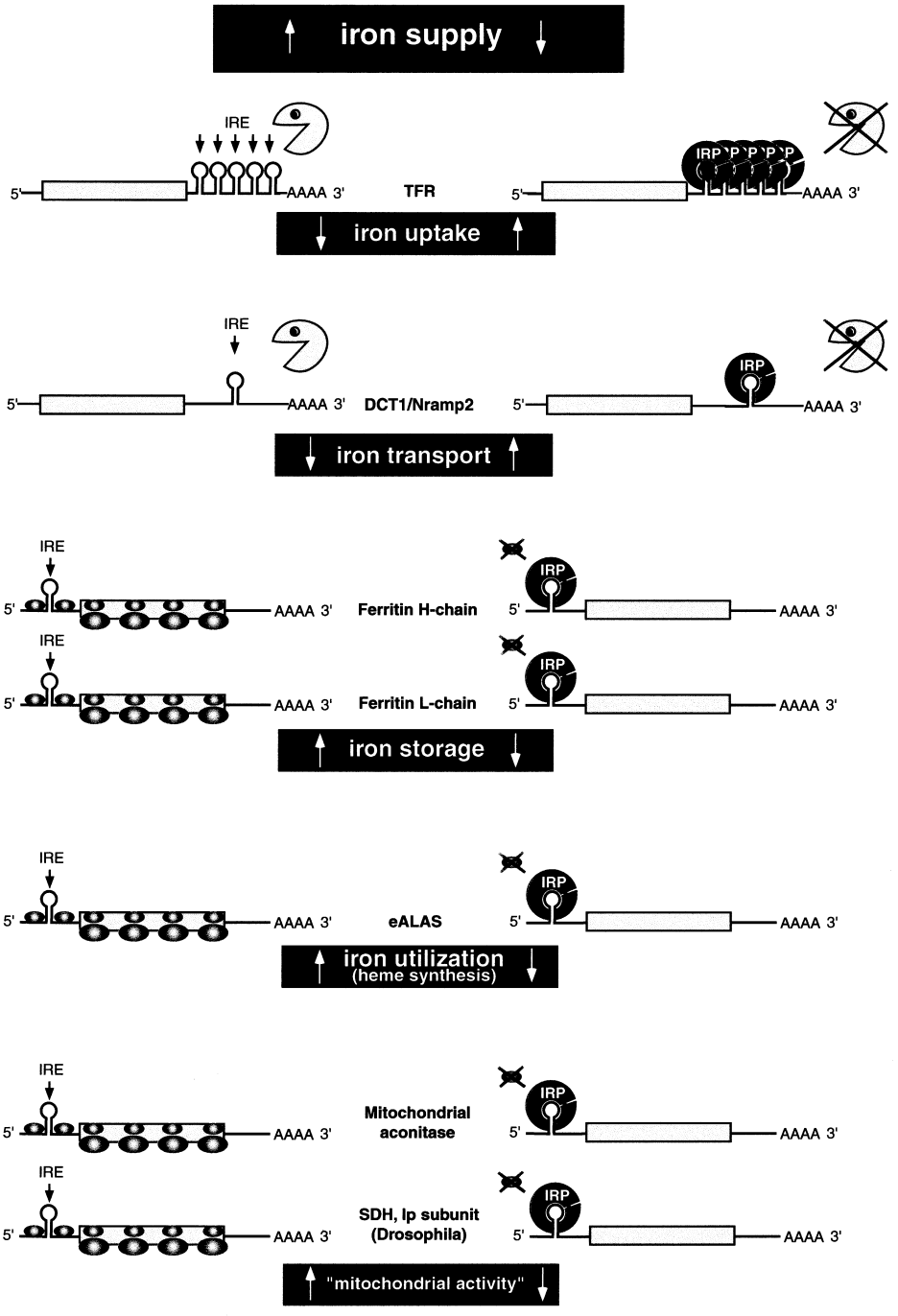


Figure 5 Homeostatic responses to cellular iron supply mediated by IRE–IRP interactions. Decreased iron supply activates binding of IRPs to IREs, resulting in stabilization of TfR and DCT1/Nramp2 mRNAs and translational inhibition of the mRNAs encoding ferritin (H- and L-chains), erythroid δ-aminolevulinate synthase (ALAS), mitochondrial aconitase, and Ip subunit of SDH (in *Drosophila*). This leads to increased iron uptake and transport, and reduced iron storage, erythroid utilization, and ''mitochondrial activity.'' Conversely, increased iron supply inactivates binding of IRPs to IREs, resulting in degradation of TfR and DCT1/Nramp2 mRNAs and translation of the mRNAs encoding ferritin (H- and L-chains), erythroid ALAS, mitochondrial aconitase, and Ip subunit of SDH (in *Drosophila*). This leads to decreased iron uptake and transport, and elevated iron storage, erythroid utilization, and ''mitochondrial activity.'' The stabilization of DCT1/Nramp2 mRNA by IRP binding is hypothetical and has not yet been directly shown.

and sufficient to confer iron-dependent regulation at the levels of mRNA translation and stability.

What is the mechanistic basis for these posttranscriptional regulatory events? To serve as a translational regulatory element, an IRE has to be positioned close to the 5' end of the mRNA, which contains an inverted 7-methylguanosine cap. This region is critical for the initial steps in translation. During translation initiation, a typical mRNA associates close to the cap with the 43S translation preinitiation complex, comprising the small ribosomal subunit and translation initiation factors. Subsequent to its binding, the preinitiation complex scans the 5' UTR to identify the initiation codon AUG. This is followed by joining of the large ribosomal subunit and polypeptide synthesis. Studies have revealed that IRP binding to IREs (Goossen and Hentze, 1992), or even other heterologous RNA–protein complexes (Stripecke *et al.,* 1994) located in close proximity to the 5' end of an mRNA, likely impedes translation by a steric hindrance mechanism (Gray and Hentze, 1994). Thus, an IRE–IRP complex located in a cap-proximal position effectively inhibits the stable association of the small ribosomal subunit with the initiation factor eIF4F and the assembly of 43S preinitiation complex (Muckenthaler *et al.,* 1998). Nevertheless, IRE–IRP complexes in cap-distal positions retain an impaired capacity to inhibit translation, mainly by ribosomal pausing and scanning arrest (Paraskeva *et al.,* 1999), indicative of a fine-tuning of the regulatory mechanism.

The mechanism underlying the regulation of mRNA stability by IRE–IRP interactions in the 3' UTR is less well understood. The long and complex 3' UTR of TfR mRNA contains five IREs. It appears that only three of them are required for regulation. In iron deficiency, IRP binding to these IREs appears to protect the TfR mRNA from an initial endonucleolytic attack (Binder *et al.,* 1994).

The early studies on the expression of ferritin and TfR suggested that the IRE–IRP system plays a fundamental role for the regulation of iron uptake and storage in the cell. Subsequently, it has become clear that additional mRNAs are under the control of the IRE–IRP system, and it now appears that this may also fulfill more comprehensive functions. One of them may be the control of iron utilization in erythroid cells, the major iron consumers in the body. The vast majority of iron in erythroid cells is utilized for the synthesis of heme as a constituent of hemoglobin (Ponka, 1997). The first reaction in the heme biosynthetic pathway is the condensation of glycine and succinyl-CoA to yield δ-aminolevulinic acid. This reaction is catalyzed by δ-aminolevulinate synthase (ALAS). In contrast to the housekeeping ALAS, the erythroid-specific eALAS is regulated translationally by the IRE–IRP system. The mRNA encoding eALAS contains an IRE in its 5' UTR (Cox *et al.,* 1991; Dandekar *et al.,* 1991). Even though the function of the IRE in eALAS mRNA has not yet been investigated in animal models, this IRE mediates translational control in erythroid cell culture models (Melefors *et al.,* 1993) and *in vitro* (Bhasker *et al.,* 1993). From the homeostatic point of view, the repression of eALAS translation in iron-deficient erythroid cells would serve to prevent accumulation of excess protoporphyrin IX.

An additional, IRE-like hairpin structure has been noted in the 3' UTR of DCT1/Nramp2 mRNA (Gunshin *et al.,* 1997). This putative IRE has not been functionally characterized yet, but it may well serve as a ''stability-type'' IRE, as DCT1/Nramp2 mRNA levels in the duodenum are significantly increased in iron-deficient animals. It is tempting to speculate that the expression of the metal cation transporter DCT1/Nramp2, which is possibly involved in intestinal iron absorption and intracellular iron release from the endosome, may be controlled by IRE–IRP interactions.

Finally, the identification of functional IREs in mRNAs encoding polypeptides whose function appears to be less directly involved in iron metabolism suggests that the IRE–IRP system may operate as a more general posttranscriptional regulatory circuit. The mRNAs of two mitochondrial enzymes of the Krebs cycle, mammalian aconitase and insect succinate dehydrogenase (iron–sulfur subunit), both contain functional IREs in their 5' UTRs (Kohler *et al.,* 1995; Gray *et al.,* 1996; Kim *et al.,* 1996). Both aconitase and succinate dehydrogenase are iron-containing proteins involved in the regulation of cellular energy metabolism. The translational arrest of these mRNAs in the iron-deficient cell may help to prevent accumulation of the nonfunctional apoproteins.

A variety of biological activities appear to be controlled by IRE–IRP interactions. One would expect that abrogation of this genetic control may lead to disease. Indeed, a genetic defect in the IRE–IRP regulatory system has been identified in the form of the hereditary hyperferritinemia-cataract syndrome (HHCS). Patients suffering from HHCS carry mutations in L-ferritin IRE (several different mutations have been described to date), which render it functionally inactive and do not allow IRP binding (Beaumont *et al.,* 1995). The failure to control L-ferritin mRNA translation by IRPs results in the overexpression of L-ferritin and a marked increase in serum L-ferritin levels (hyperferritinemia). Even though this has no profound effects on general clinical parameters of iron metabolism, the accumulation of L-rich apoferritin in the eye lens leads to cataract formation by a currently unknown mechanism.

Iron Regulatory Proteins

The iron regulatory proteins IRP1 and IRP2 serve as the iron sensors in the cell (Henderson, 1996; Hentze and Kühn, 1996; Rouault *et al.,* 1996; Richardson and Ponka, 1997; Pantopoulos and Hentze, 1999). Both are cytoplasmic polypeptides of 98 and 105 kDa, respectively, and they belong to the family of iron–sulfur cluster isomerases (Frishman and Hentze, 1996). Their primary function is to regulate IRE-containing mRNAs. IRP1 and IRP2 were initially considered to bind to IREs with similar affinities. However, their binding affinity to some artificially engineered (Butt *et al.,* 1996; Henderson *et al.,* 1996) or natural IREs (Ke *et al.,* 1998) was found to vary substantially. IRP1, the first iron regulatory

protein to be characterized (Leibold and Munro, 1988; Rouault *et al.,* 1990), accounts for the major fraction of IRE binding activity in many tissues and cultured cells. With the exception of some cell lines, IRP1 appears to be ubiquitously expressed, whereas the levels of IRP2 usually vary between different tissues.

The best-characterized member of iron–sulfur isomerases is mitochondrial aconitase. This enzyme of the Krebs (citric acid) cycle catalyzes isomerization of citrate to isocitrate via *cis*-aconitate. The predicted amino acid sequence of human IRP1 shares ~31% identity and ~56% similarity (including conservative substitutions) with that of porcine mitochondrial aconitase (Hentze and Argos, 1991; Rouault *et al.,* 1991; Frishman and Hentze, 1996). This homology spans the entire molecule and is particularly striking (100% identity) around the catalytic core of mitochondrial aconitase, which contains a cubane 4Fe–4S cluster (Fig. 6). In this unusual iron–sulfur cluster, only three iron atoms are coordinated with Cys amino acid residues in the protein, while the fourth, Fe_a, remains coordinated to a hydroxide from solvent in a ''free,'' labile form. Crystallographic and biochemical studies of mitochondrial aconitase have established a direct involvement of Fe_a in the catalytic mechanism (Lauble *et al.,* 1992; Robbins and Stout, 1989a,b).

The structural similarity between IRP1 and mitochondrial aconitase has raised the possibility that IRP1 may also display aconitase activity. A series of experiments have confirmed that IRP1 is capable of assembling an aconitase-type 4Fe–4S cluster, which renders it catalytically active (Haile *et al.,* 1992a,b; Kennedy *et al.,* 1992). Indeed, IRP1 has been reidentified as the cytoplasmic homolog of mitochondrial aconitase, which was documented to exist many years ago (Guarriero-Bobyleva *et al.,* 1973). The catalytic efficiency of cytosolic aconitase is similar to that of mitochondrial (Kennedy *et al.,* 1992). However, the physiological function of an aconitase activity in the cytoplasm still remains puzzling.

The 4Fe–4S clusters in mitochondrial aconitase and IRP1 are very similar but not identical (Kennedy *et al.,* 1992). For example, in IRP1, the 4Fe–4S cluster is more stable and resistant to oxidation. In contrast, in mitochondrial aconitase, Fe_a is very labile and can be readily lost under aerobic conditions to yield 3Fe–4S. The fundamental difference between the two aconitase isoforms lies in the fact that, unlike mitochondrial aconitase, IRP1 does not continuously carry its 4Fe–4S cluster. Instead, the presence of this cluster in IRP1 strictly depends on cellular iron availability (Haile *et al.,* 1992a,b). Iron-replete cells contain 4Fe–4S IRP1, which correlates with detectable cytoplasmic aconitase and minimal IRE-binding activity. On the other hand, apoIRP1 predominates in iron-starved cells and is associated with negligible cytoplasmic aconitase and maximal IRE binding activity. In

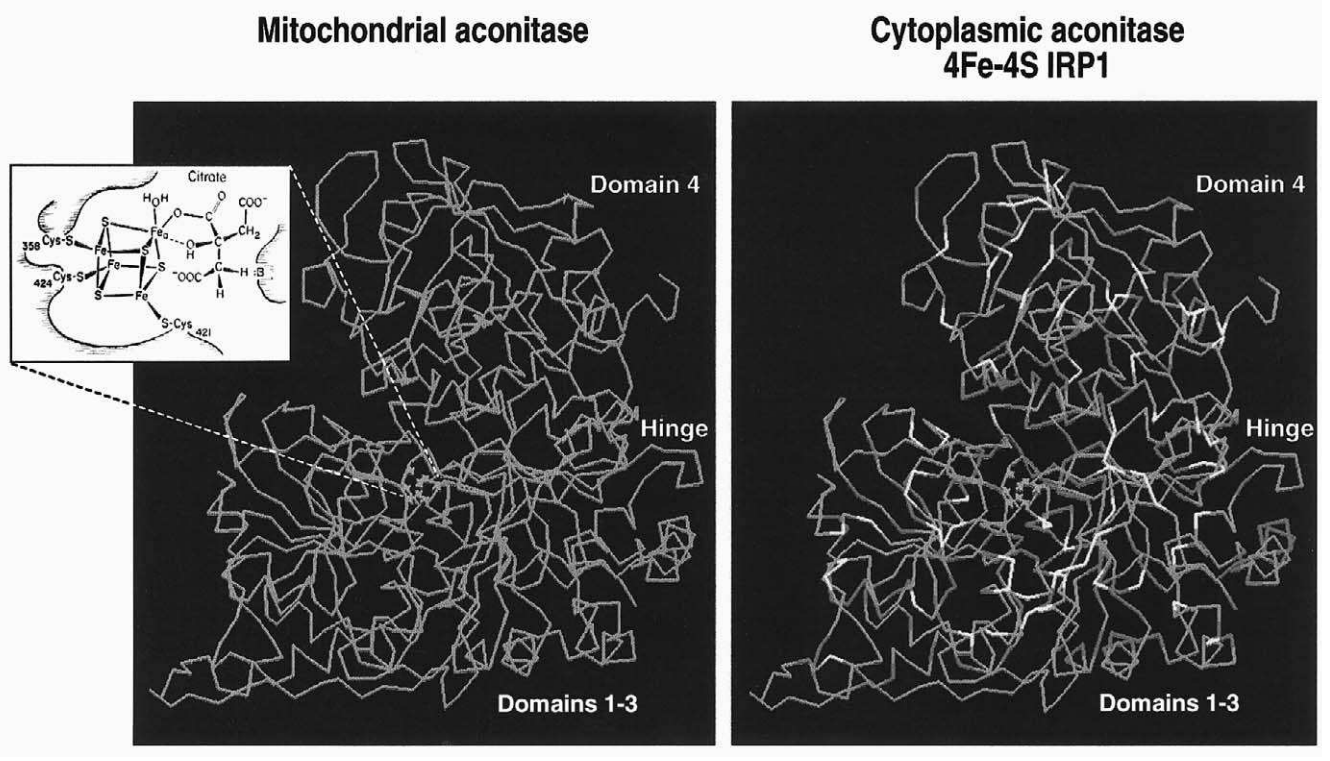


Figure 6 Mitochondrial aconitase as a structural model for IRP1. The structure of mitochondrial aconitase and its cubane 4Fe–4S cluster are shown at left. Three compact domains (1–3) are linked to a fourth (4) via a flexible hinge region. A cubane 4Fe–4S cluster in the cleft that forms between domains 1–3 and 4 interacts with substrate (citrate). IRP1 is illustrated in an analogous structure (right) based on its extensive homology with mitochondrial aconitase. Amino acids of IRP1 identical or similar to those in mitochondrial aconitase are depicted in yellow and red, respectively. The positions of the 4Fe–4S clusters in mitochondrial aconitase and IRP1 are shown in blue. See color insert.

agreement with these observations, direct experiments employing purified or recombinant protein have demonstrated that 4Fe–4S IRP1 is the cytoplasmic aconitase, and apoIRP1 is the IRE-binding protein (Constable *et al.,* 1992; Emery-Goodman *et al.,* 1993; Gray *et al.,* 1993; Basilion *et al.,* 1994a).

Thus, unlike mitochondrial aconitase, IRP1 is a bifunctional protein with two mutually exclusive activities: one gene regulatory and another enzymatic. These activities are controlled by an unusual posttranslational iron–sulfur cluster switch (Paraskeva and Hentze, 1996), and the relatively long half-life (~24 hours) of IRP1 polypeptide is not affected by iron perturbations (Tang *et al.,* 1992; Pantopoulos *et al.,* 1995). The posttranslational nature of this switch is emphasized by the fact that the induction of IRE binding in iron-deficient cells does not require ongoing protein synthesis. Moreover, in extracts of iron-loaded cells, the spontaneous IRE binding activity of 4Fe–4S IRP1 is very low, but it can be fully recovered after treatment with high concentrations of reducing agents such as 2-mercaptoethanol (Hentze *et al.,* 1989), which displaces the cluster from the polypeptide backbone.

What is the structural basis for the iron–sulfur cluster switch? The crystal structure of IRP1 has not yet been solved. However, the homology between IRP1 and mitochondrial aconitase allows one to deduce information from the well-studied example of mitochondrial aconitase (Fig. 6). The latter enzyme is composed of three compact domains, linked to a fourth by a flexible hinge region. A cleft formed between domains 1–3 and domain 4 provides access to the substrate. A similar structure, possibly facilitated by the presence of the 4Fe–4S cluster, could also account for the aconitase activity of IRP1.

How does apoIRP1 then bind to IREs? Analysis of the predicted primary amino acid sequence of IRP1 did not reveal any already characterized RNA binding motifs. However, UV cross-linking (Basilion *et al.,* 1994b; Swenson and Walden, 1994; Neupert *et al.,* 1995) and protein footprinting (Gegout *et al.,* 1999) studies have identified amino acids of IRP1 that directly interact with the IRE and that are predicted to be positioned near the cleft. The ''closed'' structure of 4Fe–4S IRP1 would not permit access of the RNA to these IRE binding sites. To accomplish IRE binding, one has to assume that the overall structure of IRP1 may acquire a more ''open'' conformation, providing sufficient space in the cleft (Fig. 7). Such a structural rearrangement may be allowed by the disassembly of the 4Fe–4S cluster.

The second iron regulatory protein, IRP2, shares substantial homology with both IRP1 and mitochondrial aconitase (Frishman and Hentze, 1996). The predicted amino acid sequence of human IRP2 is 57% identical and 75% similar to that of human IRP1 (Samaniego *et al.,* 1994). However, unlike IRP1, IRP2 does not possess aconitase enzymatic activity (Guo *et al.,* 1994). Furthermore, the regulation of its IRE binding activity does not involve an aconitase-type iron–sulfur cluster and follows completely different mechanisms, in spite of the fact that IRP2 is also iron regulated: in iron-replete cells, the IRP2 polypeptide is extremely unstable, whereas iron deficiency triggers IRP2 stabilization and, in addition, *de novo* IRP2 synthesis (Guo *et al.,* 1994, 1995; Samaniego *et al.,* 1994; Henderson and Kühn, 1995; Pantopoulos *et al.,* 1995).

A particular feature of IRP2 is the insertion of 73 amino acids in the N terminus, which is necessary and sufficient to confer its iron-dependent regulation (Iwai *et al.,* 1995). This sequence contains a cysteine-rich motif that presumably directly binds iron and functions as an iron-dependent instability element. It has been suggested that binding of iron may direct site-specific oxidation of critical amino acids, which triggers ubiquitination and proteolytic degradation of IRP2 by the proteasome pathway (Iwai *et al.,* 1998) (Fig. 8).

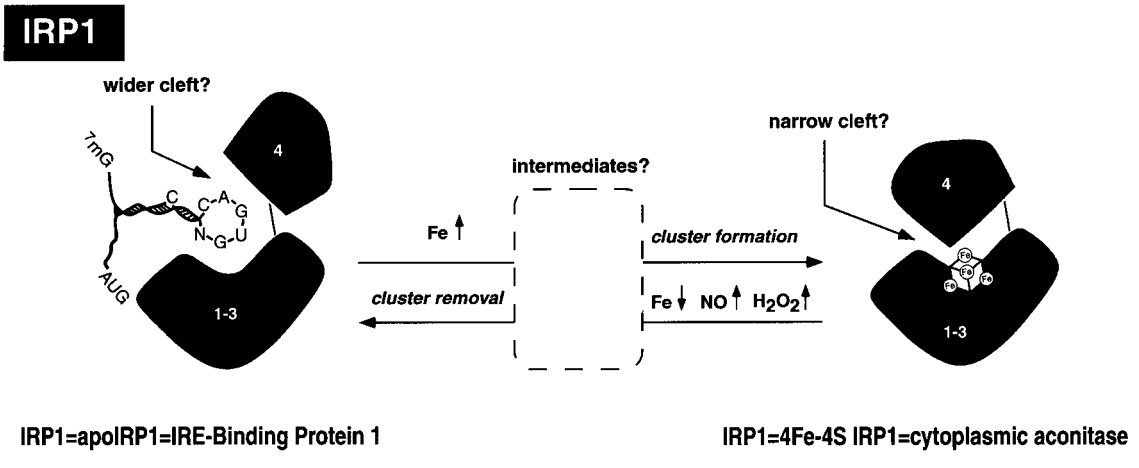


Figure 7 Posttranslational regulation of bifunctional IRP1 in response to iron, NO, and H_2O_2 is mediated by an iron–sulfur cluster switch. In iron-replete cells IRP1 assembles a cubane 4Fe–4S cluster. Iron starvation, NO, and H_2O_2 trigger the switch of 4Fe–4S to apoIRP1, resulting in the conversion of cytosolic aconitase to IRE binding protein.

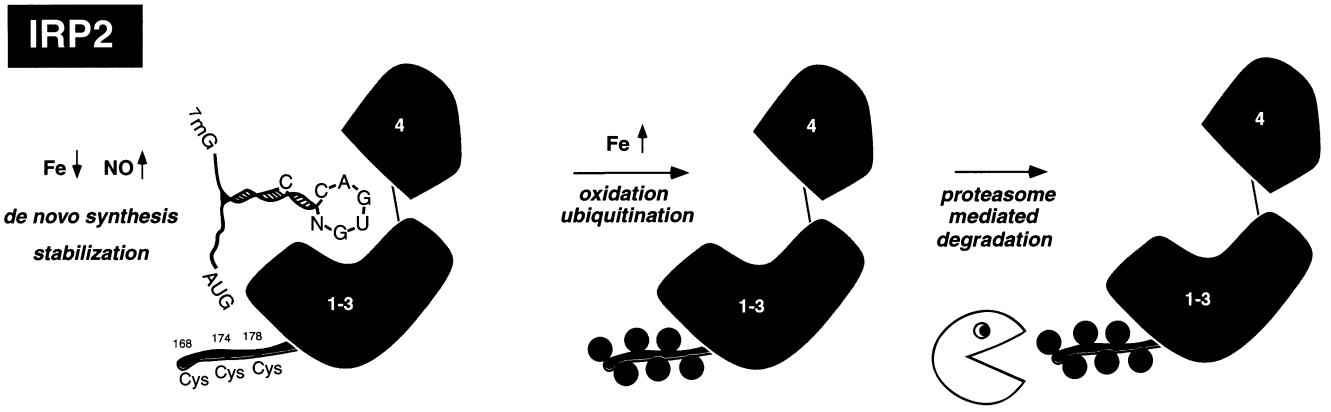


Figure 8 Regulation of IRP2 at the level of protein stability. Iron starvation and NO result in *de novo* synthesis and stabilization of IRP2. Increase in iron levels triggers oxidation of critical cysteine residues that tags IRP2 for degradation by the proteasome, following ubiquitination.

Nitric Oxide Modulates Iron Regulatory Proteins

The capacity of NO to interact with iron, among various other targets, chemically links NO biology with iron metabolism. The resulting iron–nitrosyl complexes are paramagnetic and can be studied by electron paramagnetic resonance (EPR) spectroscopy (Drapier *et al.,* 1991; Kubrina *et al.,* 1992; Henry *et al.,* 1993; Bastian *et al.,* 1994; Lee *et al.,* 1994). There is no restriction on specific forms of iron to constitute a target of NO. Thus, NO readily reacts with low-molecular-weight iron chelates, and it reacts with heme iron or iron–sulfur clusters in metalloproteins. The well-documented effect of NO to promote smooth muscle relaxation is based on the activation of a guanylyl cyclase by binding of NO to the heme iron of its catalytic site (McDonald and Murad, 1996). Presumably, NO binding elicits an allosteric change in guanylyl cyclase, which leads to increased cGMP synthesis and the concomitant activation of signaling pathways. On the other hand, NO may also negatively modulate the activity of iron-containing proteins. Stimulated macrophages release NO, generated by the inducible NOS2, to inactivate vital iron-containing proteins in target cells. The iron–sulfur clusters in complexes of the respiratory chain, NADH:ubiquinone oxidoreductase and NADH:succinate oxidoreductase, are presumed to be sites of targeted inactivation by NO (Drapier and Hibbs, 1986; Hibbs *et al.,* 1988). As an iron–sulfur protein, mitochondrial aconitase is also sensitive to inactivation by NO (Drapier and Hibbs, 1986; Gardner *et al.,* 1997; Hibbs *et al.,* 1988; Castro *et al.,* 1998).

The 4Fe–4S cluster of IRP1 is more stable than that of mitochondrial aconitase (Kennedy *et al.,* 1992). However, the overall sensitivity of iron–sulfur proteins to NO, and in addition the ability of NO (in form of NO^+) to engage in nitrosylation reactions and control the function of various proteins, raised the intriguing possibility that NO might also regulate IRP1 and therefore control cellular iron metabolism. Early experiments employing immunologically stimulated murine primary peritoneal macrophages or macrophage-like cell lines demonstrated that NO biosynthesis correlates with activation of IRP1 (to bind to IREs) and with repression of ferritin mRNA translation (Drapier *et al.,* 1993; Weiss *et al.,* 1993). At the time the initial results were obtained, IRP2 had been very poorly characterized. Nevertheless, it is noteworthy that in some of these experiments a clear activation of IRP2 can be observed (murine IRP2 migrates faster than IRP1 in electrophoretic mobility shift assays) (Weiss *et al.,* 1993). The described effects are antagonized by N^G-methyl-L-arginine, a stereospecific inhibitor of NO synthase, suggesting the involvement of NO in modulation of the IRE–IRP regulatory system.

A series of additional experimental approaches have confirmed that NO exerts a signaling activity on the IRE–IRP regulatory system. First, nonerythroid or erythroid cell lines were exposed to NO, generated intracellularly from a stably transfected NOS2 transgene (Pantopoulos and Hentze, 1995a; Rafferty *et al.,* 1996; Domachowske *et al.,* 1996). Alternatively, NO was administered to target cells from the outside, either by pharmacological donors (Oria *et al.,* 1995; Richardson *et al.,* 1995; Pantopoulos *et al.,* 1996) or by coculture with the NOS2 transfectants (Pantopoulos *et al.,* 1996). Heterologous expression of NOS2 results in a NO-dependent activation of IRP1 and IRP2 (Pantopoulos and Hentze, 1995a). As expected, this is associated with repressed ferritin biosynthesis and increased TfR mRNA levels. In addition, the erythroid cells expressing NOS2 display a profound inhibition of hemoglobin expression, most likely due to inhibition of eALAS mRNA translation (Domachowske *et al.,* 1996). Similar results have been obtained with cells treated with NO-liberating drugs (Richardson *et al.,* 1995; Pantopoulos *et al.,* 1996) and with cells cocultured with NOS2 transfectants (Pantopoulos *et al.,* 1996). Thus, NO has been demonstrated to activate IRP1 and IRP2 by intra- and intercellular means and to affect the expression of IRE-containing mRNAs. Taken together, NO modulates iron metabolism by both intracellular and intercellular signaling to the IRE–IRP regulatory system (Figs. 7 and 8).

The growing interest in the regulatory connection between the NO and IRE–IRP pathways has prompted studies

to investigate the mechanism of NO action. Exposure of highly purified bovine mitochondrial and cytosolic aconitase (4Fe–4S IRP1) preparations to authentic NO gas or to spermine NONOate results in the rapid inactivation of both enzymes, even in the presence of aconitase substrate (Kennedy *et al.,* 1997). A spectroscopic analysis by EPR showed that the 4Fe–4S clusters of aconitases are targets of NO attack (Kennedy *et al.,* 1997). The interaction with NO *in vitro* yields two major types of signals, at $g = 2.02$, corresponding to a 3Fe–4S cluster, and at $g \approx 2.04$, characteristic of dinitrosyl–iron–dithiol–protein complexes. Under these conditions, there is no strict order of appearance for the above EPR signals. Moreover, the appearance of the $g = 2.02$ signal of a 3Fe–4S cluster is not proportional to the loss of enzymatic activity. This strongly suggests that the inactivation of aconitase by NO is not exclusively based on the conversion of the 4Fe–4S cluster to 3Fe–4S by loss of Fe_a, but rather is a result of a more general cluster disassembly beyond the 3Fe–4S stage. Furthermore, this disassembly does not seem to require the formation of a 3Fe–4S intermediate. In contrast to NO, other more potent oxidants, such as peroxynitrite and superoxide, are able to remove Fe_a and yield 3Fe–4S inactive aconitases. In this case, aconitase substrates afford protection to the 4Fe–4S cluster against oxidation. As expected from previous results, the oxidative removal of the comparatively more stable Fe_a in IRP1 requires higher doses of peroxynitrite or superoxide.

In agreement with the data obtained with purified bovine aconitases, the treatment of iron-loaded human recombinant IRP1 with authentic NO gas is sufficient to completely abolish the enzymatic aconitase activity (Drapier *et al.,* 1993). However, this treatment leads to only a partial (20%) activation of IRE binding. NO-mediated partial induction of IRE binding has also been obtained with crude cytoplasmic extracts exposed to NO-releasing drugs (Jaffrey *et al.,* 1994). These *in vitro* data are fully consistent with the spectroscopic analysis and with the idea that NO itself is capable of attacking and dissociating the 4Fe–4S cluster in IRP1. However, it is still unclear whether the activity of NO per se is sufficient to completely disassemble and remove the 4Fe–4S cluster, to yield apoIRP1. The situation is more clear for peroxynitrite or superoxide: these oxidants inactivate aconitase *in vitro* (Bouton *et al.,* 1996, 1997) by converting 4Fe–4S to 3Fe–4S IRP1 (Kennedy *et al.,* 1997), an intermediate which also fails to perform as an IRE binding protein.

These *in vitro* approaches have provided some valuable insights concerning the chemical interaction of NO with IRP1, but they have not resolved the mechanism of the NO-mediated activation of IRE binding. Moreover, these studies are only partly informative with regard to the effects of NO on the regulation of iron metabolism in the living cell. Cell culture experiments with stimulated macrophages or stable NOS2 transfectants have established that NO exerts profound effects on cellular iron metabolism (Drapier *et al.,* 1993; Weiss *et al.,* 1993; Pantopoulos and Hentze, 1995a; Rafferty *et al.,* 1996; Domachowske *et al.,* 1996). However, secondary effects caused by the stimulation of macrophages or by the constitutive biosynthesis of NO caution against far-reaching mechanistic interpretations. Thus, employing a well-defined and controlled system to generate NO might be more appropriate to reconstitute the series of molecular events taking place in the IRE–IRP regulatory system in response to NO signaling.

There is a broad range of compounds that release NO in solution and have therefore been routinely used as pharmacological NO donors. The choice of a suitable NO-releasing drug is very important to address the signaling of NO to the IRE–IRP system. The minimal prerequisites for such drug would be a relative stability and linear release of NO under the experimental conditions. In addition, the metabolites arising after the liberation of NO should not affect iron metabolism. *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) is widely used as an NO donor and appears to fulfill these criteria: it is quite stable, and 100 μM SNAP yields ~1.4 μM/min NO in a near-linear fashion at 37°C (Feelisch, 1991). Moreover, NO release by SNAP in solution is rapid and can be easily followed by measuring nitrite levels. Treatment of cultured murine B6 fibroblasts with 100 μM SNAP for 4 or 8 hours results in the induction of IRP1 to bind to IREs, whereas the SNAP precursor D,L-penicillamine does not exhibit any effects (Pantopoulos *et al.,* 1996). SNAP also elicits the activation of IRP1 in other cell lines, including rat hepatoma FTO2B (Phillips *et al.,* 1996) and human erythroleukemia K562 (Richardson *et al.,* 1995). Moreover, the treatment of K562 cells with SNAP results in increased TfR mRNA levels and enhanced iron uptake from transferrin (Richardson *et al.,* 1995). Positive results on IRP1 activation have also been obtained with the NO-donors hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (NOC-18) (Pantopoulos *et al.,* 1996) and hydroxy-2-oxo-3-(*N*-ethyl-3-aminoethyl)-3-ethyl-1-triazene (NOC-12) (Castro *et al.,* 1998).

In contrast to SNAP and NOC-18, sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1) have no effect on IRP1 when administered to the B6 fibroblasts (Pantopoulos *et al.,* 1996), and the agents also fail to stimulate iron uptake and TfR mRNA stabilization in K562 cells (Richardson *et al.,* 1995). SNP is widely used as a nitrosylating agent because it liberates the nitrosonium cation NO^+, which forms nitrosothiols with cysteine sulfhydryl groups in proteins. On the other hand, the decomposition of SIN-1 yields NO and superoxide, which readily react to form peroxynitrite. These data appear to support the view that, in living cells, NO is the actual activator, whereas the redox derivatives NO^+ and peroxynitrite are ineffective in signaling to IRP1. Assuming that inside the cell 4Fe–4S IRP1 is very likely bound to citrate (present in a molar excess), the above results are consistent with the *in vitro* spectroscopic data showing that aconitase substrates protect the 4Fe–4S cluster from oxidation by peroxynitrite (or superoxide) but fail to prevent NO-mediated cluster disassembly (Kennedy *et al.,* 1997).

Another murine fibroblast cell line, Ltk^-, has been employed as a model system for studying signaling of NO to

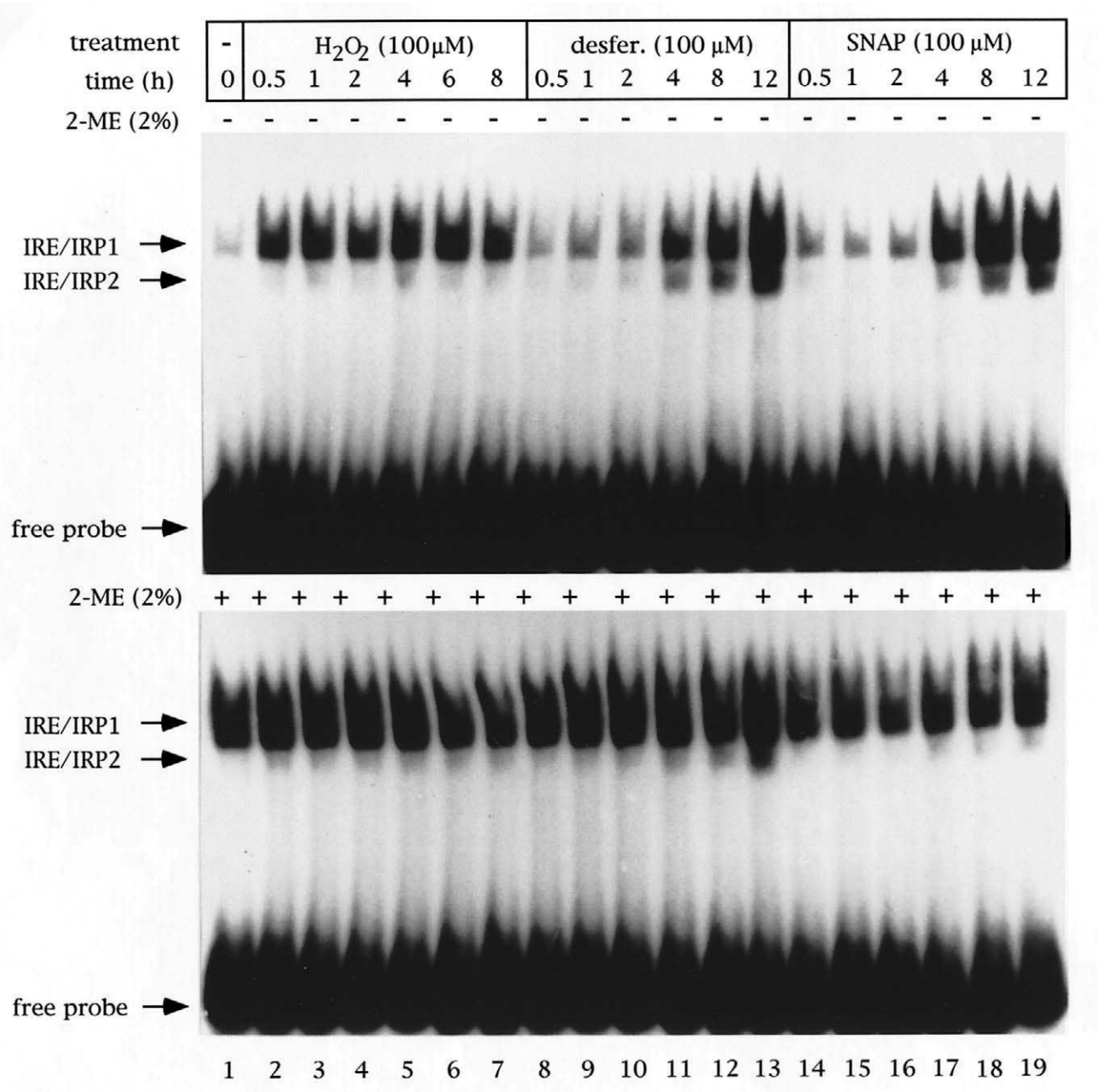


Figure 9 Kinetic analysis reveals differential responses of iron regulatory proteins to H_2O_2, iron starvation, and NO. Ltk⁻ murine fibroblasts were left untreated (lane 1) or were treated with 100 μ*M* H_2O_2 for 0.5, 1, 2, 4, 6, or 8 hours (lanes 2–7, respectively), with 100 μ*M* desferrioxamine (iron chelator) for 0.5, 1, 2, 4, 8, or 12 hours (lanes 8–13), or with 100 μ*M* SNAP (NO-releasing drug) for 0.5, 1, 2, 4, 8, or 12 hours (lanes 14–19). H_2O_2 and SNAP were replaced with fresh solutions after 4 and 6 hours, respectively. Cytoplasmic extracts (25 μg) were analyzed by electrophoretic mobility shift assay with 25,000 cpm ^{32}P-labeled IRE probe in the absence (top) or presence (bottom) of 2% 2-mercaptoethanol (2-ME). The positions of IRE/IRP1 and IRE/IRP2 complexes and of excess free IRE probe are indicated by arrows. Reproduced, with permission, from Pantopoulos, K., Weiss, G., and Hentze, M. W. (1996). Nitric oxide and oxidative stress (H_2O_2) control mammalian iron metabolism by different pathways. *Mol. Cell. Biol.* **16,** 3781–3788.

the IRE–IRP system. These cells express significant levels of IRP2, in addition to IRP1. A comparative analysis (Pantopoulos *et al.,* 1996) showed that both IRP1 and IRP2 in Ltk⁻ cells are activated in response to iron starvation and NO exposure, following pharmacological treatment with the iron chelator desferrioxamine or with SNAP, respectively. Moreover, the activation by the two stimuli displays remarkably similar kinetics: desferrioxamine and SNAP trigger a partial activation of both IRP1 and IRP2 after 4 hours of treatment, whereas maximal activation is observed within 8 to 12 hours (Fig. 9). In contrast to IRP1, IRP2 activation by iron starvation or NO is blocked by inhibitors of protein synthesis. Another interesting aspect of NO signaling to IRPs is that NO should be present during the course of the

activation pathway. A partial pulse of Ltk$^-$ cells with SNAP (or desferrioxamine) for 1 hour, following withdrawal of the drugs and incubation for up to 8 hours, does not suffice to activate IRPs.

In light of the rapid dissociation of the 4Fe–4S cluster of IRP1 by NO *in vitro* (Kennedy *et al.,* 1997), the slow activation of IRE binding activity by NO *in vivo* (Pantopoulos *et al.,* 1996; Castro *et al.,* 1998) is surprising. Considering that NO release by SNAP is immediate (Feelisch, 1991), one would predict that the activation of IRP1 would be fast, if this were largely based on a direct attack by NO on the cluster. On the other hand, these data are consistent with the incomplete activation of IRE binding by NO *in vitro,* which has also been observed after a treatment of rat cerebellar extracts with SNAP (Jaffrey *et al.,* 1994). Thus, it is tempting to speculate that, in the context of the cell, NO may be sufficient to attack the 4Fe–4S IRP1 and inactivate aconitase, possibly yielding 3Fe–4S IRP1 and dinitrosyl–iron–dithiol–IRP1 complexes (Fig. 10a). However, it is questionable whether NO per se can activate IRE binding.

In view of the above information, what is the mechanistic basis for the activation of IRE binding by NO? An attractive hypothesis would postulate that 3Fe–4S IRP1 and putative dinitrosyl–iron–dithiol–IRP1 complexes may either spontaneously lose the remaining iron–sulfur cluster, or the complexes constitute substrates for yet uncharacterized enzymatic activities that assist complete removal (Fig. 10b). An alternative assumption is based on the identical slow kinetics in the activation of IRP1 in response to NO and iron starvation (Fig. 10c). According to this, the ''delayed'' Fe–S cluster switch of IRP1 may be a result of limited iron (or sulfite) availability. It is conceivable that in the intracellular milieu, both 4Fe–4S and apoIRP1 coexist in a dynamic equilibrium, and the cluster is prone to ongoing assembly–disassembly. Conditions where intracellular iron (and sulfite) are abundant would thermodynamically favor (spontaneous?) assembly of the cluster. However, reduction of intracellular iron (or sulfite) levels below a critical threshold would lead to a slow shift of the equilibrium toward apoIRP1. This process may not necessarily require the activity of auxiliary factors. To explain the effects of NO, one has to assume that NO indirectly affects the levels of the ''regulatory iron pool'' by sequestering intracellular iron. The ability of NO to react with iron in any form and yield iron–nitrosyl complexes and the necessity of the NO signal to be sustained during the IRP activation pathway support this view. Thus, *in vivo,* NO possibly retains its potential to directly attack and modify the 4Fe–4S cluster of IRP1. However, this modification per se would be insufficient to completely remove the cluster and yield apoIRP1. The actual determinant for the complete removal of the cluster would then be the reduction in the levels of the regulatory iron pool. This would first prevent the reassembly of the partially dissociated cluster and, second, promote a slow, complete disassembly.

Another interesting aspect of this model is the rationalization of the differential ''downstream'' regulation of IRP1 and IRP2 by NO. Assuming that NO elicits iron starvation,

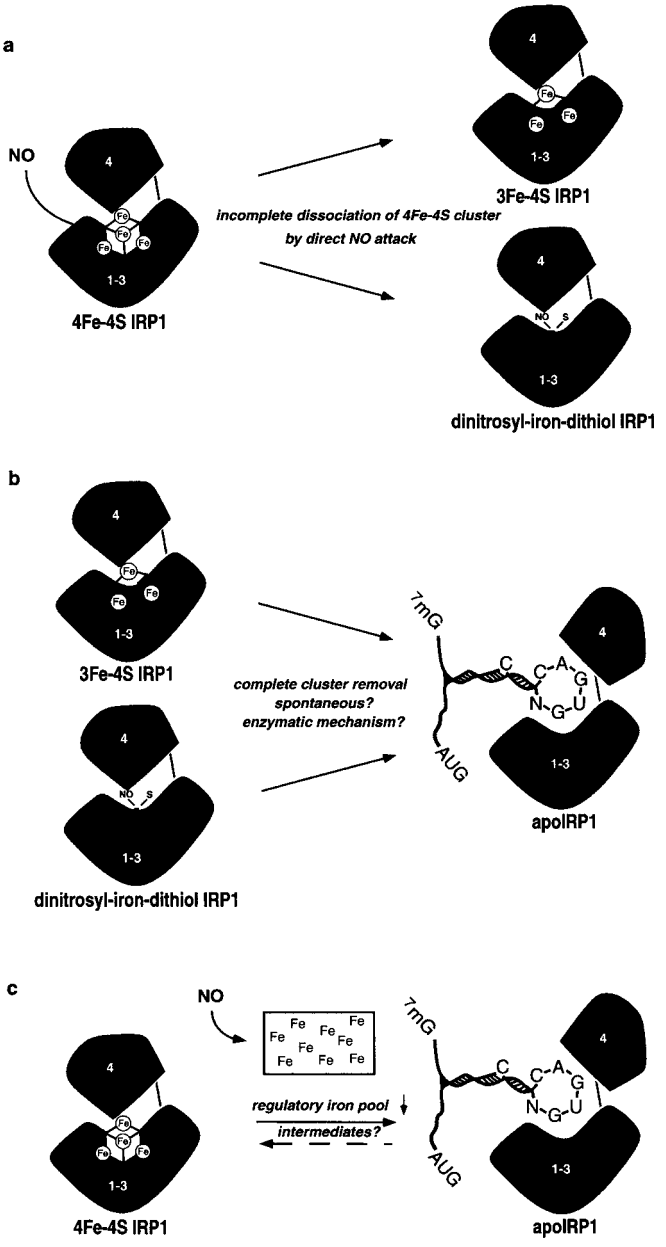


Figure 10 Models for the effects of NO on IRP1. (a) NO directly attacks the 4Fe–4S cluster of IRP1 to yield 3Fe–4S or dinitrosyl–iron–dithiol intermediates, which are inactive as enzymes or IRE-binding proteins. (b) 3Fe–4S and dinitrosyl–iron–dithiol IRP1 intermediates are liable to complete removal of the iron–sulfur cluster (spontaneous or enzymatic), resulting in formation of apoIRP1 and activation of IRE binding. (c) NO modulates the ''regulatory iron pool'' and elicits responses to iron starvation, resulting in the switch of 4Fe–4S to apoIRP1 and activation of IRE binding. These models are not mutually exclusive.

cells respond to this by activating IRP1 via the iron–sulfur cluster switch and IRP2, which does not possess an IRP1-type iron–sulfur cluster, by stabilization and *de novo* synthesis. In some instances, conflicting results concerning the effect of NO on IRP2 have been described. Although in most cases IRP2 was found to be positively regulated by NO (see also Fig. 9), some investigators found no effects of NO on IRP2 (Phillips *et al.,* 1996), and some others reported nega-

tive regulation (Recalcati *et al.,* 1998). The negative results have been obtained with cytokine-treated cell lines. These discrepancies could be explained on the basis of variabilities between different cell lines, different responses to various redox species of NO, and the pleiotropic effects of cytokines, but in some reports the lack of appropriate positive controls for IRP2 activation is also evident.

Regulation of Iron Metabolism by Oxidative Stress

The regulation of iron metabolism by NO has established that iron is not the only signal for the IRE–IRP system, which raises the possibility that additional signals may exist. On the basis of similarities with NO (discussed at the beginning of this chapter), potential other regulators can be found among the family of the reactive oxygen intermediates (ROIs). The ability of ROIs to inactivate bacterial and mitochondrial aconitases by targeting their iron–sulfur clusters (Gardner and Fridovich, 1991; Gardner *et al.,* 1995) is another feature which made them attractive as potential regulators of IRP1. Thus, in the context of oxygen-dependent iron toxicity, it was very interesting to investigate whether oxidative stress has any effects in the regulation of cellular iron homeostasis.

A first direct regulatory link between iron metabolism and oxidative stress was established with the finding that treatment of cultured cells with micromolar concentrations of H_2O_2 leads to the activation of IRP1 (Martins *et al.,* 1995; Pantopoulos and Hentze, 1995b). The induction of IRE binding is associated with a concomitant loss of aconitase activity (Fig. 7). A series of experiments have shown that H_2O_2 constitutes a distinct signal to the IRE–IRP regulatory system (Pantopoulos *et al.,* 1996). In contrast to NO and to iron chelation, H_2O_2 elicits a rapid induction of IRP1 within 30 to 60 min (Fig. 9), suggesting a different mechanistic basis for the iron–sulfur cluster switch. In addition, whereas NO and iron chelation activate both IRP1 and IRP2, the H_2O_2 signal is specific for IRP1. This stress-induction of IRP1 is biphasic, and the presence of H_2O_2 does not need to be sustained during the course of the treatment: a short pulse of cells with a bolus of 100 μM H_2O_2 for 15 min, followed by a wash and removal of the inducer, is sufficient to elicit a complete, long-lasting (over 4 hours) activation of IRP1 (Pantopoulos *et al.,* 1996). In agreement with this, a very sensitive chemiluminescence technique for H_2O_2 determination has shown that extracellular H_2O_2 decays rapidly in the medium of cultured cells, without compromising the effects on IRP1 (Pantopoulos *et al.,* 1997).

Can the switch of cytosolic aconitase to IRE-binding protein in response to oxidative stress be attributed to a direct removal of the 4Fe–4S cluster by H_2O_2 or other ROIs? As mentioned in the previous section of this chapter, reactive oxygen species (including superoxide, H_2O_2, and peroxynitrite) can oxidize 4Fe–4S to 3Fe–4S IRP1 *in vitro.* This leads to inactivation of aconitase but is not sufficient to induce IRE binding. Thus, in contrast to the effects observed in intact cells, treatment of crude cytoplasmic extracts or iron-loaded recombinant IRP1 with H_2O_2 does not activate the IRE-binding activity of IRP1 (Martins *et al.,* 1995; Pantopoulos and Hentze, 1995b; Brazzolotto *et al.,* 1999). Moreover, *in vitro* treatments with excess of ROIs may lead to complete inactivation of IRE binding, possibly as a result of IRP1 oxidation (Cairo *et al.,* 1996; Pantopoulos and Hentze, 1998).

The development of redox-sensitive probes to monitor intracellular levels of ROIs by fluorescence-activated cell sorting has helped to address these questions in cell culture models. Employment of such techniques has shown that a mere increase in intracellular levels of ROIs is not sufficient to activate IRP1. For example, pharmacological inhibition of catalase, a major H_2O_2-degrading enzyme, results in intracellular accumulation of H_2O_2, but this does not affect IRE binding activity (Pantopoulos *et al.,* 1997). An activation of IRP1 in response to intracellular oxidative stress has been observed after a treatment of cells with antimycin A, an inhibitor of complex III in the respiratory chain. Even though this treatment results in a rapid, detectable leakage of ROIs from mitochondria, IRP1 activation significantly lags behind the generation of ROIs (at least 90 min) and is not antagonized by antioxidants (Pantopoulos *et al.,* 1997). In agreement with the *in vitro* data, high levels of intracellular oxygen radicals may even completely inactivate IRP1; this is evident after treatment of cells with menadione, a redox cycling quinone. Whereas low doses of menadione partially activate IRP1 and trigger an incomplete Fe–S cluster switch, high doses cause a severe intracellular oxidative stress, which ultimately leads to the irreversible damage of both the cytoplasmic aconitase as well as the IRE binding activity of IRP1 (and IRP2) (Gehring *et al.,* 1999). The complexity of the IRP responses to intracellular oxidative stress is emphasized by the partial activation of IRP1 after a treatment of cells with paraquat, another redox cycling drug (Pantopoulos and Hentze, 1995b; Gehring *et al.,* 1999).

These data suggest that the rapid activation of IRP1 in response to extracellular H_2O_2 is not a result of a direct attack of the Fe–S cluster by ROIs. In agreement with this notion, when a bolus of H_2O_2 is administered to cells from the outside, the activation of IRP1 is not associated with a concomitant increase in intracellular levels of ROIs (Pantopoulos *et al.,* 1997). Thus, extracellular H_2O_2 most likely triggers a signaling pathway that results in the activation of IRP1. In contrast to the delayed effects of iron starvation and NO, this stress response leads to the accelerated turnover of the 4Fe–4S cluster in IRP1. Although the slow switch of 4Fe–4S to apoIRP1 by iron starvation and NO could be spontaneous, it is likely that the rapid removal of the cluster in response to H_2O_2 signaling could be assisted by other cluster-destabilizing factors.

A recently established *in vitro* system has provided more direct evidence that an oxidative stress-response program activates IRP1 (Pantopoulos and Hentze, 1998). B6 fibroblasts, permeabilized with the bacterial toxin streptolysin-O (SLO), retain their ability to activate IRP1 by H_2O_2. This activation follows the same biphasic kinetics as in intact

cells, and it requires the presence of noncytoplasmic, possibly membrane-associated components. Moreover, IRP1 activation can be blocked by addition of ATPγS and GTPγS or by treatment with calf intestinal alkaline phosphatase. Our current model for the H_2O_2-induced activation of IRP1 postulates that a (possibly plasma) membrane-associated factor senses extracellular H_2O_2 and relays the signal to the cytoplasm by a signaling pathway that results in the switch of 4Fe–4S to apoIRP1 (Fig. 11).

Conclusions

The regulation of iron metabolism by NO and ROIs via the IRPs may have significant pathophysiological implications, especially in the context of inflammation. To inactivate invading pathogens, immune effector cells such as macrophages and neutrophils are stimulated to release NO and reactive oxygen species (Baggiolini and Thelan, 1991; Hampton *et al.,* 1998). These responses are thought to directly target crucial iron-containing enzymes of the pathogens. The novel regulatory links described in this chapters suggest that NO and oxidative stress may also control iron metabolism in immune effector cells and, in addition, in tissues.

The release of NO and reactive oxygen species (including H_2O_2) under inflammatory conditions is predicted to activate IRPs and lead to an increase in cellular iron uptake via the TfR. This putative shift of iron from the circulation into cells may serve to deprive the invading bacteria of iron, which is essential for their growth. In macrophages, increased levels of intracellular iron may enhance the cytotoxic potential by Fenton/Haber–Weiss reactions in the controlled environment of the phagosome. The rapid shutoff of ferritin synthesis in response to the activation of IRPs may help to sustain cytotoxic conditions. The negative effects of some proinflammatory cytokines on TfR expression (Bourgeade *et al.,* 1992; Pantopoulos and Hentze, 1995a; Weiss *et al.,* 1997) could serve to balance iron uptake independently of the status of IRPs. Moreover, IRPs could also compromise these responses: accumulation of iron above a critical threshold inside the cells would switch apo to 4Fe–4S IRP1 and destabilize IRP2. On the other hand, it is very likely that, under certain circumstances, NO may have the potential to

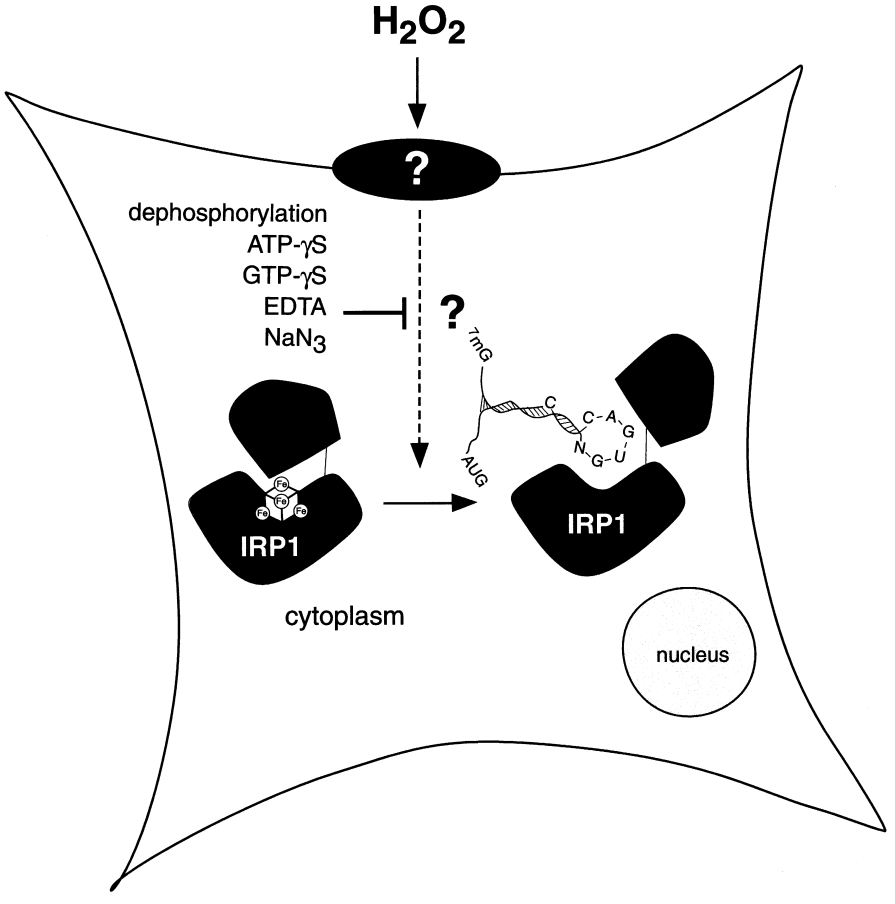


Figure 11 A model for the pathway of IRP1 activation by extracellular H_2O_2. The cell ''senses'' increased levels of extracellular H_2O_2 by a putative plasma-membrane associated factor. The relayed H_2O_2 signal leads to the activation of IRP1 by dissociation of its 4Fe–4S cluster. This signaling cascade is blocked by dephosphorylation, ATPγS, GTPγS, and sodium azide. The involved sensor, signaling factors, and mechanism of 4Fe–4S cluster removal remain to be identified.

antagonize oxygen-mediated toxicity. In tissue culture models, formation of heme or nonheme iron–nitrosyl complexes can protect cells from oxidative injury (Gorbunov *et al.,* 1997), most likely due to inactivation of the ability of iron to engage in Fenton-type reactions.

Of particular interest for the regulatory link between iron metabolism and NO biology is the control of NOS2 expression in response to iron levels. In addition to various immunological stimuli, iron starvation triggers a transcriptional activation of NOS2 biosynthesis (Weiss *et al.,* 1994). This would lead to NO-mediated activation of IRPs and enhanced cellular iron uptake. The increase in intracellular iron would not only inactivate IRPs, but also inhibit NOS2 expression in an autoregulatory loop. It has been suggested that disturbances in this control loop caused by constitutive cytokine-induced expression of NOS2 may contribute to the development of the ''anemia of chronic disease,'' which is associated with chronic inflammation (Weiss *et al.,* 1995). The hallmark of this disorder is a remarkable increase in iron stores (mirrored in serum ferritin) and reduced iron availability for erythropoiesis.

An increase in intracellular iron concentration under conditions of oxidative stress is considered to aggravate iron toxicity. To speculate on the contribution of the stress regulation of IRP1 to these phenomena, a distinction between extra- and intracellular oxidative stress should be made. IRP1 is clearly activated in response to extracellular H_2O_2. This may have important consequences in the context of inflammation. Moreover, in pathological conditions associated with oxidative stress, such as the ischemia–reperfusion injury that accompanies heart attacks and strokes, IRP1 activation may contribute to iron-mediated tissue damage. The decreased oxygen supply during hypoxia causes the inactivation of many enzymes involved in detoxification of oxygen radicals. A lag between reoxygenation and upregulation of these enzymes in the reperfusion phase results in the uncontrolled intracellular generation of oxygen radicals (Zweier *et al.,* 1988, 1994) and, possibly, extracellular release of readily diffusible H_2O_2. Experimental data suggest that a transient (10–15 min) increase in extracellular H_2O_2 levels above 10 μM would lead to a prolonged state of IRP1 activation (Pantopoulos *et al.,* 1996). Moreover, IRP1 and IRP2 were found to be activated in response to hypoxia/reperfusion in different culture models (Hanson and Leibold, 1998; Hanson *et al.,* 1999; Toth *et al.,* 1999). An IRP-mediated inhibition of ferritin biosynthesis would then reduce iron storage and detoxification and thus may promote cell damage by favoring Fenton chemistry.

The high sensitivity of IRP1 to extracellular H_2O_2 emphasizes that ROIs not only are toxic by-products, but also exert signaling functions that potentially affect additional targets. Some of them have already been identified. An additional example is the transcription factor NF-κB, which responds to a variety of environmental stimuli, including oxidative stress, and activates the transcription of multiple genes (Baeuerle and Henkel, 1994). Thus, the consequences of IRP1 activation by H_2O_2 should not be viewed in isolation but considered in a more general context. Nevertheless, the conservation of the IRP1 stress-response to extracellular H_2O_2 in different cell types and species suggests that this pathway may have important physiological implications for the regulation of iron metabolism in the body.

The regulation of iron metabolism is intimately connected with the biochemistry of NO and oxygen radicals. As in numerous other examples, dissection and elucidation of these biochemical pathways at the molecular level are prerequisite to understanding the pathophysiological implications.

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References

Aisen, P. (1994). *In* ''Iron Metabolism in Health and Disease'' (J. H. Brock, J. W. Halliday, M. J. Pippard, and L. W. Powell, eds.), pp. 1–30. Saunders, London.

Aisen, P., and Listowski, I. (1980). Iron transport and storage proteins. *Annu. Rev. Biochem.* **49,** 357–393.

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7915–7922.

Andrews, N. C., and Levy, J. E. (1998). Iron is hot: An update on the pathophysiology of hemochromatosis. *Blood* **92,** 1845–1851.

Andrews, N. C. (1999). Disorders of iron metabolism. *N. Engl. J. Med.* **341,** 1986–1995.

Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994). The FET3 gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76,** 403–410.

Askwith, C. C., de Silva, D., and Kaplan, J. (1996). Molecular biology of iron acquisition in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **20,** 27–34.

Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997). Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science* **276,** 1709–1712.

Baeuerle, P. A., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* **12,** 141–179.

Baeuerle, P. A., Rupec, R. A., and Pahl, H. L. (1996). Reactive oxygen intermediates as second messengers of a general pathogen response. *Pathol. Biol.* **44,** 29–35.

Baggiolini, M., and Thelan, M. (1991). *In* ''Oxidative Stress: Oxidants and Antioxidants'' (H. Sies, ed.), pp. 399–420. Academic Press, London.

Basilion, J. P., Kennedy, M. C., Beinert, H., Massinople, C. M., Klausner, R. D., and Rouault, T. A. (1994a). Overexpression of iron-responsive element-binding protein and its analytical characterization as the RNA-binding form, devoid of an iron-sulfur cluster. *Arch. Biochem. Biophys.* **311,** 517–522.

Basilion, J. P., Rouault, T. A., Massinople, C. M., and Klausner, R. D. (1994b). The iron-responsive element-binding protein: Localization of the RNA-binding site to the aconitase active-site cleft. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 574–578.

Bastian, N. R., Yim, C.-Y., Hibbs, J. B., and Samlowski, W. E. (1994). Induction of iron-derived EPR signals in murine cancers by nitric oxide. Evidence for multiple intracellular targets. *J. Biol. Chem.* **269,** 5127–5131.

Beal, M. F. (1998). Mitochondrial dysfunction in neurodegenerative diseases. *Biochim. Biophys. Acta* **1366,** 211–23.

Beaumont, C., Leneuve, P., Devaux, I., Scoazec, J.-Y., Berthier, M., Loiseau, M.-N., Grandchamp, B., and Bonneau, D. (1995). Mutation in the iron responsive element of the L-ferritin mRNA in a family with dominant hyperferritinaemia and cataract. *Nat. Genet.* **11,** 444–446.

Beinert, H., Holm, R. H., and Münck, E. (1997). Iron-sulfur clusters: Nature's modular, multipurpose structures. *Science* **277,** 653–659.

Beutler, E. (1998). Targeted disruption of the HFE gene. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2033–2034.

Bhasker, C. R., Burgiel, G., Neupert, B., Emery-Goodman, A., Kühn, L. C., and May, B. K. (1993). The putative iron-responsive element in the human erythroid 5-aminolevulinate synthase mRNA mediates translational control. *J. Biol. Chem.* **268,** 12699–12705.

Binder, R., Horowitz, J. A., Basilion, J. P., Koeller, D. M., Klausner, R. D., and Harford, J. B. (1994). Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. *EMBO J.* **13,** 1969–1980.

Bourgeade, M.-F., Silbermann, F., Kühn, L. C., Testa, U., Peschle, C., Memet, S., Thang, M. N., and Besancon, F. (1992). Post-transcriptional regulation of transferrin receptor mRNA by IFNγ. *Nucleic Acids Res.* **20,** 2997–3003.

Bouton, C., Raveau, M., and Drapier, J.-C. (1996). Modulation of iron regulatory protein functions. Further insights into the role of nitrogen- and oxygen-derived reactive species. *J. Biol. Chem.* **271,** 2300–2306.

Bouton, C., Hirling, H., and Drapier, J.-C. (1997). Redox modulation of iron regulatory proteins by peroxynitrite. *J. Biol. Chem.* **272,** 19969–19975.

Braun, V. (1997). Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol. Chem.* **378,** 779–786.

Brazzolotto, X., Gaillard, Z., Pantopoulos, K., Hentze, M. W., and Moulis, J. M. (1999). Human cytoplasmic aconitase (iron regulatory protein 1) is converted into its [3Fe–4S] form by hydrogen peroxide *in vitro* but is not activated for iron-responsive element binding. *J. Biol. Chem.* **274,** 21625–21630.

Butt, J., Kim, H. Y., Basilion, J. P., Cohen, S., Iwai, K., Philpott, C. C., Altschul, S., Klausner, R. D., and Rouault, T. A. (1996). Differences in the RNA binding sites of iron regulatory proteins and potential target diversity. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4345–4349.

Cadenas, E. (1989). Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* **58,** 79–110.

Cairo, G., Castrusini, E., Minotti, G., and Bernelli-Zazzera, A. (1996). Superoxide and hydrogen peroxide-dependent inhibition of iron regulatory protein activity: A protective stratagem against oxidative injury. *FASEB J.* **10,** 1326–1335.

Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D., and Harford, J. B. (1989). Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *EMBO J.* **8,** 3693–3699.

Castro, L. A., Robalinho, R. L., Cayota, A., Meneghini, R., and Radi, R. (1998). Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. *Arch. Biochem. Biophys.* **359,** 215–224.

Constable, A., Quick, S., Gray, N. K., and Hentze, M. W. (1992). Modulation of the RNA-binding activity of a regulatory protein by iron *in vitro:* Switching between enzymatic and genetic function? *Proc. Natl. Acad. Sci. U.S.A.* **89,** 4554–4558.

Cox, T. M., and Kelly, A. L. (1998). Haemochromatosis: An inherited metal and toxicity syndrome. *Curr. Opin. Gen. Dev.* **8,** 274–281.

Cox, T. C., Bawden, M. J., Martin, A., and May, B. K. (1991). Human erythroid 5-aminolevulinate synthase: Promoter analysis and identification of an iron-responsive element in the mRNA. *EMBO J.* **10,** 1891–1902.

Dancis, A. (1998). Genetic analysis of iron uptake in the yeast *Saccharomyces cerevisiae. J. Pediatr.* **132,** S-24–S-29.

Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G., and Klausner, R. D. (1992). Ferric reductase of *Saccharomyces cerevisiae:* Molecular characterization, role in iron uptake, and transcriptional control by iron. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 3869–3873.

Dandekar, T., Stripecke, R., Gray, N. K., Goossen, B., Constable, A., Johansson, H. E., and Hentze, M. W. (1991). Identification of a novel iron-responsive element in murine and human erythroid δ-aminolevulinic acid synthase mRNA. *EMBO J.* **10,** 1903–1909.

DeMaeyer, E., and Adiels-Tegman, M. (1985). The prevalence of anemia in the world. *World Health Stat. Q.* **38,** 302–316.

de Silva, D. M., Askwith, C. C., and Kaplan, J. (1996). Molecular mechanisms of iron uptake in eukaryotes. *Physiol. Rev.* **76,** 31–47.

de Sousa, M., Reimao, R., Lacerda, R., Hugo, P., Kaufmann, S. H. E., and Porto, G. (1994). Iron overload in β_2-microglobulin-deficient mice. *Immunol. Lett.* **39,** 105–111.

Dix, D. R., Bridgham, J. T., Broderius, M. A., Byersdorfer, C. A., and Eide, D. J. (1994). The *FET4* gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae. J. Biol. Chem.* **269,** 26092–26099.

Domachowske, J. B., Rafferty, S. P., Singhania, N., Mardiney III, M., and Malech, H. L. (1996). Nitric oxide alters the expression of γ-globin, H-ferritin, and transferrin receptor in human K562 cells at the posttranscriptional level. *Blood* **88,** 2980–2988.

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S. J., Moynihan, J., Paw, B. H., Drejer, A., Barut, B., Zapata, A., Law, T. C., Brugnara, C., Lux, S. E., Pinkus, G. S., Pinkus, J. L., Kingsley, P. D., Palis, J., Fleming, M. D., Andrews, N. C., and Zon, L. I. (2000). Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403,** 776–781.

Drapier, J.-C., and Hibbs, J. B., Jr. (1986). Murine cytotoxic activated macrophages inhibit aconitase in tumour cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin. Invest.* **78,** 790–797.

Drapier, J. C., Pellat, C., and Henry, Y. (1991). Generation of EPR-detectable nitrosyl–iron complexes in tumor target cells cocultured with activated macrophages. *J. Biol. Chem.* **266,** 10162–10167.

Drapier, J. C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kühn, L. C. (1993). Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *EMBO J.* **12,** 3643–3649.

Dreosti, I. E. (1998). Nutrition, cancer, and aging. *Ann. N.Y. Acad. Sci.* **854,** 371–377.

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5624–5628.

Emery-Goodman, A., Hirling, H., Scarpellino, L., Henderson, B., and Kühn, L. C. (1993). Iron regulatory factor expressed from recombinant baculovirus: Conversion between the RNA-binding apoprotein and Fe-S cluster containing aconitase. *Nucleic Acids Res.* **21,** 1457–1461.

Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., and Cabantchik, I. (1997). Fluorescence analysis of the labile iron pool of mammalian cells. *Anal. Biochem.* **248,** 31–40.

Feder, J. N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D. A., Basava, A., Dormishian, F., Domingo, R., Jr., Ellis, M. C., Fullan, A., Hinton, L. M., Jones, N. L., Kimmel, B. E., Kronmal, G. S., Lauer, P., Lee, V. K., Loeb, D. B., Mapa, F. A., McClelland, E., Meyer, N. C., Mintier, G. A., Moeller, N., Moore, T., Morikang, E., Prass, C. E., Quintana, L., Starnes, S. M., Schatzman, R. C., Brunke, K. J., Drayna, D. T., Risch, N. J., Bacon, B. R., and Wolff, R. K. (1996). A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat. Genet.* **13,** 399–408.

Feelisch, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: Appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.* **17**(Suppl. 3), S25–S33.

Ferrari, R., Agnoletti, L., Comini, L., Gaia, G., Bachetti, T., Cargnoni, A., Ceconi, C., Curello, S., and Visioli, O. (1998). Oxidative stress during myocardial ischaemia and heart failure. *Eur. Heart J.* **19** (Suppl. B), 2–11.

Fleming, M. D., Trenor, C. C. I., Su, M. A., Foernzler, D., Beier, D. R., Dietrich, W. F., and Andrews, N. C. (1997). Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat. Genet.* **16,** 383–386.

Fleming, M. D., Romano, M. A., Su, M. A., Garrick, L. M., Garrick, M. D., and Andrews, N. C. (1998). Nramp2 is mutated in the anemic Belgrade

(b) rat: Evidence of a role for Nramp2 in endosomal iron transport. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 1148–1153.

Fleming, R. E., Migas, M. C., Zhou, X., Jiang, J., Britton, R. S., Brunt, E. M., Tomatsu, S., Waheed, A., Bacon, B. R., and Sly, W. S. (1999). Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: Increased duodenal expression of the iron transporter DMT1. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 3143–3148.

Frausto da Silva, J. J. R., and Williams, R. J. P. (1991). ''The Biological Chemistry of the Elements. The Inorganic Chemistry of Life,'' pp. 319–369. Oxford Univ. Press (Clarendon), Oxford.

Frishman, D., and Hentze, M. W. (1996). Conservation of aconitase residues revealed by multiple sequence analysis. Implications for structure/function relationships. *Eur. J. Biochem.* **239,** 197–200.

Gardner, P. R., and Fridovich, I. (1991). Superoxide sensitivity of the *Escherichia coli* aconitase. *J. Biol. Chem.* **266,** 19328–19333.

Gardner, P. R., Raineri, I., Epstein, L. B., and White, C. W. (1995). Superoxide radical and iron modulate aconitase activity in mammalian cells. *J. Biol. Chem.* **270,** 13399–13405.

Gardner, P. R., Constantino, G., Szabo, C., and Salzman, A. L. (1997). Nitric oxide sensitivity of the aconitases. *J. Biol. Chem.* **272,** 25071–25076.

Garrick, M. D., Gniecko, K., Liu, Y., Cohan, D. S., and Garrick, L. M. (1993). Transferrin and the transferrin cycle in Belgrade rat reticulocytes. *J. Biol. Chem.* **268,** 14867–14874.

Gegout, V., Schlegl, J., Schläger, B., Hentze, M. W., Reinbolt, J., Ehresmann, B., Ehresmann, C., and Romby, P. (1999). Ligand-induced structural alterations in human iron regulatory protein-1 revealed by protein footprinting. *J. Biol. Chem.* **274,** 15052–15058.

Gehring, N., Hentze, M. W., and Pantopoulos, K. (1999). Menadione-induced oxidative stress leads to inactivation of both RNA-binding and aconitase activities of iron regulatory protein-1. *J. Biol. Chem.* **274,** 6219–6225.

Georgatsou, E., and Alexandraki, D. (1994). Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14,** 3065–3073.

Gitlin, J. D. (1998). Aceruloplasminemia. *Pediatr. Res.* **44,** 271–276.

Goossen, B., and Hentze, M. W. (1992). Position is the critical determinant for function of iron-responsive elements as translational regulators. *Mol. Cell. Biol.* **12,** 1959–1966.

Gorbunov, N. V., Yalowich, J. C., Gaddam, A., Thampatty, P., Ritov, V. B., Kisin, E. R., Elsayed, N. M., and Kagan, V. E. (1997). Nitric oxide prevents oxidative damage produced by *tert*-butyl hydroperoxide in erythroleukemia cells via nitrosylation of heme and non-heme iron. Electron paramagnetic resonance evidence. *J. Biol. Chem.* **272,** 12328–12341.

Gray, N. K., and Hentze, M. W. (1994). Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J.* **13,** 3882–3891.

Gray, N. K., Quick, S., Goossen, B., Constable, A., Hirling, H., Kühn, L. C., and Hentze, M. W. (1993). Recombinant iron regulatory factor functions as an iron-responsive element-binding protein, a translational repressor and an aconitase. A functional assay for translational repression and direct demonstration of the iron switch. *Eur. J. Biochem.* **218,** 657–667.

Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A. C., and Hentze, M. W. (1996). Translational regulation of mammalian and *Drosophila* citric acid cycle enzymes via iron-responsive elements. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4925–4930.

Gross, C. N., Irrinki, A., Feder, J. N., and Enns, C. A. (1998). Co-trafficking of HFE, a nonclassical major histocompatibility complex class I protein, with the transferrin receptor implies a role in intracellular iron regulation. *J. Biol. Chem.* **273,** 22068–22074.

Guarriero-Bobyleva, V., Volpi-Becchi, M. A., and Masini, A. (1973). Parallel partial purification of cytoplasmic and mitochondrial aconitase hydratases from rat liver. *Eur. J. Biochem.* **34,** 455–458.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., and Hediger, M. A. (1997). Cloning and characterization of a mammalian protein-coupled metal-ion transporter. *Nature* **388,** 482–488.

Guo, B., Yu, Y., and Leibold, E. A. (1994). Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity. *J. Biol. Chem.* **269,** 24252–24260.

Guo, B., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995). Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J. Biol. Chem.* **270,** 21645–21651.

Haile, D. J., Rouault, T. A., Harford, J. B., Kennedy, M. C., Blondin, G. A., Beinert, H., and Klausner, R. D. (1992a). Cellular regulation of the iron-responsive element binding protein: Disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11735–11739.

Haile, D. J., Rouault, T. A., Tang, C. K., Chin, J., Harford, J. B., and Klausner, R. D. (1992b). Reciprocal control of RNA-binding and aconitase activity in the regulation of the iron-responsive element binding protein: Role of the iron-sulfur cluster. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7536–7540.

Halliwell, B., and Gutteridge, J. M. C. (1990). The role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol.* **186,** 1–85.

Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998). Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood* **92,** 3007–3017.

Hanson, E. S., and Leibold, E. A. (1998). Regulation of iron regulatory protein 1 during hypoxia and hypoxia/reoxygenation. *J. Biol. Chem.* **273,** 7588–7593.

Hanson, E. S., Foot, L. M., and Leibold, E. A. (1999). Hypoxia post-translationally activates iron-regulatory protein 2. *J. Biol. Chem.* **274,** 5047–5052.

Harris, Z. L., Takahashi, Y., Miyajima, H., Serizawa, M., MacGillivray, R. T., and Gitlin, J. D. (1995). Aceruloplasminemia: Molecular characterization of this disorder of iron metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 2539–43.

Henderson, B. R. (1996). Iron regulatory proteins 1 and 2. *BioEssays* **18,** 739–746.

Henderson, B. R., and Kühn, L. C. (1995). Differential modulation of the RNA-binding proteins IRP-1 and IRP-2 in response to iron. IRP-2 inactivation requires translation of another protein. *J. Biol. Chem.* **270,** 20509–20515.

Henderson, B. R., Menotti, E., and Kühn, L. C. (1996). Iron regulatory proteins 1 and 2 bind distinct sets of RNA target sequences. *J. Biol. Chem.* **271,** 4900–4908.

Henry, Y., Lepoivre, M., Drapier, J.-C., Ducrocq, C., Bucher, J.-L., and Guissani, A. (1993). EPR characterization of molecular targets for NO in mammalian cells and organelles. *FASEB J.* **7,** 1124–1134.

Hentze, M. W., and Argos, P. (1991). Homology between IRE-BP, a regulatory RNA-binding protein, aconitase and isopropylmalate isomerase. *Nucleic Acids Res.* **19,** 1739–1740.

Hentze, M. W., and Kühn, L. C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8175–8182.

Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., Harford, J. B., and Klausner, R. D. (1987). Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science* **238,** 1570–1573.

Hentze, M. W., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1989). Oxidation–reduction and the molecular mechanism of a regulated RNA–protein interaction. *Science* **244,** 357–359.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94.

Iwai, K., Klausner, R. D., and Rouault, T. A. (1995). Requirements for iron-regulated degradation of the RNA binding protein, iron regulatory protein 2. *EMBO J.* **14,** 5350–5357.

Iwai, K., Drake, S. K., Wehr, N. B., Weissman, A. M., LaVaute, T., Minato, N., Klausner, R. D., Levine, R. L., and Rouault, T. A. (1998). Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: Implications for degradation of oxidized proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 4924–4928.

Jaffrey, S. R., Cohen, N. A., Rouault, T. A., Klausner, R. D., and Snyder, S. H. (1994). The iron-responsive element binding protein: A target for synaptic actions of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12994–12998.

Kaplan, J., and O'Halloran, T. V. (1996). Iron metabolism in eukaryotes: Mars and Venus at it again. *Science* **271,** 1510–1512.

Ke, Y., Wu, J., Leibold, E. A., Walden, W. E., and Theil, E. C. (1998). Loops and bulge/loops in iron-responsive element isoforms influence iron regulatory protein binding. *J. Biol. Chem.* **273,** 23637–23640.

Kennedy, M. C., Mende-Mueller, L., Blondin, G. A., and Beinert, H. (1992). Purification and characterization of cytosolic aconitase from beef liver and its relationship to the iron-responsive element binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11730–11734.

Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997). An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. *J. Biol. Chem.* **272,** 20340–20347.

Kim, H.-Y., LaVaute, T., Iwai, K., Klausner, R. D., and Rouault, T. A. (1996). Identification of a conserved and functional iron-responsive element in the 5'-untranslated region of mammalian mitochondrial aconitase. *J. Biol. Chem.* **271,** 24226–24230.

Klausner, R. D., and Dancis, A. (1994). A genetic approach to elucidating eukaryotic iron metabolism. *FEBS Lett.* **355,** 109–113.

Klausner, R. D., Rouault, T., and Harford, J. B. (1993). Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell* **72,** 19–28.

Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L.-N. L., Klausner, R. D., and Harford, J. B. (1989). A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3574–3578.

Koenig, M., and Mandel, J. L. (1997). Deciphering the cause of Friedreich ataxia. *Curr. Opin. Neurobiol.* **7,** 689–694.

Kohler, S. A., Henderson, B. R., and Kühn, L. C. (1995). Succinate dehydrogenase b mRNA of *Drosophila melanogaster* has a functional iron-responsive element in its 5'-untranslated region. *J. Biol. Chem.* **270,** 30781–30786.

Kubrina, L. N., Caldwell, W. S., Mordvintcev, P. I., Malenkova, I. V., and Vanin, A. F. (1992). EPR evidence for nitric oxide production from guanidino nitrogens of L-arginine in animal tissues in vivo. *Biochim. Biophys. Acta* **1099,** 233–237.

Kühn, L. C. (1994). Molecular regulation of iron proteins. *Baillieres Clin. Haematol.* **7,** 763–785.

Kyriakis, J. M., and Avruch, J. (1996). Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays* **18,** 567–577.

Lauble, H., Kennedy, M. C., Beinert, H., and Stout, C. D. (1992). Crystal structures of aconitase with isocitrate and nitroisocitrate bound. *Biochemistry* **31,** 2735–2748.

Lebrón, J. A., Bennet, M. J., Vaughn, D. E., Chirino, A. J., Snow, P. M., Mintier, G. A., Feder, J. N., and Bjorkman, P. J. (1998). Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. *Cell* **93,** 111–123.

Lee, M., Arosio, P., Cozzi, A., and Chasteen, N. D. (1994). Identification of the EPR-active iron–nitrosyl complexes in mammalian ferritins. *Biochemistry* **33,** 3679–3687.

Leibold, E. A., and Munro, H. N. (1988). Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5' untranslated regions of ferritin heavy- and light-subunit mRNAs. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2171–2175.

McDonald, L. J., and Murad, F. (1996). Nitric oxide and cyclic GMP signaling. *Proc. Soc. Exp. Biol. Med.* **211,** 1–6.

McKie, A. T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T. J., Farzaneh, F., Hediger, M. A., Hentze, M. W., and Simpson, R. J. (2000). A novel duodenal iron-regulated transporter IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol. Cell* **5,** 299–309.

Martins, E. A. L., Robalinho, R. L., and Meneghini, R. (1995). Oxidative stress induces activation of a cytosolic protein responsible for control of iron uptake. *Arch. Biochem. Biophys.* **316,** 128–134.

Melefors, Ö., Goossen, B., Johansson, H. E., Stripecke, R., Gray, N. K., and Hentze, M. W. (1993). Translational control of 5-aminolevulinate synthase mRNA by iron-responsive elements in erythroid cells. *J. Biol. Chem.* **268,** 5974–5978.

Muckenthaler, M., Gray, N. K., and Hentze, M. W. (1998). IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol. Cell* **2,** 383–388.

Mukhopadhyay, C., Attieh, Z. K., and Fox, P. L. (1998). Role of ceruloplasmin in cellular iron uptake. *Science* **279,** 714–717.

Müllner, E. W., and Kühn, L. C. (1988). A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. *Cell* **53,** 815–825.

Neupert, B., Menotti, E., and Kühn, L. C. (1995). A novel method to identify nucleic acid binding sites in proteins by scanning mutagenesis: Application to iron regulatory protein. *Nucleic Acids Res.* **23,** 2579–2583.

Oria, R., Sánchez, L., Houston, T., Hentze, M. W., Liew, F. Y., and Brock, J. H. (1995). Effect of nitric oxide on expression of transferrin receptor and ferritin and on cellular iron metabolism in K562 human erythroleukemia cells. *Blood* **85,** 2962–2966.

Pantopoulos, K., and Hentze, M. W. (1995a). Nitric oxide signaling to iron-regulatory protein (IRP): Direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1267–1271.

Pantopoulos, K., and Hentze, M. W. (1995b). Rapid responses to oxidative stress mediated by iron regulatory protein. *EMBO J.* **14,** 2917–2924.

Pantopoulos, K., and Hentze, M. W. (1998). Activation of iron regulatory protein-1 by oxidative stress *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10559–10563.

Pantopoulos, K., and Hentze, M. W. (1999). *In* Inorganic Biochemistry and Regulatory Mechanisms of Iron Metabolism (G. C. Ferreira, J. J. G. Moura, and R. Franco, eds.), pp. 131–152. Wiley-VCH, Weinheim, Germany.

Pantopoulos, K., Gray, N., and Hentze, M. W. (1995). Differential regulation of two related RNA-binding proteins, iron regulatory protein (IRP) and IRP_B. *RNA* **1,** 155–163.

Pantopoulos, K., Weiss, G., and Hentze, M. W. (1996). Nitric oxide and oxidative stress (H_2O_2) control mammalian iron metabolism by different pathways. *Mol. Cell. Biol.* **16,** 3781–3788.

Pantopoulos, K., Mueller, S., Atzberger, A., Ansorge, W., Stremmel, W., and Hentze, M. W. (1997). Differences in the regulation of iron regulatory protein 1 (IRP1) by *extra-* and *intra*cellular oxidative stress. *J. Biol. Chem.* **272,** 9802–9808.

Paraskeva, E., and Hentze, M. W. (1996). Iron-sulphur clusters as genetic regulatory switches: The bifunctional iron regulatory protein-1. *FEBS Lett.* **389,** 40–43.

Paraskeva, E., Gray, N. K., Schläger, B., Wehr, K., and Hentze, M. W. (1999). Ribosomal pausing and scanning arrest as mechanisms of translational regulation from cap-distal iron-responsive elements. *Mol. Cell. Biol.* **19,** 807–816.

Parkkila, S., Waheed, A., Britton, R. S., Bacon, B. R., Zhou, X. Y., Tomatsu, S., Fleming, R. E., and Sly, W. S. (1997). Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 13198–13202.

Phillips, J. D., Kinikini, D. V., Yu, Y., Guo, B., and Leibold, E. A. (1996). Differential regulation of IRP-1 and IRP-2 by nitric oxide in rat hepatoma cells. *Blood* **87,** 2983–2992.

Pippard, M. J. (1994). *In* ''Iron Metabolism in Health and Disease'' (J. H. Brock, J. W. Halliday, M. J. Pippard, and L. W. Powell, eds.), pp. 271–309. Saunders, London.

Ponka, P. (1997). Tissue-specific regulation of iron metabolism and heme synthesis: Distinct control mechanisms in erythroid cells. *Blood* **89,** 1–25.

Ponka, P., Beaumont, C., and Richardson, D. R. (1998). Function and regulation of transferrin and ferritin. *Semin. Hematol.* **35,** 35–54.

Poulsen, H. E., Prieme, H., and Loft, S. (1998). Role of oxidative DNA damage in cancer initiation and promotion. *Eur. J. Cancer Prev.* **7,** 9–16.

Radisky, D. C., and Kaplan, J. (1998). Iron in cytosolic ferritin can be recycled through lysosomal degradation in human fibroblasts. *Biochem. J.* **336,** 201–205.

Radisky, D., and Kaplan, J. (1999). Regulation of transition metal transport across the yeast plasma membrane. *J. Biol. Chem.* **274,** 4481–4484.

Radisky, D. C., Babcock, M. C., and Kaplan, J. (1999). The yeast frataxin homologue mediates mitochondrial iron efflux. Evidence for a mitochondrial iron cycle. *J. Biol. Chem.* **274,** 4497–4499.

Rafferty, S. P., Domachowske, J. B., and Malech, H. L. (1996). Inhibition of hemoglobin expression by heterologous production of nitric oxide synthase in the K562 erythroleukemic cell line. *Blood* **88,** 1070–1078.

Raja, K. B., Simpson, R. J., and Peters, T. J. (1992). Investigation of a role for reduction in ferric iron uptake by mouse duodenum. *Biochem. Biophys. Acta* **1135,** 141–146.

Recalcati, S., Taramelli, D., Conte, D., and Cairo, G. (1998). Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: Role of selective iron regulatory protein-2 downregulation. *Blood* **91,** 1059–1066.

Richardson, D. R., and Ponka, P. (1997). The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochem. Biophys. Acta* **1331,** 1–40.

Richardson, D. R., Neumannova, V., Nagy, E., and Ponka, P. (1995). The effect of redox-related species of nitrogen monoxide on transferrin and iron uptake and cellular proliferation of erythroleukemia (K562) cells. *Blood* **86,** 3211–3219.

Riedel, H. D., Remus, A. J., Fitscher, B. A., and Stremmel, W. (1995). Characterization and partial purification of a ferrireductase from human duodenal microvillus membranes. *Biochem. J.* **309,** 745–748.

Robbins, A. H., and Stout, C. D. (1989a). The structure of aconitase. *Proteins* **5,** 289–312.

Robbins, A. H., and Stout, C. D. (1989b). Structure of activated aconitase: Formation of the [4Fe-4S] cluster in the crystal. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3639–3643.

Rothenberg, B. E., and Voland, J. R. (1996). β_2 knockout mice develop parenchymal iron overload: A putative role for class I genes of the major histocompatibility complex in iron metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1529–1534.

Rouault, T. A., Hentze, M. W., Caughman, S. W., Harford, J. B., and Klausner, R. D. (1988). Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA. *Science* **241,** 1207–1210.

Rouault, T. A., Tang, C. K., Kaptain, S., Burgess, W. H., Haile, D. J., Samaniego, G., McBride, O. W., Harford, J. B., and Klausner, R. D. (1990). Cloning of the cDNA encoding an RNA regulatory protein- the human iron-responsive element-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7958–7962.

Rouault, T. A., Stout, C. D., Kaptain, S., Harford, J. B., and Klausner, R. D. (1991). ''Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase: Functional implications. *Cell* **64,** 881–883.

Rouault, T., Klausner, R. D., and Harford, J. B. (1996). *In* ''Translational Control'' (J. W. B. Hershey, M. B. Mathews, and N. Sonenberg, eds.), pp. 335–362. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Samaniego, F., Chin, J., Iwai, K., Rouault, T. A., and Klausner, R. D. (1994). Molecular characterization of a second iron-responsive element binding protein, iron regulatory protein 2. *J. Biol. Chem.* **269,** 30904–30910.

Shigenaga, M. K., Hagen, T. M., and Ames, B. N. (1994). Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 10771–10778.

Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *Exp. Physiol.* **82,** 291–295.

Skikne, B., and Baynes, R. D. (1994). *In* ''Iron Metabolism in Health and Disease'' (J. H. Brock, J. W. Halliday, M. J. Pippard, and L. W. Powell, eds.), pp. 151–187. Saunders, London.

Stearman, R., Yuan, D. S., Yammaguchi-Iwai, Y., Klausner, R. D., and Dancis, A. (1996). A permease–oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271,** 1552–1557.

Stevens, R. G., Jones, Y. D., Micozzi, M. S., and Taylor, P. R. (1988). Body iron stores and the risk of cancer. *N. Engl. J. Med.* **319,** 1047–1052.

Stripecke, R., Oliveira, C. C., McCarthy, J. E. G., and Hentze, M. W. (1994). Proteins binding to 5' untranslated region sites: A general mechanism for translational regulation of mRNAs in human and yeast cells. *Mol. Cell. Biol.* **14,** 5898–5909.

Su, M. A., Trenor III, C. C., Fleming, J. C., Fleming, M. D., and Andrews, N. C. (1998). The G185R mutation disrupts function of the iron transporter Nramp2. *Blood* **92,** 2157–2163.

Sun, A. Y., and Chen, Y. M. (1998). Oxidative stress and neurodegenerative disorders. *J. Biomed. Sci.* **5,** 401–414.

Swenson, G. R., and Walden, W. E. (1994). Localization of an RNA binding element of the iron responsive element binding protein within a proteolytic fragment containing iron coordination ligands. *Nucleic Acids Res.* **22,** 2627–2633.

Takagi, H., Shi, D., Ha, Y., Allewell, N. M., and Theil, E. C. (1998). Localized unfolding at the junction of three ferritin subunits. A mechanism for iron release? *J. Biol. Chem.* **273,** 18685–18688.

Tang, C. K., Chin, J., Harford, J. B., Klausner, R. D., and Rouault, T. A. (1992). Iron regulates the activity of the iron-responsive element binding protein without changing its rate of synthesis or degradation. *J. Biol. Chem.* **267,** 24466–24470.

Theil, E. C. (1987). Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem.* **56,** 289–315.

Theil, E. C. (1994). Iron regulatory elements (IREs): A family of mRNA non-coding sequences. *Biochem. J.* **304,** 1–11.

Toth, I., Yuan, L., Rogers, J. T., Boyce, H., and Bridges, K. R. (1999). Hypoxia alters iron-regulatory protein-1 binding capacity and modulates cellular iron homeostasis in human hepatoma and erythroleukemia cells. *J. Biol. Chem.* **274,** 4467–4473.

Vulpe, C. D., Kuo, Y. M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., Gitschier, J., and Anderson, G. J. (1999). Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat. Genet.* **21,** 195–199.

Wächtershäuser, G. (1992). Groundworks for an evolutionary biochemistry: The iron–sulphur world. *Prog. Biophys. Mol. Biol.* **58,** 85–201.

Waheed, A., Parkkila, S., Saarnio, J., Fleming, R. E., Zhou, X. Y., Tomatsu, S., Britton, R. S., Bacon, B. R., and Sly, W. S. (1999). Association of HFE protein with transferrin receptor in crypt enterocytes of human duodenum. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 1579–1584.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., and Hentze, M. W. (1993). Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *EMBO J.* **12,** 3651–3657.

Weiss, G., Werner-Felmayer, G., Werner, E. R., Grünewald, K., Wachter, H., and Hentze, M. W. (1994). Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J. Exp. Med.* **180,** 969–976.

Weiss, G., Wachter, H., and Fuchs, D. (1995). Linkage of cell-mediated immunity to iron metabolism. *Immunol. Today* **16,** 495–500.

Weiss, G., Bogdan, C., and Hentze, M. W. (1997). Pathways for the regulation of macrophage iron metabolism by the anti-inflammatory cytokines IL-4 and IL-13. *J. Immunol.* **158,** 420–425.

Wooldridge, K. G., and Williams, P. H. (1993). Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol. Rev.* **12,** 325–348.

Zhou, X. Y., Tomatsu, S., Fleming, R. E., Parkkila, S., Waheed, A., Jiang, J., Fei, Y., Brunt, E., Ruddy, D. A., Prass, C. E., Schatzman, R. C., O'Neill, R., Britton, R. S., Bacon, B. R., and Sly, W. S. (1998). HFE gene knockout produces mouse model of hereditary hemochromatosis. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2492–2497.

Zoller, H., Pietrangelo, A., Vogel, W., and Weiss, G. (1999). Duodenal metal-transporter (DMT-1, NRAMP-2) expression in patients with hereditary haemochromatosis. *Lancet* **353,** 2120–2123.

Zweier, J. L., Kuppusamy, P., and Lutty, G. A. (1988). Measurement of endothelial cell free radical generation: Evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 4046–4050.

Zweier, J. L., Broderick, R., Kuppusamy, P., Thompson-Gorman, S., and Lutty, G. A. (1994). Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *J. Biol. Chem.* **269,** 24156–24162.

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CHAPTER 20

Redox Modulation of Iron Regulatory Proteins by Nitric Oxide and Peroxynitrite

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IRON REGULATORY PROTEINS (IRP1 AND IRP2) BIND TO IRON-RESPONSIVE ELEMENT(S) (IRE) IN THE UNTRANSLATED REGION OF SEVERAL MRNAS ENCODING PROTEINS INVOLVED IN IRON METABOLISM AND ENERGY PRODUCTION. IRP1 IS A BIFUNCTIONAL PROTEIN THAT ALSO DISPLAYS ACONITASE ACTIVITY WHEN IT CONTAINS AN INTACT [4FE–4S] CLUSTER. IRP2 HAS NO FE–S CLUSTER BUT EXHIBITS SEVERAL REDOX-SENSITIVE CYSTEINES. IN THIS REVIEW, WE DESCRIBE, COMPARE, AND EXPRESS OUR VIEWS ON THE RESPECTIVE EFFECTS OF NITRIC OXIDE (NO) AND PEROXYNITRITE ON THESE TWO CHEMOREACTIVE PROTEINS. DATA ARE DISCUSSED IN THE MORE GENERAL CONTEXT OF THE SENSITIVITY OF IRPS TO CHEMICAL MESSENGERS INCLUDING OXYGEN-DERIVED SPECIES. WE DESCRIBE TWO LATENT FORMS OF IRP1 WHOSE EXISTENCE FURTHER COMPLICATES THE PICTURE, AND WE REVIEW THE REQUIREMENT FOR REDUCTION PRIOR TO BINDING IRE. ENDOGENOUS PRODUCTION OF NO READILY TRIGGERS THE ACONITASE–RNA BINDING SWITCH. *IN VITRO* STUDIES REVEALED THAT IN SO DOING NO REACTS RAPIDLY WITH IRP1 BUT REQUIRES COOPERATION WITH THE THIOREDOXIN SYSTEM. PEROXYNITRITE, AS A STRONG OXIDANT, MOSTLY LIMITS ITS CAPACITIES TO INACTIVATION OF ACONITASE ACTIVITY, WITHOUT SIMPLE TRIGGERING OF IRE BINDING. WE OBSERVED THAT IRP2 REGULATION IS OPPOSITE TO THAT OF IRP1 IN STIMULATED MACROPHAGES. PEROXYNITRITE ALSO DECREASES IRP2 ACTIVITY AT A POSTTRANSLATIONAL LEVEL. DOWNREGULATION OF IRP2 MAY BE RELEVANT FOR BALANCING IRP1 INTERFERENCE IN IRON HOMEOSTASIS.

Introduction

The notion of nitric oxide (NO)-mediated redox modulation has emerged in the mid-1990s. NO and related species are prototypical effectors of this distinctive signaling pathway, which proceeds through covalent bonding and involves redox biochemistry (Stamler, 1994; Drapier and Bouton, 1996). This novel type of signaling relies on the interplay between two partners: a reactive effector and a sensor molecule (hormone or protein) able to respond to the chemical signal by adapting its function to the covalent modifications and/or structural change. In most cases, the submolecular targets that perceive the chemical message are redox active sulfhydryl groups and transition metals, especially iron. The list of proteins sensitive to redox modulation by NO and related species is growing rapidly (Broillet, 1999). Here, we will focus on the interplay between NO or peroxynitrite with iron regulatory protein 1 (IRP1) and IRP2, two RNA-binding proteins that regulate vertebrate cell iron metabolism (see also Bouton, 1999). Iron is essential for survival of most

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living organisms, but it has two major drawbacks: low solubility and production of harmful products via the Fenton reaction. It is therefore essential for cells to fine-tune transport, uptake, and storage of iron. In mammals, the transferrin receptor and ferritin are chiefly responsible for iron uptake and sequestration, respectively. Cellular iron status is controlled by sound expression of transferrin receptor and ferritin through expression and activity of IRPs. Regulation of IRP activities has been the subject of numerous studies. We shall outline the background of such studies, and we compare the effects of NO and peroxynitrite.

IRPs and Aconitases: Two Merging Stories

IRP1 and IRP2 maintain the homeostasis of intracellular iron through a posttranscriptional mechanism. They recognize specific sequences called iron responsive elements (IREs) on distinctive mRNA (Leibold and Munro, 1988). IREs are stem–loops made up of ~30 nucleotides and consist of a stable stem about 10 nucleotides long with a bulged cytosine residue and a loop with the conserved sequence CAGUGN (Theil, 1998). Functional IREs have been found in the 5' untranslated region of mRNA of human ferritin H- and L-chains (Aziz and Munro, 1987; Hentze *et al.,* 1987), δ-aminolevulinate synthase of erythroid cells (Dandekar *et al.,* 1991), mitochondrial aconitase (Zheng *et al.,* 1992; Kim *et al.,* 1996), and subunit b of *Drosophila melanogaster* succinate dehydrogenase (Kohler *et al.,* 1995; Gray *et al.,* 1996; Meleförs, 1996). IRP–IRE interaction in the 5' untranslated region inhibits translation (Gray and Hentze, 1994). In contrast, when IRPs bind to IRE(s) at the 3' end of mRNA, the binding stabilizes the message, probably by preventing endonucleotide degradation (Fig. 1) (see Klausner *et al.,* 1993, Rouault and Klausner, 1997, and Kühn, 1998, for reviews). Transferrin receptor mRNA contains five IREs, all located at its 3' end, and the interaction between IRPs and IREs increases the stability of its mRNA. An iron transporter named ''divalent-metal transporter'' (DMT1, also known as DCT1/Nramp2) has been characterized (Gushin *et al.,* 1997). It is expressed ubiquitously but preferentially in the duodenum. DCT1/Nramp2 mRNA contains an IRE-like sequence in its 3' untranslated region, which strongly suggests that it could also be controlled by IRPs.

In the late 1980s, IRP1, which has previously been referred to as FRP, P90, IRF, or IRE-BP, was identified as an RNA-binding protein that regulates vertebrate cell iron metabolism. A few years later, comparison of the amino acid sequences of pig heart aconitase and IRP1 revealed high identity (~30%) between porcine mitochondrial aconitase and human IRP1. Eventually, it was disclosed that the same polypeptide carries both activities. IRP1 was therefore acknowledged as a bifunctional protein, either cytosolic aconitase or RNA-binding protein (Hentze and Argos, 1991; Rouault *et al.,* 1991; Kaptain *et al.,* 1991; Kennedy *et al.,* 1992). IRP1 thus belongs to the iron–sulfur (Fe–S) isomerase family, which contains fungal and bacterial isomerases

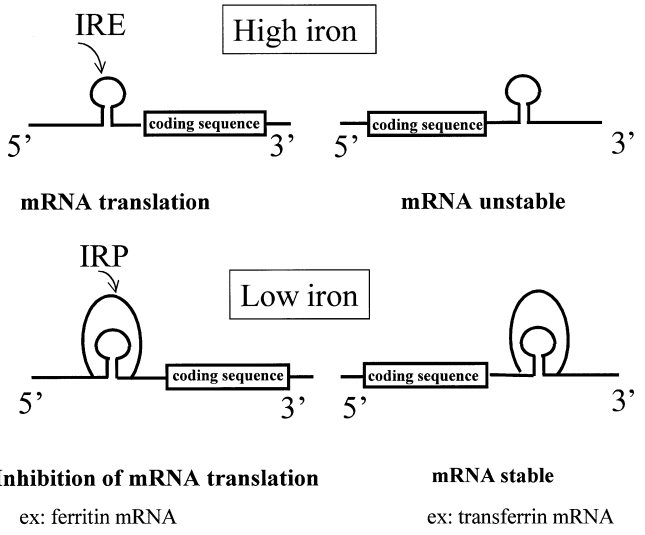


Figure 1 Schematic representation of the posttranscriptional regulation mediated by IRPs. In cells depleted of iron, IRP1 and IRP2 bind equally well to the IRE stem–loop structures found in the untranslated regions of different mRNA. The binding of IRP to an IRE in the 5' end of mRNAs represses their translation. In contrast, the binding of IRP to at least three of the five IREs at the 3' end of transferrin receptor mRNAs protects it from endonucleolytic degradation.

involved in leucine biosynthesis and aconitases (Frishman and Hentze, 1996; Gruer *et al.,* 1997).

Fe–S proteins were identified and recognized in the 1960s. They participate in several basic metabolic pathways of living organisms including photosynthesis, nitrogen fixation, and oxidative phosphorylation. Fe–S clusters are very ancient prosthetic groups that, in many cases, are involved in oxidoreduction reactions. Aconitases are monomeric proteins (M_r 68,000–120,000) containing a single Fe–S cluster that stands out because it binds substrate at the active site (Beinert and Kennedy, 1989). They convert citrate to isocitrate, with *cis*-aconitate as an intermediate. Porcine heart mitochondrial aconitase was crystallized by Stout and coworkers (Robbins and Stout, 1989; Lauble *et al.,* 1992) (Fig. 2). It thus became the prototypic aconitase, and valuable information on the active site was derived from these studies. This aconitase will hereafter be referred to as mt-aco. As IRP1 resembles mt-aco in the predicted domain structure, we will digress on what we can learn from the crystal structure of the latter. Mt-aco is an 83-kDa monomer arranged in four domains, with three structural domains packed around a [4Fe–4S] cluster and connected to the fourth domain by a linker peptide. The [4Fe–4S] cluster is thus located within a cleft which divides the packed domains 1–3 from domain 4. One specific iron of the cluster (Fe_a) is not connected to the protein backbone but is held by the α (isocitrate) or β (citrate) carboxyl of the hydrated substrates. In addition, the hydroxyl of citrate or isocitrate also binds Fe_a. This iron atom of the cluster is thus involved in the enzymatic catalysis, and its removal is sufficient to abolish activity (Beinert and Kennedy, 1989). Such a cluster is exposed to the solvent

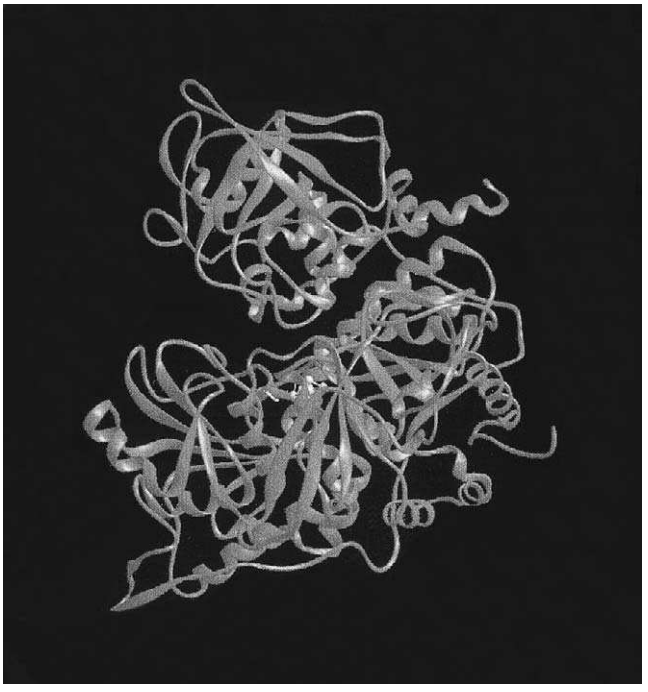


Figure 2 Structure of the [3Fe–4S] cluster-containing pig mitochondrial aconitase crystallized by Robbins and Stout (1989). From SWISS-PROT protein database. See color insert.

and hence vulnerable to environmental signals such as NO (Drapier, 1997). Analysis combining electron paramagnetic resonance (EPR) and Mössbauer spectroscopy revealed that $[4Fe–4S]^{2+}$-containing mt-aco is the enzymatically active form. The three other iron atoms are held to the protein by cysteine residues at positions 358, 421, and 424.

IRP1 and mt-aco exhibit approximately 31% identity and about 56% similarity. As deduced from sequence alignment and computer prediction, IRP1, like mt-aco, comprises four domains, three at the N-terminal end which form a compact core (domains 1–3), to which the fourth (domain 4) is connected by a peptide linker. The flexibility of this hinge allows fields 1–3 and 4 to come together around a narrow cleft corresponding to the active site of the enzyme. The [4Fe–4S] center is located in the cleft and is linked to domain 3 by cysteines 437, 503, and 506 (Fig. 3). Fe_a interacts with substrates, for example, citrate, which itself binds to at least four arginines (R536, R541, R699, R780) in human IRP1 belonging to the four domains. Thus the presence of the substrate at the catalytic site contributes to the stabilization of the protein structure and to the connection of domains 1–3 to domain 4 (Beinert and Kennedy, 1993). IRP1 in this closed conformation (holo-IRP1) exhibits aconitase activity in the cytosol.

IRP2, whose sequence in humans exhibits 57% identity with and 75% similarity to that of IRP1 (Henderson, 1996), was also categorized as an aconitase, even though it has no Fe–S cluster and hence no enzymatic activity.

Measurement of IRP Activities

Aconitase assays have been extensively described elsewhere (Fansler and Löwenstein, 1969; Drapier and Hibbs, 1996). Two spectrophotometric assays are routinely used. One relies on the UV absorbance of *cis*-aconitate at 240 nm. The other is performed in the presence of isocitrate dehydrogenase, which catalyzes the oxidation of citrate to α-ketoglutarate coupled with the reduction of NADP at 340 nm. RNA-binding activity of IRPs is determined by an electrophoreticmobility shift assay, which was originally described by Leibold and Munro (1998) and has since been modified (Müllner *et al.,* 1989; Drapier and Hibbs, 1996). Briefly, a [^{32}P]CTP-labeled IRE probe is prepared, generally from a plasmid containing the IRE sequence of human ferritin H-chain mRNA. After incubation of a molecular *excess* of probe (this is a crucial point) with cell lysate or purified protein, complexes between IRPs and IRE are separated from unbound probe on a 6% polyacrylamide gel. The two IRPs from rat, or mouse, migrate separately (Fig. 4). When human cells are analyzed, IRP1 and IRP2 comigrate and can be discriminated only by a supershift assay. Two percent 2-mercaptoethanol (2-Me) is routinely added to each sample prior to the gel shift assay, since high concentrations of reductant allow full expression of IRP RNA-binding activity (Hentze *et al.,* 1989). This constitutes a convenient way of checking equal loading of protein on the gel and of expressing RNA binding as percentage of total activity. It should be stressed that maximal binding activity of IRP2 is not visualized at 2% 2-Me but rather at 0.2–0.5% (Bouton *et al.,* 1998). This may explain why, originally, it was sometimes reported that IRP2 was barely sensitive to reductants. Last, the great sensitivity of IRP to reducing agents, especially some cluster-free forms of IRP1 (see later), means that concentrations of reducers like dithiothreitol (DTT) must be kept low in the binding assay buffer.

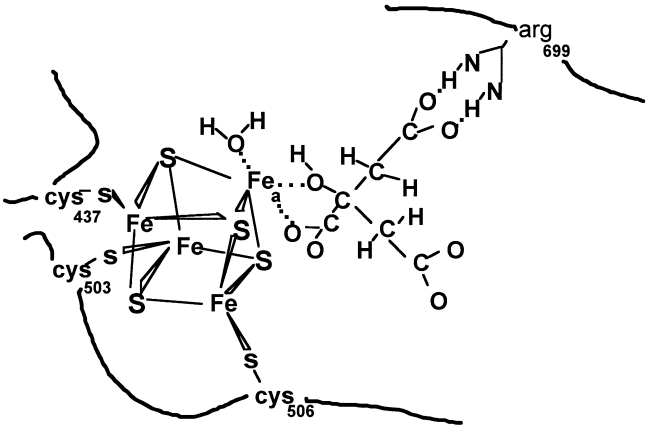


Figure 3 Representation of the solvent-exposed [4Fe–4S] cluster cytosolic IRP1 with citrate bound. Based on the crystal structure of mitochondrial aconitase determined by Robbins and Stout (1989) and adapted from Hirling *et al.* (1994), *EMBO J.* **13,** 453–461, by permission of Oxford University Press.

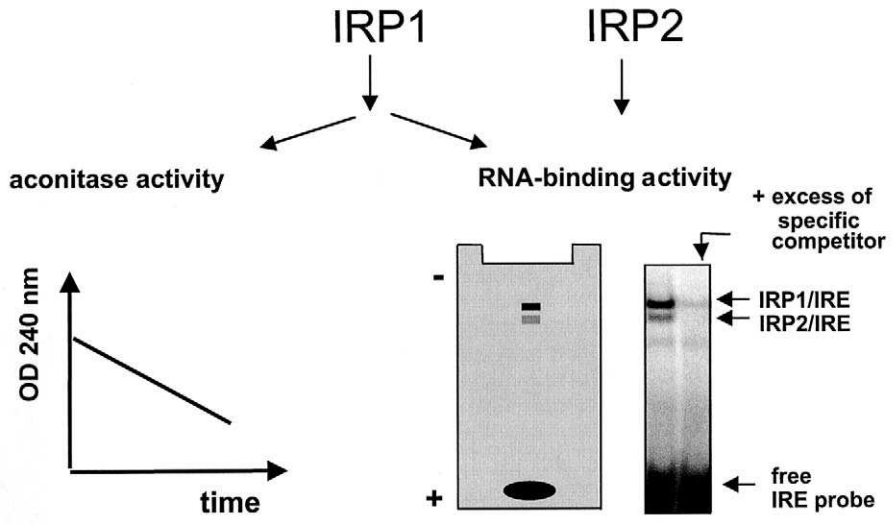


Figure 4 Measurement of IRP activities. IRP1 can display two activities: aconitase activity and IRE binding activity. IRP2 is active only as an IRE-binding protein. In our experiments, aconitase activity was followed spectrophotometrically by the disappearance of *cis*-aconitate at 240 nm. IRE binding activity was measured by an electrophoretic mobility shift assay (EMSA) with excess ^{32}P-labeled IRE probe in the absence and presence of 2% 2-mercaptoethanol which reveals full IRE-binding activity (Hentze *et al.,* 1989). Two IRP–IRE complexes (indicated by the arrows) are resolved in extracts of rodent cells.

Modulation of Iron Regulatory Proteins

A Salient Feature for Posttranslational IRP1 Regulation: A Versatile Fe-S Cluster

Several studies have shown that the entire [Fe–S] center hampers access of the IRE sequence to the residues involved in the RNA-binding domain, and that loss of Fe_a, which induces loss of aconitase activity, is not sufficient to gain IRE-binding capacity (Haile *et al.,* 1992). According to the model proposed by Klausner and Rouault (1993), cluster assembly–disassembly involves the three other iron atoms of the cluster. In the absence of the Fe–S center, the fourth domain of the apo-IRP1 could move away from packed domains 1–3, due to the flexible hinge, to allow an ''open'' configuration and make the binding site accessible to IREs. Mutation of cysteines 437, 503, and 506 definitively proved the crucial role of the Fe–S center in IRP1 functions. Indeed, IRP1 devoid of Cys-437, or of both Cys-503 and Cys-506, cannot insert the Fe–S center and therefore constitutively binds to IRE sequences (Philpott *et al.,* 1993; Hirling *et al.,* 1994). Accordingly, the form of IRP1 able to bind IRE sequences with a high affinity is thought to be entirely devoid of its Fe–S center (Fig. 5) (Haile *et al.,* 1992; Hirling *et al.,* 1994).

The way IRP1 alternates between the two functions has been widely investigated *in vitro* using sources of iron or iron chelators as well as oxidants and reductants. However, the physiological mechanisms by which IRP1 adapts its structure in response to functional needs are still poorly understood. To understand how IRP1 activities are modulated, it is crucial to elucidate the mechanism whereby [Fe–S] is assembled in living organisms. *In vitro* experiments show that in solution Fe–S clusters spontaneously assemble in the presence of iron, sulfide, and a reducing agent like DTT. In bacteria, enzymes catalyzing sulfur transfer from cysteine to a Fe–S cluster have been characterized. These sulfur-transferases, originally found to participate in assembly of the Fe–S cluster of *Azotobacter vinelandii* nitrogenase, are termed NifS proteins (Zheng *et al.,* 1993). In yeast, a yeast ortholog (Nfs1p) has been characterized in the mitochondrial matrix (Kispal *et al.,* 1999). Moreover, other data indicate that NifS-like proteins also exist in mammals (Land and Rouault, 1998). It is likely that as yet undefined iron-carrying proteins also participate in Fe–S assembly. Once these putative proteins are fully characterized, it will be interesting to investigate their sensitivity to changes in intracellular iron concentration and to environmental redox messengers.

The Key Role of Cysteine-437

Diamide, a sulfhydryl group blocker, inhibits the *trans*-regulating activity of the apo-IRP1 (Hirling *et al.,* 1994). Apo-IRP1 thus requires free sulfhydryls for its interaction with IRE sequences. Mutation of Cys-437, 503, or 506 to serine showed that only the Cys-437 mutant was fully active in the absence of reductant, whereas IRP1 from cells transfected with Cys-503 or Cys-506 mutant required the presence of low concentrations of 2-Me to be fully active as an RNA-binding protein (Hirling *et al.,* 1994). These experiments confirmed the existence of an oxidized apoprotein form of IRP1. As anticipated by computer prediction based on the structure of mt-aco, the only likely partner to yield a

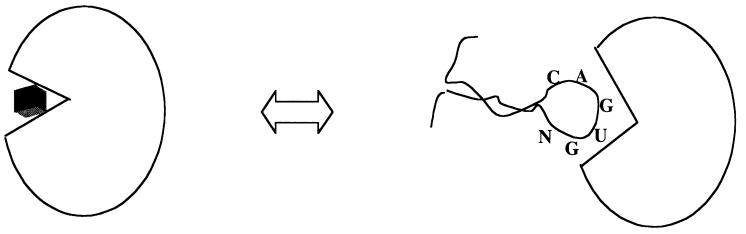


Figure 5 IRP1: one polypeptide, two functions. Loss of the [4Fe–4S] cluster induces an allosteric switch which causes conversion of the cytosolic aconitase into an IRE-binding protein (Klausner and Rouault, 1993).

disulfide bond between Cys-503 or Cys-506 is Cys-437 (Hirling *et al.,* 1994). In brief, a salient feature to remember is that disulfide bridging between Cys-437 and either Cys-503 or Cys-506 prevents interaction with IRE.

IRP1, a Multifaceted Molecule

The [4Fe–4S] cluster-containing form of IRP1 is active as aconitase, but IRE binding is prevented. In aconitases, 4Fe to 3Fe conversion is accompanied by loss of enzymatic activity. IRP1 obeys the rule, as testified by the *in vitro* experiments of Haile *et al.* (1992) in which a lysate of iron-replete cells was titrated with ferricyanide. At low concentrations of oxidant, aconitase activity was lost, and RNA binding was detectable only in the presence of 2% 2-Me. Substrates that bind 3Fe- as well as 4Fe-clusters prevent activation by 2% 2-Me (Haile *et al.,* 1992). There is evidence that this form also exists in intact cells, as testified by ascorbate induction of a latent aconitase activity in K562 erythroleukemia cells (Toth and Bridges, 1995). This [3Fe–4Fe]$^{+}$-containing form is paramagnetic and emits a typical $g = 2.02$ signal detected by EPR spectroscopy (Basilion *et al.,* 1994; Kennedy *et al.,* 1992).

In vitro experiments showed that at high ferricyanide concentrations (>100 μM), a cluster-free (apo) IRP1 is formed, as testified by the absence of protection of substrates against 2% 2-Me (Haile *et al.,* 1992). In this conformation, the absence of the 4Fe–4S cluster prevents the bridging between the packed domains 1–3 and domain 4. This relaxed structure can accommodate IRE, but it is worth noting that minute amounts of thiol are necessary to maintain the RNA-binding capability of apo-IRP1 (Haile *et al.,* 1992; Beinert and Kennedy, 1993). In the absence of reducing conditions, this form is biologically inactive.

IRP2, More Than a Spare Wheel

A second protein, first named IRP_B then iron regulatory protein-2 (IRP2), was characterized in rodents and in humans (Henderson *et al.,* 1993; Samaniego *et al.,* 1994; Guo *et al.,* 1994, 1995). IRP2 cDNA was isolated both in humans and rats, and there is 93% identity between the two species (Guo *et al.,* 1995). IRP2 binds consensus IRE sequences with the same affinity as IRP1 (Kim *et al.,* 1995). IRP2 has a molecular mass of 105 kDa due to an additional 73-amino acid domain near the N-terminal part (Guo *et al.,* 1995; Iwai *et al.,* 1995). Although IRP2 has 16 of the 18 active site amino acids present in IRP1, including cysteines-512, -578, and -581, which are equivalent to those which hold the Fe–S center of IRP1, it does not exhibit aconitase activity. This lack of enzymatic activity is not explained by insertion of the 73-amino acid sequence (Phillips *et al.,* 1996). Moreover, attempts at *in vitro* reconstitution of an Fe–S center in IRP2 were unsuccessful (Phillips *et al.,* 1996). Other studies have focused on the proper role of IRP2 in intracellular iron homeostasis of cells not expressing IRP1 (Schalinske *et al.,* 1997). Other potential peculiar roles include regulation of cellular iron in neurodegenerative disorders (Smith *et al.,* 1998) and c-MYC-dependent control of proliferation/transformation (Wu *et al.,* 1999). Intrinsic role of IRP2 was confirmed by studies with IRP1- and IRP2-deficient mice (T. Rouault, 1999, personal communication).

In contrast to IRP1, IRP2 is degraded in iron-replete cells (Guo *et al.,* 1994; Henderson and Kühn, 1995). This fast degradation depends on the presence of the aforementioned 73-amino acid sequence, which was termed the ''iron-dependent degradation domain'' (Iwai *et al.,* 1995). Three characteristics deserve attention:

1. The additional sequence contains five cysteines that are potential targets for redox reactions. Mutation of the three cysteines of the CX5CX3C motif to serines abolishes the iron-mediated fast degradation of the protein (Iwai *et al.,* 1995).
2. Degradation was prevented by specific proteasome inhibitors, thus suggesting that IRP2 is degraded by the 26S complex of the proteasome (Iwai *et al.,* 1995; Guo *et al.,* 1995).
3. IRP2 oxidation is a prerequisite for degradation. Many of these oxidized residues, most of which are represented in

the iron-dependent degradation domain, are carbonylated then ubiquitinated prior to degradation by the proteasome (Iwai *et al.,* 1998).

Sensitivity of IRPs to Oxygen and Oxygen Derivatives

It was long believed that activity/expression of IRPs was regulated only by intracellular iron fluxes. However, evidence indicates that physiological redox effectors also play an important part in IRP modulation. As mentioned above, [4Fe–4S] cluster-containing dehydratases, especially aconitases, are susceptible to environmental signals mostly because one of the iron atoms of the [4Fe–4S] cluster lacks a cysteine *S*-ligand. Even though it is less sensitive to oxygen than its mitochondrial counterpart (Beinert and Kennedy, 1993), IRP1 also reacts to variation in oxygen tension. It was first reported that IRP1 IRE-binding activity of rat hepatoma cells (Hanson and Leibold, 1998) and murine macrophages (Kuriyama-Matsumura *et al.,* 1998) decreased after exposure to hypoxia, probably through stabilization of the 4Fe–4S cluster. In the latter case, the ferritin level was found to be higher than under normoxic conditions. However, data obtained with a human hepatoma cell line were somewhat divergent: on hypoxia, IRE binding was higher, cytosolic aconitase activity was decreased, and ferritin level was reduced (Toth *et al.,* 1999). The effect of oxygen tension on IRP1 might therefore be cell specific.

Alternatively, another explanation can stem from the fact that the electromobility shift assay of IRP RNA-binding capacity does not discriminate between IRP1 and IRP2 in human cells, in contrast to mouse or rat cells. This may explain some discrepancies, because IRP2 expression and activity were later shown to increase under hypoxia in human cells (Hanson *et al.,* 1999). Therefore, hypoxia conversely regulates IRP1 and IRP2 (Hanson and Leibold, 1999). Accordingly, the net result of a change in oxygen tension on ferritin and transferrin expression will likely depend on which IRP predominates with respect to IRE binding. The response of cells to hypoxia provides one of the clues indicating that IRP1 and IRP2 are differentially regulated by environmental messages.

Modulation of IRP1 activities by oxidative stress is outside the main scope of this chapter, but a brief review is apposite. It has long been reported that bacterial $[4Fe–4S]^{2+}$-containing dehydratases including aconitase are very sensitive to oxygen-derived reactive species or hyperbaric conditions (Gardner and Fridovich, 1991; Flint *et al.,* 1993). There are also several data sets indicating that mammalian aconitases are sensitive to superoxide anion (O_2^-) (Hausladen and Fridovich, 1994; Castro *et al.,* 1994). As shown by Gardner *et al.* (1995), endogenously produced O_2^- inactivates mt-aco and cytosolic aconitase–IRP1 in various cell lines. Moreover, aconitases are also inhibited by O_2^- in cell-free systems. Thus, Hausladen and Fridovich (1994) reported that pure mt-aco and cytosolic aco–IRP1 are inactivated by a flux of O_2^- generated by the xanthine–xanthine oxidase reaction with a constant rate of $\sim 0.8 \times 10^7$ and $\sim 3 \times 10^7$ M^{-1} s^1, respectively. Whereas O_2^- directly inhibits the aconitase activity of IRP1, it does not activate its RNA-binding capacity. Data showed no significant increase in IRE binding of cell lysates in response to the xanthine oxidase-dependent O_2^--generating system (Bouton *et al.,* 1996) and to paraquat (Pantopoulos *et al.,* 1996). Downregulation was even reported after exposure of pure IRP1 to a flux of O_2^- (Cairo *et al.,* 1996) or treatment of cells by the oxidative stress inducer menadione (Gehring *et al.,* 1999). There is a debate as to whether oxygen-derived oxidants can directly destabilize the IRP1 cluster chemically or whether a more sophisticated signaling pathway is involved (Rouault and Klausner, 1996; Hentze, 1996). A clue may be given by studies showing that phosphoryation of serine-138, which is in the region required for RNA binding, makes the cluster of IRP1 more sensitive to oxidative or nitrosative stress (Brown *et al.,* 1998). In brief, much remains to be clarified if we are to delineate the molecular mechanism sustaining oxidant-dependent cluster disruption. Here we shall just draw attention to some pointers.

There is a consensual view that O_2^- reacts with ''cationic'' [4Fe–4S] clusters like IRP1's, but the resulting loss of enzymatic activity is not accompanied by a gain in RNA binding capacity. The most likely explanation is that reaction of O_2^- with IRP1 is limited to the conversion of the 4Fe form to the 3Fe form, which has no biological activity.

Hydrogen peroxide (H_2O_2) does not activate IRP1 binding activity directly (Bouton *et al.,* 1996; Pantopoulos and Hentze, 1995a; Brazzolotto *et al.,* 1999), but it induces a rapid activation of IRE binding capacity in intact cells when applied extracellularly (Martins *et al.,* 1995; Pantopoulos and Hentze, 1995a; Pantopoulos *et al.,* 1997). H_2O_2-dependent activation of IRP1 proceeds through a multistep signaling pathway that is phosphorylation-, temperature- and possibly energy-dependent (Pantopoulos and Hentze, 1998). Activation of IRP1 by H_2O_2 is accompanied by inhibition of ferritin synthesis and by stabilization of transferrin receptor mRNA. IRP2 activity does not seem to be modulated in response to H_2O_2 (Pantopoulos *et al.,* 1996).

NO and the so-called reactive oxygen species are sometimes all tarred with the same brush and are sometimes given the general term of ''oxidant'' or ''perturbant.'' Actually, each of these species has its own chemical reactivity, and they likely exert a specific effect on IRP functions.

Modulation of IRP1 by NO

The production of NO from L-arginine was the first biological pathway shown to conversely modulate the two activities of IRP1. This regulation was first observed in activated macrophages and then in other cell types expressing either constitutive or inducible NO synthase (iNOS, NOS-2) (Drapier *et al.,* 1993; Weiss *et al.,* 1993; Jaffrey *et al.,* 1994). It must be stressed that the stimuli that manage induction of NOS-2 in turn control IRP1 functions. γ-Interferon (IFN-γ) and tumor necrosis factor (TNF) upregulate IRE binding

(Drapier *et al.,* 1993; Weiss *et al.,* 1993; Bouton *et al.,* 1998), whereas the two Th2 cytokines interleukin 4 (IL-4) and IL-13 repress it (Weiss *et al.,* 1997). These data establish a connection between the cytokine network (and beyond, i.e., the cell-mediated immunity) and cellular iron homeostasis (Weiss *et al.,* 1995).

Two hypotheses have been advanced for the mechanism by which NO reacts with IRP1. First, NO reacts directly with IRP1, and more precisely its Fe–S center. This assumption was based on three sets of results: (a) the rapidity of the effect of NO both on a cell-free system (Bouton *et al.,* 1996) and on a reporter target cell withdrawn from the NO-generating cell monolayer (Bouton *et al.,* 1998); (b) the prevention of the NO-mediated increase in IRP1 IRE binding on addition of substrates (Bouton *et al.,* 1996); and (c) the appearance of an EPR $g = 2.04$ signal originating from a protein-bound dinitrosyl–iron–dithiol complex on exposure of pure mt-aco and IRP1 to NO (Kennedy *et al.,* 1997). Second, modulation by NO results from reduced intracellular iron pool availability, leading to cellular iron deficiency. In this case, NO would have a delayed and indirect effect on the modulation of IRP1 (Pantopoulos *et al.,* 1996).

NO, Quick or Slow?

One oft-debated question is how fast is NO? This question is central to progress in the understanding of the mechanism by which NO activates the RNA-binding capacity of IRP1. Kinetic arguments argue that NO exerts a slow and indirect effect on IRP1 (Pantopoulos *et al.,* 1996; Hentze and Kühn, 1996). As NO would activate IRP1 as slowly as desferrioxamine-mediated iron depletion, the proposed mechanism involves mobilization of iron by NO which would prevent assembly of the cluster into the neosynthesized IRP1. This hypothesis is supported by the fact that NO yields EPR-detectable complexes with iron within cells (Pellat *et al.,* 1990: Lancaster and Hibbs, 1990). The origin of iron bound to NO in such nitrosyl–iron complexes is ill-defined, but it appears that ferritin, the main iron storage protein, is not involved (P. Lipinski and J.-C. Drapier, 1997, unpublished). One possibility is that NO targets some ''transit'' and chelatable iron pool, which is believed to be a source of iron available for Fe–S cluster insertion. Two sets of results underpin this hypothesis:

1. IRP1 activation measured in murine primary macrophages, stimulated for endogenous NO production by a combination of IFN-γ and lipopolysaccharide (LPS), increased significantly up to 8 hours and leveled off at 12 hours (Drapier *et al.,* 1993).
2. Rat fibroblasts exposed *in vitro* to chemicals which release NO (''NO donors'') exhibited maximal activity after 8–12 hours (Pantopoulos *et al.,* 1996).

Before interpreting these results, several considerations should be kept in mind. In the first set of experiments, three different measurements were performed in parallel: cytosolic aconitase activity, IRE-binding activity, and NO production indirectly assessed by nitrite accumulation in culture medium. It is important to remember that NO produced by macrophages is synthesized by NOS-2, which is induced transcriptionally and thus requires time before it is able to produce NO. Care must therefore be taken not to include this delay in the time allotted to NO to activate IRP1. To avoid this bias, we performed coculture experiments by activating adherent macrophages for a steady-state production of NO and then covering macrophages with nonadherent target cells unable to produce NO. These cells were then withdrawn from the macrophage monolayer, and their cytosol was analyzed for IRE-binding capacity. Bouton *et al.* (1998) showed that IRE binding was significantly increased in target cells within 1 hour of contact with NO-producing cells (Fig. 6). We confirmed the rapidity of NO in a cell-free system in which cell lysates were exposed to the mixture 1,3-morpholino-sydnoniminehydrochloride/superoxide dismutase (SIN-1/SOD). Time-course experiments revealed that IRE binding was significantly increased after only 15 min (Bouton *et al.,* 1996; Oliveira *et al.,* 1999). Furthermore, when reduced thioredoxin was added to IRP1 previously exposed to SIN-1/SOD (see below), an increase in RNA binding was detected after only 5 min of exposure to the NO-generating system and was maximal after 30 min (Oliveira *et al.,* 1999).

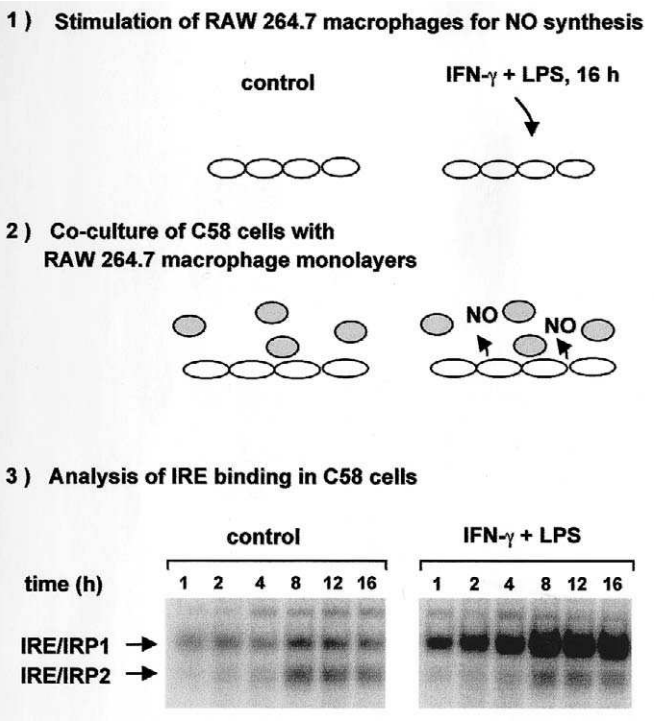


Figure 6 Rapid upregulation of IRP1 in target cells cocultured with NO-producing cells. Murine RAW 264.7 macrophages were left untreated (control) or stimulated with 10 U/ml IFN-γ and 50 ng/ml LPS for 16 hours. Macrophage monolayers were washed off, and C58 pre-T cells were added in a fresh medium at an effector-to-target ratio of 1 and were analyzed for IRE binding activity by EMSA. Adapted from Bouton *et al.* (1998) with permission.

With regard to the second set of experiments, cells were exposed to exogenous NO released from *S*-nitroso-*N*-acetylpenicillamine (SNAP), or 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (NOC-18) also termed DETA-NO. They both triggered RNA binding, but maximal activity was observed after several hours. However, the authors did not provide information about the rate of NO release under their experimental conditions, and this casts doubt on the efficacy of these chemicals at releasing NO quickly. Indeed, NOC-18 is a diazeniumdiolate held to be fairly stable, with a $t_{1/2} = 20$ hours at 37°C at pH 7.4 (Keefer *et al.,* 1996). SNAP was also successfully used to activate IRP1 in cells in culture (Pantopoulos *et al.,* 1996). SNAP is generally regarded as a fast NO releaser, but as a thionitrite its decomposition rate is highly dependent on the composition of the medium, and release of NO often relies on the presence of trace amounts of copper or iron (McAninly *et al.,* 1993). Therefore, its capacity to release NO may vary from one experiment to another.

Another caveat deserves attention: one of the arguments that supports the supposed slowness of NO is the need to take into account the duration necessary to obtain the maximal RNA binding activity. It may be more relevant to consider the time required to detect the first significant change. Indeed, the switch that allows conversion of the aconitase form (or a null form) to the form that accommodates an IRE is an all-or-nothing mechanism. Accordingly, it seems to us that the time needed for NO to completely achieve the biochemical/structural process which transforms a single molecule of IRP1/aconitase into an IRE-binding protein would be the most relevant parameter to take into account if it were possible to measure it. In brief, if we consider the time corresponding to the start of the rise of RNA binding rather than that of the plateau, NO is unequivocally fast. The lag required to obtain full IRE-binding activity, that is, to have all the molecules of IRP1 able to bind IRE, may be due to suboptimal experimental conditions. Also, it may be physiologically important not to convert every molecule of IRP1 into RNA-binding molecules ''in a flash.'' A milder effect may be more suitable with respect to cell physiology. It is worth recalling that maximal activity is routinely obtained by adding high concentrations of 2-Me to IRP1. Under these conditions, all forms of IRP1 are converted to RNA-binding forms. There is no reason to assume that a physiological effector like NO has to perform as well. It is probably safe for the cell to keep some of the IRP1 molecules in readiness at least part of the time, especially storage or dormant forms (e.g., the 3 Fe-IRP1) that are rapidly convertible back to aconitase.

Role of Thioredoxin

The notion that a cellular component was probably necessary for an optimal NO-mediated IRP1 activation emerged from the observation that, *in vitro,* activation of IRP1 by NO was far from maximal. Indeed, when cytosolic extracts are exposed to NO donors, IRP1 activation is consistently lower than that observed in living cells stimulated for NO synthesis. Moreover, treatment of purified recombinant IRP1 with NO donors or with NO gas (Drapier *et al.,* 1993; Bouton *et al.,* 1996) resulted in an even weaker IRP1 activation. In addition, as discussed above, several sets of *in vitro* studies have demonstrated the existence of oxidized IRP1, which, in the absence of a reducing environment, is biologically inactive. Altogether, these observations prompted us to investigate the role of endogenous reducers in the activation of IRP1 mediated by NO, particularly thioredoxin. Thioredoxin is a 12-kDa multifunctional protein that participates in redox reactions through dithiol–disulfide exchange reactions. Oxidized thioredoxin is reduced by NADPH and thioredoxin reductase. In its reduced form, thioredoxin has been implicated in the regulation of the DNA binding activity of several redox-sensitive transcription factors including NF-κB, activator protein 1 (AP-1) via reduction of Ref-1, glucocorticoid receptor, and heat shock factor-1 (Holmgren, 1985; Nakamura *et al.,* 1997).

Oliveira *et al.* (1999) showed that reduced thioredoxin strongly potentiated the IRE-binding activity of IRP1 in cytosolic extracts that had been concentrated on a membrane with an M_r cutoff of 30,000 and exposed to NO (Fig. 7). Optimal IRP1–IRE binding activity could also be observed by exposing NO-treated recombinant IRP1 to the thioredoxin system. Furthermore, we demonstrated that neutralization of endogenous thioredoxin with an anti-thioredoxin monoclonal antibody abolished the increase in RNA-binding activity of IRP1 in cytosolic extracts exposed to NO. These results point to endogenous thioredoxin as a cellular companion of NO in accelerating IRP1–IRE binding.

Modulation of IRP2 by NO

Whether or not IRP2 is modulated by NO and with what consequences is still controversial. The IRE-binding activity of IRP2 was increased in a B6 fibroblast cell line transfected to overexpress NOS-2 mRNA (Pantopoulos and Hentze, 1995b). On the other hand, in a rat hepatoma cell line, neither induction of NOS-2 by cytokines nor exposure of the cells to NO donors modulated IRP2 activity or expression (Phillips *et al.,* 1996). Moreover, two independent studies reported that expression of *trans*-regulating activity of IRP2 was decreased in macrophages stimulated *in vitro* by the combination of IFN-γ and LPS. In one case the authors claimed that the downregulation observed was mediated by NO synthesis (Recalcati *et al.,* 1998), whereas in the other case the authors concluded that it was not (Bouton *et al.,* 1998). However, the main point is that IRP2 is regulated differently than IRP1 in murine macrophages activated by IFN-γ and LPS. In brief, if IRP2 is regulated differently or even oppositely to IRP1, it could help balance IRP1-mediated control of iron metabolism.

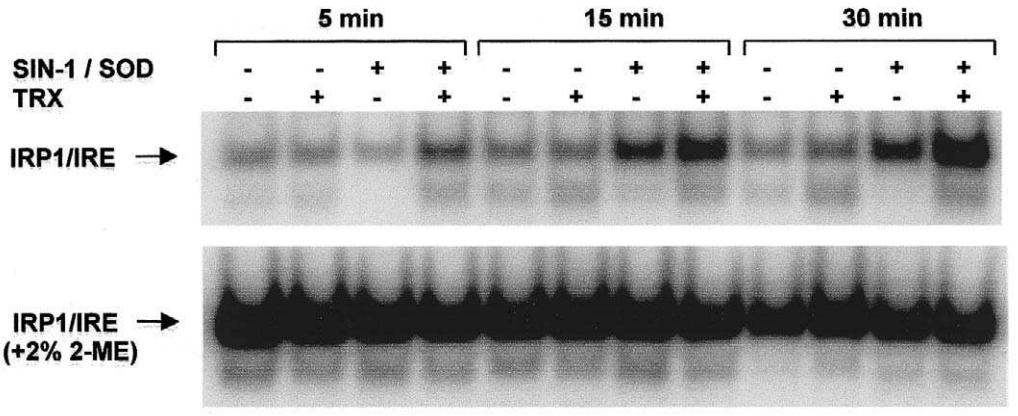


Figure 7 Cooperation between NO and thioredoxin in activating IRP1. Cytosolic extracts from RAW 264.7 cells were exposed to 5 m*M* SIN-1 in the presence of 3000 U/ml superoxide dismutase for the time indicated. After filtration on a P-6 Bio-Spin column, extracts were incubated with 5 μ*M* thioredoxin (TRX), 1 n*M* thioredoxin reductase, and 0.4 m*M* NADPH for 20 min prior to analysis by EMSA in the presence and absence of 2% 2-mercaptoethanol. Adapted from Oliveira *et al.* (1999) with permission.

Physiological Consequences of NO-Dependent Modulation of IRPs

Because IRP binding to IRE results in repression of ferritin translation and transferrin receptor mRNA stability, NO synthesis, by activating IRP1, was expected to decrease ferritin expression and increase transferrin receptor synthesis. Several lines of evidence indicate that ferritin translation is repressed in response to NO-mediated activation of IRP1. As shown by Weiss *et al.* (1993), ferritin H- and L-chains were downregulated in the J774 macrophage cell line stimulated for NO synthesis, whereas the ferritin mRNA level was unchanged. Furthermore, the same authors confirmed the regulation of ferritin translation by NO using a fibroblast cell line stably transformed to constitutively express NOS-2 (Pantopoulos and Hentze, 1995b). However, not all data agree with a NO-mediated reduction in ferritin expression. Indeed, exposure of K562 erythroleukemia cells to the NO-generator SNAP had no effect on the intracellular ferritin level, despite IRP1 activation (Oria *et al.,* 1995). Moreover, as reported by Recalcati *et al.* (1998), stimulation of J774 cells by cytokines and LPS induced an increase in ferritin synthesis. These conflicting results may be explained by the intrinsic, and at times predominant, role of IRP2, which, as mentioned above, is often regulated in an opposite way to IRP1.

At first glance, the situation regarding NO modulation of the IRP–IRE system and transferrin receptor expression is also somewhat puzzling. Cairo and Pietrangelo (1995) showed that during experimental inflammation, transferrin receptor mRNA content was increased in response to enhancement of NOS-2 expression. In several independent *in vitro* studies, it was shown that treatment of cells by SNAP increases IRP1 activity and transferrin receptor expression (Pantopoulos and Hentze, 1995b; Oria *et al.,* 1995; Richardson *et al.,* 1995). Moreover, in two of these studies, it was reported that sodium nitroprussiate, which releases an NO^+-like molecule, had no effect or even downregulated both IRP1 activity and transferrin receptor expression (Richardson *et al.,* 1995). Mechanistically, it makes sense that molecules with an NO^+ character are more efficient at reacting with thiolate than with iron. Actually, as long as the critical cysteines of IRP1 (Cys-437, Cys-503, and Cys-506) are bound to (and therefore shielded by) the Fe–S cluster, the compact structure of IRP1 is hardly reachable by IRE. Thus, depending on the redox environment, NO release may have different consequences for IRP1 activity and, in turn, for transferrin receptor expression.

As pointed out by two groups, stimulation of cells by IFN-γ and LPS lowers transferrin receptor mRNA levels, whether NO is produced or not (Phillips *et al.,* 1996; Pantopoulos and Hentze, 1995b). As cells transformed to overexpress NOS-2 exhibit a severalfold increase in transferrin receptor mRNA mediated by the IRP–IRE interaction (Pantopoulos and Hentze, 1995b), it was proposed that downregulation of transferrin receptor expression in response to IFN-γ and/or LPS exceeds the positive effect of NO. It is possible that despite activation of IRP1 (via NO), IFN-γ/LPS-mediated downregulation of IRP2 governs posttranscriptional regulation of transferrin receptor.

Regulation by Peroxynitrite

It is believed that peroxynitrite attacks the Fe–S clusters (Keyer and Imlay, 1997), and it was not surprising that IRP1 became a likely member of the fast-growing list of its targets. Hausladen and Fridovich (1994) first studied the effect of peroxynitrite on IRP1, and they reported inactivation of aconitase activity of pure IRP1 by bolus addition of peroxynitrite. The experiment was performed under special *in vitro* conditions (i.e., pH 9) in which peroxynitrite is relatively (and unphysiologically) stable. However, RNA-binding activity was not tested. Inactivation of mt-aco or cyt-aco/IRP1 by peroxynitrite was reported by other groups (Castro *et al.,*

1994; Bouton *et al.,* 1997; Kennedy *et al.,* 1997). There is a consensus view that exposure of aconitases (as crude preparation or pure) to bolus addition of peroxynitrite leads to loss of activity even though the efficacy of peroxynitrite is assessed differently. Indeed, as reported by Castro *et al.* (1994) peroxynitrite has a strong affinity for mt-aco ($k = 1.4 \times 10^{-5}$ $M^{-1}s^{-1}$). On the other hand, Kennedy *et al.* (1997) noted a relative resistance of both mt-aco and cytosolic aconitase/IRP1 to peroxynitrite.

With regard to the IRE-binding capacity of IRP1 on exposure of peroxynitrite, the situation was far from explicit. Whereas treatment of cells or cell lysates with NO donors (SNAP, NONOates, SIN1 plus SOD) increased IRE-binding activity of IRP1, treatments that produce peroxynitrite formation (SIN-1 alone, SNAP plus paraquat) did not (Bouton *et al.,* 1996; Pantopoulos and Hentze, 1995a; Richardson *et al.,* 1995). Radi and co-workers analyzed the effect of NO and peroxynitrite on the activity of mt-aco and on both aconitase and RNA-binding activities of IRP1 in intact fibroblasts. When intact cells were exposed to exogenous NO released from a diazeniumdiolate, hydroxy-2-oxo-3-(*N*-ethyl-3-aminoethyl)-3-ethyl-1-triazene (NOC-12), they exhibited lower aconitase activity and higher IRE-binding activity. The latter activity was significantly increased after a 30-min exposure to 1 μM/min NO and reached 100% after 4 hours. In the same set of experiments, peroxynitrite, either applied to cells as a bolus or endogenously produced by cells both exposed to NOC-12 and treated with antimycin A, also triggered an increase in RNA binding (Castro *et al.,* 1998).

Before addressing the question of whether (and how) such a powerful oxidant as peroxynitrite could modulate IRP1, it is worth recalling some important indicators. As mentioned above, for IRP1, one function precludes the other. This postulate wrongly led to the deduction that loss of aconitase activity of peroxynitrite-exposed IRP1 necessarily conferred a gain in RNA-binding activity. In fact, one of the clues to comprehending this issue is to realize that the ''one polypeptide, two mutually exclusive functions'' concept, as regards IRP1, is probably rather simplistic. Indeed at least *in vitro,* two other forms can also exist: a 3Fe-containing form and an oxidized ''apo'' form. None of these forms has any biological activity, and therefore they are barely detectable. Hence four forms can interconvert, of which only the two canonical forms, that is, the aconitase and the IRE binding protein, are generally detected. The two ''null'' forms may be used as storage proteins, which are rapidly convertible to biologically active forms (Toth and Bridges, 1995; Drapier and Bouton, 1996).

Another basic clue in delineating the role of redox active species like peroxynitrite on IRP1 is to admit that IRP1 binds IRE on reduction. This assumption, it is very important to recall, has been documented by many reports. Early studies determined that exposure of IRP1 to 2% 2-Me fully activated its IRE-binding activity (Hentze *et al.,* 1989). Above all, a series of careful studies showed that in cluster-free IRP1, Cys-437 is a reactive residue whose redox status is crucial for IRE binding (Philpott *et al.,* 1993; Hirling *et al.,* 1994). As mentioned previously, Hirling *et al.* (1994) provided evidence that Cys-503 in IRP1 lacking Cys-506 and Cys-506 in IRP1 lacking Cys-503 are linked to another cysteine by a disulfide bond, and they concluded that the Cys-437 was the only partner for the remaining cysteine. In short, it is important to draw attention to the fact that removal of the cluster from IRP1 is necessary but not sufficient to achieve RNA binding. A reducing environment is also crucial to prevent Cys-437 from forming a bridge with Cys-503 or Cys-506.

Taken together, these considerations led us to evaluate the effect of peroxynitrite on IRP1 IRE binding. We confirmed that peroxynitrite inhibits the enzymatic activity of IRP1 without degrading the protein. However, IRP1 did not gain RNA-binding activity (Bouton *et al.,* 1997). To solve this somewhat mystifying question, the mechanism of action of peroxynitrite on IRP1 was further studied *in vitro* by exposing IRP1 previously treated with peroxynitrite to suboptimal concentrations of 2-Me. It was thus concluded that not only does peroxynitrite disrupt the Fe–S center of IRP1, but it also promotes formation of an oxidized apo-IRP1 exhibiting neither aconitase activity nor *trans*-regulatory activity. Furthermore, site-directed mutagenesis showed that peroxynitrite allows formation of a disulfide bridge involving Cys-437 (Bouton *et al.,* 1997). In such an oxidized form, IRP1 has no biological activity. It was concluded that peroxynitrite may predispose IRP1 to bind IRE sequences only if the protein is then placed in a very slightly reducing environment. Such a narrow dependence on reducing systems may explain some paradoxical results reported in the literature.

It is known that IRP2 activity is lost on exposure to diamide or 5,5-dithiobis(2-nitrobenzoic acid), but can be recovered in the presence of 2-Me or DTT (Phillips *et al.,* 1996; Henderson and Kühn, 1995). The effect of peroxynitrite on its RNA-binding activity was studied by Bouton *et al.* (1997). RNA-binding capacity was lost in response to peroxynitrite, but the protein was not degraded, as shown by recovery of full activity in the presence of 2-Me. Thus, IRP2 can also be modulated posttranslationally in response to redox influence.

Aconitase Inhibition: The Respective Roles of NO and Peroxynitrite

The debate concerning modulation of aconitase and IRP1 activities relates to whether NO or one of its higher oxides inhibits enzymatic activity of aconitases in general and more specifically that of IRP1. Let us first consider the data.

In the mid 1980s, mt-aco activity loss was noted in cells activated to synthesize NO or in cells adjacent to NO-producing cells (Drapier and Hibbs, 1986, 1988). Ten years later, several independent studies showed that cells stimulated to produce NO or cytosolic extracts exposed to NO donors exhibited increased RNA-binding capacity and loss of cytosolic aconitase activity (Drapier *et al.,* 1993; Weiss *et al.,* 1993; Bouton *et al.,* 1996). In one of these reports, it was stated that pure NO gas diluted in buffer could inhibit the aconitase activity of purified recombinant IRP1 and increase

its RNA binding activity, thus pointing to a direct effect of NO (Drapier *et al.,* 1993). Two groups compared the effect of NO and peroxynitrite on aconitases *in vitro.* Castro *et al.* (1994) found that NO under anaerobic conditions moderately inhibited commercial porcine mitochondrial aconitase, whereas Hausladen and Fridovich (1994) did not find any effect of NO on either mt-aco or purified cytosolic aconitase/IRP1 enzymatic activity. Consequently, both groups concluded that peroxynitrite rather than NO was the molecule responsible for aconitase inhibition on NOS activation.

The issue was then addressed by Kennedy *et al.* (1997). They found that NO, either applied anaerobically as a buffered solution of NO gas or aerobically released from a precursor, was able to inactivate both mitochondrial and cytosolic aconitase/IRP1. Inactivation of both aconitases was accompanied by the formation of an EPR-detectable signal typical of dinitrosyl–iron–thiol complexes.

More recently, Gardner *et al.* (1997) reinvestigated the issue by exposing bacterial culture or mammalian mt-aco to a solution of NO gas. They found that moderate amounts of NO (120 ppm) applied to *Escherichia coli* culture under anaerobic conditions were sufficient to cause ~70% loss of aconitase activity in 2 hours. Furthermore, by using MnSOD-overexpressing or glutathione-deficient *E. coli* mutant strains, they determined that neither O_2^- nor peroxynitrite was necessary in NO-mediated inhibition of aconitase. In *in vitro* experiments under various conditions (pH, salts, etc.), they noted that enhancing ionic strength or the presence of *cis*-aconitate increased inhibition. Above all, they also reported that lowering pH favors inhibition. This feature may reconcile most, if not all, of the discrepancies mentioned above. Indeed, in Gardner's experiments, mt-aco exposed to NO at pH 7.5 was fairly resistant to inactivation, which is consistent with the data of Castro *et al.* (1994) and of Hausladen and Fridovich (1994) with mt-aco. However, when the experiments were performed at pH 6.5–6.8, activity was inhibited. In brief, according to Gardner *et al.* (1997), a few tenths of a pH unit are crucial to determine resistance or sensitivity of mt-aco to NO *in vitro.* This observation may likely be extrapolated to IRP1. Indeed, it is striking that the experiments of Kennedy *et al.* (1997) demonstrating inhibition of mammalian mt-aco and cytosolic aconitase/IRP1 by spermine-NONOate (sper-NO) were also conducted at pH 6.6. The prolonged debate about the sensivity of aconitases to NO may therefore simply be solved by careful examination and control of the buffering conditions. This precaution would be particularly suitable when NO is added to protein under not strictly anaerobic conditions, because NO and oxygen in aqueous solution can yield nitrous acid which may lower the pH of buffers.

Taken together, these data indicate that exposure of *intact* bacteria or mammalian cells to NO applied as gas in buffered solution or released from NO synthase or chemicals, *does* lead to aconitase inhibition (Drapier and Hibbs, 1996; Gardner *et al.,* 1997; Castro *et al.,* 1998). At this juncture, it is important to bear in mind that under such experimental conditions (which approximate the physiological situation), the efficacy of NO has not been contested. The situation is more debatable with regard to the *in vitro* experiments. Gardner's data provided a valuable clue to better understanding of the issue by revealing the sharp pH dependence of NO in its inhibition of aconitases (Gardner *et al.,* 1997). Yet the molecular mechanism explaining why aconitases would be more sensitive to NO at slightly acidic pH and possibly in the presence of *cis*-aconitate is still a matter of speculation. It has been proposed that a change of active site conformation following ionization of the side chains of the active site amino acid or an alteration of the ligand field of Fe_a may affect NO reactivity (Gardner *et al.,* 1997).

Concluding Remarks

In the NO versus IRP play, the cast is auspicious for at least two reasons: (1) physiologically, NO, at least produced by the cytokine-induced NOS-2, can opportunely appear and ambush at the juncture between innate immunity and cellular iron metabolism, and (2) biochemically, NO is a reactive messenger that faces two very receptive proteins. IRP1 possesses a watchful Fe–S cluster that senses and sorts the chemical signals. Several lines of evidence indicate that NO quickly reacts with IRP1. As expected from the Lewis acid character of Fe_a, NO probably interacts with the Fe–S cluster. Indeed, it leaves a footprint, that is, an EPR-detectable nitrosyl–iron complex. Yet, to complete binding to IRE sequences, it relies on an endogenous reducing system. Thioredoxin is a likely partner in this regard. Peroxynitrite is fast at inactivating the aconitase activity of IRP1, but, as a potent oxidant, it would hardly activate IRE binding because it oxidizes Cys-437.

Peroxynitrite-exposed IRP1 would accommodate IRE in the situation where a reducing system (thioredoxin again?) would intervene once peroxynitrite has vanished. IRP2 is also sensitive to oxidoreduction. It has no Fe–S cluster, but several cysteines are likely to be S-nitrosylated or oxidized. Consequently, dissecting the mechanisms that underlie the modifications of IRPs by NO and accomplices such as peroxynitrite is a worthwhile objective in striving to improve knowledge of the actions of NO.

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References

Aziz, N., and Munro, H. N. (1987). Iron regulates ferritin mRNA translation through a segment of its 5' untranslated region. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 8478–8482.

Basilion, J. P., Rouault, T. A., Massinople, C. M., Klausner, R. D., and Burgess, W. H. (1994). The iron-responsive element-binding protein: Localization of the RNA-binding site to the aconitase active-site cleft. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 574–578.

Beinert, H., and Kennedy, M. C. (1989). 19th Sir Hans Krebs lecture. Engineering of protein bound iron-sulfur clusters. A tool for the study of protein and cluster chemistry and mechanism of iron-sulfur enzymes. *Eur. J. Biochem.* **186,** 5–15.

Beinert, H., and Kennedy, M. C. (1993). Aconitase, a two-faced protein: Enzyme and iron regulatory factor. *FASEB J.* **7,** 1442–1449.

Bouton, C. (1999). Nitrosative and oxidative modulation of iron regulatory proteins. *Cell Mol. Life Sci.* **55,** 1043–1053.

Bouton, C., Raveau, M., and Drapier, J. C. (1996). Modulation of iron regulatory protein functions. Further insights into the role of nitrogen- and oxygen-derived reactive species. *J. Biol. Chem.* **271,** 2300–2306.

Bouton, C., Hirling, H., and Drapier, J. C. (1997). Redox modulation of iron regulatory proteins by peroxynitrite. *J. Biol. Chem.* **272,** 19969–19975.

Bouton, C., Oliveira, L., and Drapier, J. C. (1998). Converse modulation of IRP1 and IRP2 by immunological stimuli in murine RAW 264.7 macrophages. *J. Biol. Chem.* **273,** 9403–9408.

Brazzolotto, X., Gaillard, J., Pantopoulos, K., Hentze, M. W., and Moulis, J. M. (1999). Human cytoplasmic aconitase (Iron regulatory protein 1) is converted into its [3Fe-4S] form by hydrogen peroxide in vitro but is not activated for iron-responsive element binding. *J. Biol. Chem.* **274,** 21625–21630.

Broillet, M. C. (1999). S-nitrosylation of proteins. *Cell Mol. Life Sci.* **55,** 1036–1042.

Brown, N. M., Anderson, S. A., Steffen, D. W., Carpenter, T. B., Kennedy, M. C., Walden, W. E., and Eisenstein, R. S. (1998). Novel role of phosphorylation in Fe-S cluster stability revealed by phosphomimetic mutations at Ser-138 of iron regulatory protein 1. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15235–15240.

Cairo, G., and Pietrangelo, A. (1995). Nitric-oxide-mediated activation of iron-regulatory protein controls hepatic iron metabolism during acute inflammation. *Eur. J. Biochem.* **232,** 358–363.

Cairo, G., Castrusini, E., Minotti, G., and Bernelli-Zazzera, A. (1996). Superoxide and hydrogen peroxide-dependent inhibition of iron regulatory protein activity: A protective stratagem against oxidative injury. *FASEB J.* **10,** 1326–1335.

Castro, L., Rodriguez, M., and Radi, R. (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.* **269,** 29409–29415.

Castro, L. A., Robalinho, R. L., Cayota, A., Meneghini, R., and Radi, R. (1998). Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. *Arch. Biochem. Biophys.* **359,** 215–224.

Dandekar, T., Stripecke, R., Gray, N. K., Goossen, B., Constable, A., Johansson, H. E., and Hentze, M. W. (1991). Identification of a novel iron-responsive element in murine and human erythroid δ-aminolevulinic acid synthase mRNA. *EMBO J.* **10,** 1903–1909.

Drapier, J. C. (1997). Interplay between NO and [Fe-S] clusters: Relevance to biological systems. *Methods* (*San Diego*) **11,** 319–329.

Drapier, J. C., and Bouton, C. (1996). Modulation by nitric oxide of metalloprotein regulatory activities. *BioEssays* **18,** 549–556.

Drapier, J. C., and Hibbs, J. B., Jr. (1986). Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin. Invest.* **78,** 790–797.

Drapier, J. C., and Hibbs, J. B., Jr. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in sml-nmarginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J. Immunol.* **140,** 2829–2838.

Drapier, J. C., and Hibbs, J. B., Jr. (1996). Aconitases: A class of metalloproteins highly sensitive to nitric oxide synthesis. *Meth. Enzymol.* **269,** 26–36.

Drapier, J. C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kuhn, L. C. (1993). Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *EMBO J.* **12,** 3643–3649.

Fansler, B., and Lowenstein, J. M. (1969). Aconitase from pig heart. *Meth. Enzymol.* **13,** 26–30.

Flint, D. H., Smyk-Randall, E., Tuminello, J. F., Draczynska-Lusiak, B., and Brown, O. R. (1993). The inactivation of dihydroxy-acid dehydratase in *Escherichia coli* treated with hyperbaric oxygen occurs because of the destruction of its Fe-S cluster, but the enzyme remains in the cell in a form that can be reactivated. *J. Biol. Chem.* **268,** 25547–25552.

Frishman, D., and Hentze, M. W. (1996). Conservation of aconitase residues revealed by multiple sequence analysis. Implications for structure/function relationships. *Eur. J. Biochem.* **239,** 197–200.

Gardner, P. R., and Fridovich, I. (1991). Superoxide sensitivity of the *Escherichia coli* aconitase. *J. Biol. Chem.* **266,** 19328–19333.

Gardner, P. R., Raineri, I., Epstein, L. B., and White, C. W. (1995). Superoxide radical and iron modulate aconitase activity in mammalian cells. *J. Biol. Chem.* **270,** 13399–13405.

Gardner, P. R., Costantino, G., Szabo, C., and Salzman, A. L. (1997). Nitric oxide sensitivity of the aconitases. *J. Biol. Chem.* **272,** 25071–25076.

Gehring, N. H., Hentze, M. W., and Pantopoulos, K. (1999). Inactivation of both RNA binding and aconitase activities of iron regulatory protein-1 by quinone-induced oxidative stress. *J. Biol. Chem.* **274,** 6219–6225.

Gray, N. K., and Hentze, M. W. (1994). Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J.* **13,** 3882–3891.

Gray, N. K., Pantopoulous, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996). Translational regulation of mammalian and *Drosophila* citric acid cycle enzymes via iron-responsive elements. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4925–4930.

Gruer, M. J., Artymiuk, P. J., and Guest, J. R. (1997). The aconitase family: Three structural variations on a common theme. *Trends Biochem. Sci.* **22,** 3–6.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., and Hediger, M. A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388,** 482–488.

Guo, B., Yu, Y., and Leibold E. A. (1994). Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity. *J. Biol. Chem.* **269,** 24252–24260.

Guo, B., Brown, F. M., Phillips, J. D., Yu, Y., and Leibold E. A. (1995). Characterization and expression of iron regulatory protein 2 (IRP2): Presence of multiple IRP2 transcripts regulated by intracellular iron levels. *J. Biol. Chem.* **270,** 16529–16535.

Haile, D. J., Rouault, T. A., Harford, J. B., Kennedy, M. C., Blondin, G. A., Beinert, H., and Klausner, R. D. (1992). Cellular regulation of the iron-responsive element binding protein: Disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11735–11739.

Hanson, E. S., and Leibold, E. A. (1998). Regulation of iron regulatory protein 1 during hypoxia and hypoxia/reoxygenation. *J. Biol. Chem.* **273,** 7588–7593.

Hanson, E. S., and Leibold, E. A. (1999). Regulation of the iron regulatory proteins by reactive nitrogen and oxygen species *Gene Express.* **7,** 367–376.

Hanson, E. S., Foot, L. M., and Leibold, E. A. (1999). Hypoxia post-translationally activates iron-regulatory protein 2. *J. Biol. Chem.* **274,** 5047–5052.

Hausladen, A., and Fridovich, I. (1994). Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.* **269,** 29405–29408.

Henderson, B. R. (1996). Iron regulatory proteins 1 and 2. *BioEssays* **18,** 739–746.

Henderson, B. R., and Kühn, L. C. (1995). Differential modulation of the RNA-binding proteins IRP-1 and IRP-2 in response to iron. IRP-2 inactivation requires translation of another protein. *J. Biol. Chem.* **270,** 20509–20515.

Henderson, B. R., Seiser, C., and Kühn, L. C. (1993). Characterization of a second RNA-binding protein in rodents with specificity for iron-responsive elements. *J. Biol. Chem.* **268,** 27327–27334.

Hentze, M. W. (1996). Iron-sulfur clusters and oxidant stress responses. *Trends Biochem. Sci.* **8,** 282–283.

Hentze, M. W., and Argos, P. (1991). Homology between IRE-BP, a regulatory RNA-binding protein, aconitase, and isopropylmalate isomerase. *Nucleic Acids Res.* **19,** 1739–1740.

Hentze, M. W., and Kühn, L. C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8175–8182.

Hentze, M. W., Rouault T. A., Caughman, S. W., Dancis, A., Harford, J. B., and Klausner, R. D. (1987). A cis-acting element is necessary and sufficient for translational regulation of human ferritin expression in response to iron. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 6730–6734.

Hentze, M. W., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1989). Oxidation–reduction and the molecular mechanism of a regulatory RNA–protein interaction. *Science* **244,** 357–359.

Hirling, H., Henderson, B. R., and Kuhn, L. C. (1994). Mutational analysis of the [4Fe-4S]-cluster converting iron regulatory factor from its RNA-binding form to cytoplasmic aconitase. *EMBO J.* **13,** 453–461.

Holmgren, A. (1985). Thioredoxin. *Annu. Rev. Biochem.* **54,** 237–271.

Iwai, K., Klausner, R. D., and Rouault, T. A. (1995). Requirements for iron-regulated degradation of the RNA binding protein, iron regulatory protein 2. *EMBO J.* **14,** 5350–5357.

Iwai, K., Drake, S. K., Wehr, N. B., Weissman, A. M., LaVaute, T., Minato, N., Klausner, R. D., Levine, R. L., and Rouault, T. A. (1998). Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: Implications for degradation of oxidized proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 4924–4928.

Jaffrey, S. R., Cohen, N. A., Rouault, T. A., Klausner, R. D., and Snyder, S. H. (1994). The iron-responsive element binding protein: A target for synaptic actions of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12994–12998.

Kaptain, S., Downey, W. E., Tang, C., Philpott, C., Haile, D., Orloff, D. G., Harford, J. B., Rouault, T. A., and Klausner, R. D. (1991). A regulated RNA binding protein also possesses aconitase activity. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10109–10113.

Keefer, L. K., Nims, R. W., Davies, K. M., and Wink, D. A. (1996). ''NONOates'' (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: Convenient nitric oxide dosage forms. *Meth. Enzymol.* **268,** 281–293.

Kennedy, M. C., Mende-Mueller, L., Blondin, G. A., and Beinert, H. (1992). Purification and characterization of cytosolic aconitase from beef liver and its relationship to the iron-responsive element binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11730–11734.

Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997). An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. *J. Biol. Chem.* **272,** 20340–20347.

Keyer, K., and Imlay, J. A. (1997). Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J. Biol. Chem.* **272,** 27652–27659.

Kim, H. Y., Klausner, R. D., and Rouault, T. A. (1995). Translational repressor activity is equivalent and is quantitatively predicted by in vitro RNA binding for two iron-responsive element-binding proteins, IRP1 and IRP2. *J. Biol. Chem.* **270,** 4983–4986.

Kim, H. Y., LaVaute, T., Iwai, K., Klausner, R. D., and Rouault, T. A. (1996). Identification of a conserved and functional iron-responsive element in the 5'-untranslated region of mammalian mitochondrial aconitase. *J. Biol. Chem.* **271,** 24226–24230.

Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999). The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J.* **18,** 3981–3989.

Klausner, R. D., and Rouault, T. A. (1993). A double life: Cytosolic aconitase as a regulatory RNA binding protein. *Mol. Biol. Cell* **4,** 1–5.

Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993). Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell* **72,** 19–28.

Kohler, S. A., Henderson, B. R., and Kuhn, L. C. (1995). Succinate dehydrogenase b mRNA of *Drosophila melanogaster* has a functional iron-responsive element in its 5'-untranslated region. *J. Biol. Chem.* **270,** 30781–30786.

Kühn, L. C. (1998). Iron and gene expression: Molecular mechanisms regulating cellular iron homeostasis. *Nutr. Rev.* **56,** s11-9; discussion s54-75.

Kuriyama-Matsumura, K., Sato, H., Yamaguchi, M., and Bannai, S. (1998). Regulation of ferritin synthesis and iron regulatory protein 1 by oxygen in mouse peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **249,** 241–246.

Lancaster, J. R., Jr., and Hibbs, J. B., Jr. (1990). EPR demonstration of iron–nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1223–1227.

Land, T., and Rouault, T. A. (1998). Targeting of a human iron-sulfur cluster assembly enzyme, nifs, to different subcellular compartments is regulated through alternative AUG utilization. *Mol. Cell* **2,** 807–815.

Lauble, H., Kennedy, M. C., Beinert, H., and Stout, C. D. (1992). Crystal structures of aconitase with isocitrate and nitroisocitrate bound. *Biochemistry* **31,** 2735–2748.

Leibold, E. A., and Munro, H. N. (1988). Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2171–2175.

McAninly, D., Williams, D. L. H., Askew, S. C., Butler, A. R., and Russell, C. (1993). Metal ion catalysis in nitrosothiol (RSNO) decomposition. *J. Chem. Soc. Chem. Commun.,* 1758–1759.

Martins, E. A. L., Robalinho, R. L., and Meneghini, R. (1995). Oxidative stress induces activation of a cytosolic protein responsible for control of iron uptake. *Arch. Biochem. Biophys.* **316,** 128–134.

Meleförs, O. (1996). Translational regulation in vivo of the *Drosophila melanogaster* mRNA encoding succinate dehydrogenase iron protein via iron responsive elements. *Biochem. Biophys. Res. Commun.* **221,** 437–441.

Müllner, E. W., Neupert, B., and Kühn, L. C. (1989). A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. *Cell* **58,** 373–382.

Nakamura, H., Nakamura, K., and Yodoi, J. (1997). Redox regulation of cellular activation. *Annu. Rev. Immunol.* **15,** 351–369.

Oliveira, L., Bouton, C., and Drapier, J. C. (1999). Thioredoxin activation of iron regulatory proteins. Redox regulation of RNA binding after exposure to nitric oxide. *J. Biol. Chem.* **274,** 516–521.

Oria, R., Sanchez, L., Houston, T., Hentze, M. W., Liew, F. Y., and Brock, J. H. (1995). Effect of nitric oxide on expression of transferrin receptor and ferritin and on cellular iron metabolism in K562 human erythroleukemia cells. *Blood* **85,** 2962–2966.

Pantopoulos, K., and Hentze, M. W. (1995a). Rapid responses to oxidative stress mediated by iron regulatory protein. *EMBO J.* **14,** 2917–2924.

Pantopoulos, K., and Hentze, M. W. (1995b). Nitric oxide signaling to iron-regulatory protein: Direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1267–1271.

Pantopoulos, K., and Hentze, M. W. (1998). Activation of iron regulatory protein-1 by oxidative stress *in vitro. Proc. Natl. Acad. Sci. U.S.A.* **95,** 10559–10563.

Pantopoulos, K., Weiss, G., and Hentze, M. W. (1996). Nitric oxide and oxidative stress (H_2O_2) control mammalian iron metabolism by different pathways. *Mol. Cell. Biol.* **16,** 3781–3788.

Pantopoulos, K., Mueller, S., Atzberger, A., Ansorge, W., Stremmel, W., and Hentze, M. W. (1997). Differences in the regulation of iron regulatory protein-1 (IRP-1) by extra- and intracellular oxidative stress. *J. Biol. Chem.* **272,** 9802–9808.

Pellat, C., Henry, Y., and Drapier, J. C. (1990). IFN-γ-activated macrophages: Detection by electron paramagnetic resonance of complexes between sml-nmarginine-derived nitric oxide and non-heme iron proteins. *Biochem. Biophys. Res. Commun.* **166,** 119–125.

Phillips, J. D., Guo, B., Yang, Y., Brown, F. M., and Leibold, E. A. (1996). Expression and biochemical characterization of iron regulatory proteins 1 and 2 in *Saccharomyces cerevisiae. Biochemistry* **35,** 15704–15714.

Philpott, C. C., Haile, D., Rouault, T. A., and Klausner, R. D. (1993). Modification of a free Fe-S cluster cysteine residue in the active iron-responsive element-binding protein prevents RNA binding. *J. Biol. Chem.* **268,** 17655–17658.

Recalcati, S., Taramelli, D., Conte, D., and Cairo, G. (1998). Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: Role of selective iron regulatory protein-2 down-regulation. *Blood* **91,** 1059–1066.

Richardson, D. R., Neumannova, V., Nagy, E., and Ponka, P. (1995). The effect of redox-related species of nitrogen monoxide on transferrin and iron uptake and cellular proliferation of erythroleukemia (K562) cells. *Blood* **86,** 3211–3219.

Robbins, A. H., and Stout, C. D. (1989). The structure of aconitase. *Proteins* **5,** 289–312.

Rouault, T. A., and Klausner, R. D. (1996). Iron-sulfur clusters as biosensors of oxidants and iron [published erratum appears in *Trends Biochem. Sci.* (1996) July; **21**(7); 246]. *Trends Biochem. Sci.* **21,** 174–177.

Rouault, T., and Klausner, R. (1997). Regulation of iron metabolism in eukaryotes. *Curr. Top. Cell Regul.* **35,** 1–19.

Rouault, T. A., Stout, C. D., Kaptain, S., Harford, J. B., and Klausner, R. D. (1991). Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase: Functional implications. *Cell* **64,** 881–883.

Samaniego, F., Chin, J., Iwai, K., Rouault, T. A., and Klausner, R. D. (1994). Molecular characterization of a second iron-responsive element binding protein, iron regulatory protein 2. Structure, function, and post-translational regulation. *J. Biol. Chem.* **269,** 30904–30910.

Schalinske, K. L., Blemings, K. P., Steffen, D. W., Chen, O. S., and Eisenstein, R. S. (1997). Iron regulatory protein 1 is not required for the modulation of ferritin and transferrin receptor expression by iron in a murine pro-B lymphocyte cell line. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10681–10686.

Smith, M. A., Wehr, K., Harris, P. L. R., Siedlak, S. L., Connor, J. R., and Perry, G. (1998). Abnormal localization of iron regulatory protein in Alzheimer's disease. *Brain Res.* **788,** 232–236.

Stamler, J. S. (1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78,** 931–936.

Theil, E. C. (1998). The iron responsive element (IRE) family of mRNA regulators. Regulation of iron transport and uptake compared in animals, plants, and microorganisms. *Met. Ions Biol. Syst.* **35,** 403–434.

Toth, I., and Bridges, K. R. (1995). Ascorbic acid enhances ferritin mRNA translation by an IRP/aconitase switch. *J. Biol. Chem.* **270,** 19540–19544.

Toth, I., Yuan, L., Rogers, J. T., Boyce, H., and Bridges, K. R. (1999). Hypoxia alters iron-regulatory protein-1 binding capacity and modulates cellular iron homeostasis in human hepatoma and erythroleukemia cells. *J. Biol. Chem.* **274,** 4467–4473.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., and Hentze, M. W. (1993). Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *EMBO J.* **12,** 3651–3657.

Weiss, G., Wachter, H., and Fuchs, D. (1995). Linkage of cell-mediated immunity to iron metabolism. *Immunol. Today* **16,** 495–500.

Weiss, G., Bogdan, C., and Hentze, M. W. (1997). Pathways for the regulation of macrophage iron metabolism by the anti-inflammatory cytokines IL-4 and IL-13. *J. Immunol.* **158,** 420–425.

Wu, K. J., Polack, A., and Dalla-Favera, R. (1999). Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC. *Science* **283,** 676–679.

Zheng, L., Kennedy, M. C., Blondin, G. A., Beinert, H., and Zalkin, H. (1992). Binding of cytosolic aconitase to the iron responsive element of porcine mitochondrial aconitase mRNA. *Arch. Biochem. Biophys.* **299,** 356–360.

Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993). Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2754–2758.

CHAPTER 21

Nitric Oxide, Oxidative Stress, and Signal Transduction

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WHEREAS THE PARTICIPATION OF NITRIC OXIDE (NO), A FREE RADICAL, IN SIGNAL TRANSDUCTION IS WELL ESTABLISHED, THE ROLES OF SUPEROXIDE ($O_2^{\cdot-}$), HYDROGEN PEROXIDE (H_2O_2), AND PEROXYNITRITE ($ONOO^-$) ARE LESS WELL UNDERSTOOD. THIS CHAPTER DESCRIBES THE PROPERTIES OF SECOND MESSENGERS AND HOW THESE OXIDANTS FIT INTO THIS ESSENTIAL ROLE IN SIGNAL TRANSDUCTION. AN IMPORTANT ASPECT OF THIS DISCUSSION IS THE CAVEATS THAT MUST BE CONSIDERED, INCLUDING AN EXAMINATION OF THE USE OF ANTIOXIDANTS. THE EVIDENCE FOR INVOLVEMENT OF OXIDANTS IN SIGNALING IS SUBSTANTIAL, AND THIS CHAPTER DESCRIBES THE VARIOUS SIGNALING PATHWAYS TRIGGERED BY OXIDANTS. TRANSIENT CHANGES IN INTRACELLULAR FREE CALCIUM CONCENTRATION, ACTIVATION OF PHOSPHOLIPASES, AND MODULATION OF PROTEIN KINASE C (PKC), THE MITOGEN-ACTIVATED PROTEIN KINASES, AND PROTEIN TYROSINE PHOSPHATASES ARE EXAMINED. BOTH THE ESSENTIAL ELEMENTS IN THE SIGNALING PATHWAYS AND THE SITE WHERE OXIDANTS MAY ACT ARE DESCRIBED. THE MAJOR ROLE OF PROTEIN CYSTEINE RESIDUES AS SITES FOR INTERACTION WITH OXIDANTS IN TRANSMISSION OF SIGNALING IS EXAMINED. FINALLY, THE TRANSCRIPTION FACTORS AND *CIS*-ACTING ELEMENTS THAT RESPOND TO OXIDANT-INDUCED SIGNALING OF GENE EXPRESSION ARE BRIEFLY EXAMINED. THE PICTURE THAT EMERGES IS SOMEWHAT FUZZY BUT IS BECOMING INCREASINGLY CLEAR.

General Introduction

Free radicals and related oxidizing species, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), were long considered to be agents of cell and tissue damage only through random oxidation of tissue components. Even where these reactive species had been demonstrated to have a beneficial role in biology, such as in the microbicidal action of phagocytes, their action was ultimately destructive. This chapter describes some of the chemistry involved in the destructive aspect of oxidative stress; however, the main focus is on the role of reactive oxygen and nitrogen species in signal transduction.

A Brief Review of Reactive Oxygen and Nitrogen Biochemistry

Before the role of reactive oxygen and nitrogen species in signaling pathways can be discussed, it is important to understand how these molecules are generated in cells, some of their chemical properties, and the nonenzymatic reactions that produce cellular damage. More than 95% of the O_2 consumption of tissues is the result of its reduction to H_2O by cytochrome oxidase in mitochondria, a reaction that does not release any intermediates. The remaining consumption of O_2 involves univalent and divalent reduction in which the reactive oxygen species superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide are produced, respectively:

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$$O_2 + e^- \rightarrow O_2^{\cdot -} \quad (1)$$

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \quad (2)$$

The univalent reduction of O_2 [reaction (1)] results in the formation of $O_2^{\cdot -}$ and occurs under various conditions: the respiratory burst NAD(P)H oxidase in phagocytes and related oxidases (see later); leak of electrons from the mitochondrial electron transport chain, which is most likely due to autooxidation of ubisemiquinone and is probably the largest source of superoxide generation in nonphagocytosing cells; reactions of some monooxygenases; reactions catalyzed by dehydrogenases (when ''leaking'' electrons to oxygen); and metal-catalyzed autooxidation of small molecules and oxyhemoglobin. Superoxide is both a reducing and oxidizing agent but is not a particularly reactive species with organic compounds; however, it does react with iron–sulfur centers, such as in aconitase, which it inactivates. At acidic pH, superoxide is protonated to form hydroperoxyl ($HO_2\cdot$), and it is more reactive with membrane lipids because of its greater lipophilicity and reactivity. Most of the ''danger'' from superoxide appears to come from species derived from it.

The divalent reduction of oxygen yields the nonradical species hydrogen peroxide [reaction (2)]. Sources of hydrogen peroxide that catalyze such divalent reduction are monooxygenases, namely, glucose oxidase or D-amino acid oxidase. Some monooxygenases release both superoxide and hydrogen peroxide. Another major source of hydrogen peroxide is the dismutation of superoxide:

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Dismutation occurs nonenzymatically and is rapid ($\sim 10^5$ $mol^{-1}\ s^{-1}$) at physiological pH, but it is markedly accelerated by the superoxide dismutases ($>10^9$ $mol^{-1}\ s^{-1}$). Hydrogen peroxide is fully protonated at physiological pH, has a relatively long half-life, is membrane permeable, and may cause damage at sites distant from its origin. Iron bound to proteins may be reduced by superoxide, which may simultaneously cause release of the metal, enhancing its reactivity [reaction (4a)]. In the presence of reduced transition metals, such as iron and copper, hydrogen peroxide is reduced to hydroxyl radical ($\cdot OH$) and hydroxyl ion (OH^-), a reaction referred to as the Haber–Weiss reaction or the superoxide-driven Fenton reaction [reaction (4b)]:

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (4a)$$

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+} \quad (4b)$$

Hydroxyl radical is extremely reactive and will react with any organic molecule, with the rate of reaction near diffusion limitation. Therefore, it modifies molecules immediately adjacent to its site of generation, usually by hydrogen abstraction. This can result in a chain reaction, such as lipid peroxidation:

$$LH + \cdot OH \rightarrow L\cdot + H_2O \quad (5)$$

$$L\cdot + O_2 \rightarrow LO_2^{\cdot} \quad (6)$$

$$LO_2^{\cdot} + LH \rightarrow LOOH + L\cdot \quad (7)$$

where LH is an unsaturated fatty acid.

Much of the toxicity of superoxide may be mediated through its reaction with nitric oxide (nitrogen monoxide, NO), the main subject of this book, the chemistry of which is covered far more extensively in other chapters. Peroxynitrite ($ONOO^-$) is formed very rapidly (6.7×10^9 $mol^{-1}\ s^{-1}$) [reaction (8)]:

$$O_2^{\cdot -} + \cdot NO \rightarrow ONOO^- \quad (8)$$

Like superoxide, peroxynitrite is not a particularly reactive species, whereas the protonated form, peroxynitrous acid, is unstable and has the potential to react with organic molecules with chemistry similar to both nitrogen dioxide ($NO_2\cdot$) and hydroxyl radical. Activated macrophages, which can produce both superoxide and nitric oxide, can use peroxynitrite formation to destroy harmful organisms, and reaction (8) may play a role in chronic inflammation and tissue injury as well. The physiological actions of nitric oxide in vasodilation and neurotransmission are well established and are among the clearest examples of a role for free radicals in normal physiology. These physiological effects are largely mediated through the activation of guanylate cyclase by nitric oxide. The formation of and signaling by nitric oxide are extensively described in other chapters. Thus, here we will only address the potential participation of peroxynitrite in oxidative signaling.

General Concepts of Signal Transduction

Signal transduction is a regulated sequence of biochemical steps through which a stimulant conveys a message resulting in a physiological response. The key words here are ''regulated'' and ''biochemical steps.'' In signal transduction, regulation usually involves transient changes in the steady-state concentrations of metabolites or ions and phosphorylation of proteins. With few exceptions, most of these changes involve the rapid ''turning on'' and then ''turning off'' of enzymatic or ion channel activities. This, however, does not require the process to be a reversible reaction, as the return to the previous state can be through a different pathway. Examples of this rapid cycling are seen in the free intracellular calcium ion concentration, which increases severalfold for a few seconds to minutes, the phosphorylation of protein kinases that are then dephosphorylated, and the transient 10-fold elevation in the concentration of cyclic AMP (cAMP) that lasts less than a minute. The duration and extent of such changes vary with stimulant and cell type. The biochemical steps involved in signal transduction are mostly enzyme-catalyzed reactions that are regulated by either allosteric modulation due to binding of small molecules (second messengers, such as cAMP) or to reversible phosphorylation.

Signal transduction cascades begin with the binding of an agonist (stimulant) to its receptor protein. The largest class of receptors are the cell surface transmembrane proteins, with binding domains for the agonist on the outside of the cell and transducing domains on the inside. Nonetheless, there are numerous receptors also found in the cytosol (i.e.,

steroid hormone receptors) and on other membranes [i.e., the inositol-1,3,4-trisphosphate (IP_3) receptor on the endoplasmic reticulum]. Figure 1 shows the general scheme for signaling through a type of membrane receptor that is coupled to heterotrimeric G proteins. Figure 2 shows a more detailed scheme for the activation of Ca^{2+}-dependent signaling.

Figures 1 and 2 show only the steps involved in the initiation of signaling that result in a change in cell function but not the steps that result in ending or reversing these changes. For signaling through heterotrimeric G proteins, this occurs when the Gα subunit, which is a GTPase, hydrolyzes GTP to GDP and inorganic phosphate and then dissociates from the effector. Many receptors have been found to be inactivated by phosphorylation, catalyzed by one of the protein kinases activated through that receptor. To stop signaling at other steps, second messengers are decreased in concentration. For an ion such as Ca^{2+}, this occurs through activation of calcium-ATPases that move the ion into storage (endoplasmic reticulum or sarcoplasmic reticulum in muscle) or out of the cell. For second messengers such as cAMP, the concentration is lowered by enzymatic degradation. The decreases in second messengers cause dissociation and inactivation of effectors. Dephosphorylation by a protein phosphatase can decrease the activity of an effector that was activated by a protein kinase. Some effectors, however, may be initially activated by removal of phosphate and then inactivated by phosphorylation. Once the initial processes are reversed, the cell can return to its prestimulation state.

Reactive Oxygen and Nitrogen Species as Second Messengers: Characteristics

Second messengers have four basic characteristics that allow regulation. (1) They are either enzymatically generated or regulated by channels and pumps. (2) They are enzymatically degraded. (3) Their concentration rises and falls within a short period. (4) They are specific in action.

How do the reactive oxygen and nitrogen species fit into signaling? Superoxide and hydrogen peroxide have all four of the characteristics of second messengers just defined. They are generated enzymatically as described above, degraded by superoxide dismutases or catalase and glutathione peroxidases, are present at very low concentrations but can transiently be elevated to measurable amounts, and do not react randomly.

Peroxynitrite is not made in the active site of an enzyme but depends on the generation of superoxide and nitric oxide, which is regulated. Therefore, as the reaction that produces peroxynitrite is diffusion limited, its generation is as enzymatically regulated as are its precursors. Peroxynitrite is not itself highly reactive; however, its acid form, peroxynitrous acid, is very unstable and reactive. S-Nitrosation, that is, the formation of *S*-nitrosothiols by reaction of peroxynitrite with the cysteine residues of proteins or glutathione, provides a source of bioavailable nitric oxide, but it has also been proposed to regulate the activity of enzymes, thereby representing a possible signaling mechanism. Peroxynitrite also irreversibly modifies tyrosine residues, producing nitrotyrosines. The process of tyrosine nitration may also play a role in signaling by altering the activity of critical enzymes through covalent dimerization, as shown with the EGF receptor. Tyrosine nitration of proteins occurs in neutrophils treated with peroxynitrite, resulting in priming of the NADPH oxidase. These effects are observed with exogenous peroxynitrite over a large range of concentrations; whether endogenous peroxynitrite production produces similar effects is still a matter of debate. Peroxynitrite may participate in signaling processes at low concentrations but, at greater concentrations, it will produce toxicity (presumably through

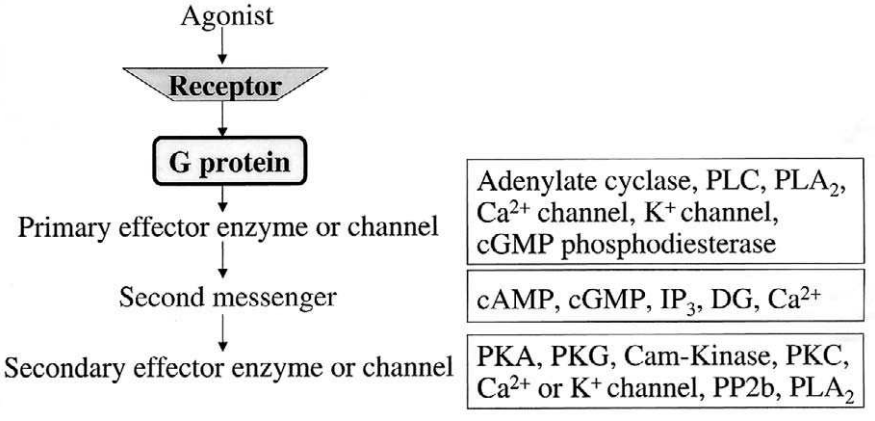


Figure 1 General scheme for heterotrimeric G protein coupled receptor signaling. An agonist binds to a specific receptor and changes its conformation. This signals two changes in the heterotrimeric G proteins. Initially, GDP is bound to the Gα subunit of the heterotrimer. GDP dissociates from Gα when GTP binds to this protein. The GTP-bound Gα then dissociates from the Gβγ subunits. The GTP-bound Gα and/or the Gβγ dimer then binds to another protein called an effector. This effector may be a channel that opens, allowing ion flow, or it may be an enzyme that is activated as long as the G protein is bound to it. The effector enzyme produces a small molecule that acts as a second messenger. This second messenger then binds to another effector, causing a conformational change that activates its activity.

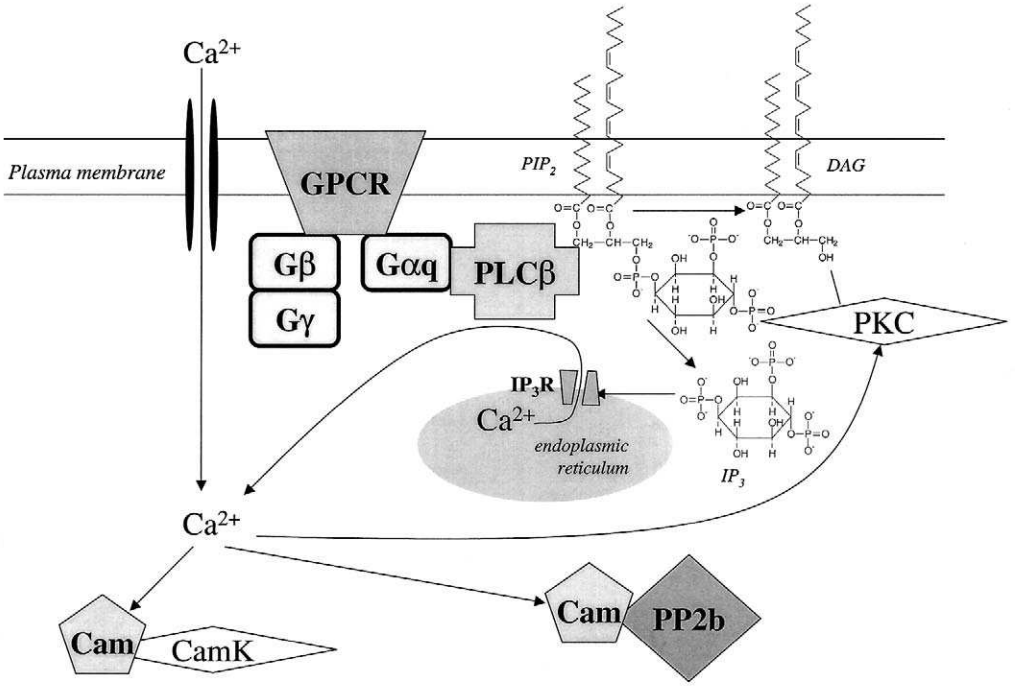


Figure 2 Calcium-dependent signaling. A heterotrimeric G protein coupled receptor (GPCR) activates Gαq (a subtype of the Gα class), which binds to and activates phospholipase Cβ (PLCβ). This enzyme then cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to form two second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to a receptor channel on the endoplasmic reticulum, which opens and allows Ca^{2+} to flow down a concentration gradient into the cytosol. The increase in cytosolic Ca^{2+} concentration activates Ca^{2+}-dependent enzymes, such as the α, β, and γ isoforms of protein kinase C (PKC), and binds to a protein called calmodulin, allowing it to activate enzymes such as calmodulin-dependent kinase (CamK) and the calmodulin-dependent protein phosphatase (PP2b or calcineurin). DAG also is necessary for activation of several protein kinase C isoforms.

formation of peroxynitrous acid or metal-catalyzed reaction). The fate of nitric oxide, namely, conversion to nitrite and nitrate, is well established. Peroxynitrite can isomerize to nitrate or decompose to form nitrite. Nonetheless, there is no known enzymatic pathway for degradation of peroxynitrite or nitric oxide.

Hydroxyl radical formation depends on random encounters between hydrogen peroxide and reduced transition metals. Hydroxyl radical is so reactive that it has no chance for specific action because it reacts with almost every organic molecule at nearly the same rate. Instead, hydroxyl radical is responsible for causing random damage to cellular constituents, including DNA. If the damage is not lethal, DNA repair systems are signaled, but this is actually a response to injury rather than a specific response to hydroxyl radical. Obviously, this free radical does not fit the criteria for a second messenger.

Evidence for Participation of Oxidants in Signaling

Because of their chemical reactivity, giving oxidants the ability to randomly oxidize cellular constituents, it is easy to conceive how oxidants could interfere with signaling or any other cellular function. The preceding discussion suggests that some oxidants have characteristics of second messengers; however, the evidence that they function as such is not yet as clear, in part due to difficulties in ascertaining their production and site of action. The following section discusses some of the caveats associated with this rapidly developing area of investigation.

One of the major problems is that the analytical tools used for measuring the generation of oxidants in cells either are not sensitive to transient fluctuations in radical concentrations (spin trapping) or are not specific for a particular oxidant. A second difficulty in identifying how oxidants act in signaling is to determine the effectors that recognize these reactive species in a specific and reversible manner. This makes establishing quantitative and temporal relationships between oxidant production and signaling quite difficult. This has not been addressed rigorously for many of the pathways. A third problem is that the majority of the studies have relied on the addition of exogenous oxidants to the cells to demonstrate their effects on cell function. Fortunately, there has been a marked decline in the use of ''thermonuclear'' concentrations of oxidants in studying the effects; however, the literature has many examples of studies where cells have been ''bleached'' rather than gently provoked. Although the prudent use of physiologically relevant concentrations of exogenous oxidants is valuable, the results must be carefully considered and interpreted to reach proper conclusions. Finally, a problem inherent to all signal transduction studies is the use of purportedly specific inhibitors, which can result in

misinterpretation. In this context, molecules with antioxidant properties are often used to implicate oxidants in a particular pathway. The rationale is that a reductant will act as an inhibitor of signaling by reacting with the oxidant (commonly called scavenging). Thus, using these molecules allows the demonstration of a possible correlation between diminished oxidant steady-state concentration and diminished signaling, such as activity of a protein kinase. Unfortunately, antioxidants are not particularly specific in their action, and many of the commonly used agents are not actually directly reactive with oxidants under physiological conditions. For example, *N*-acetylcysteine (NAC), which is wdely used as an antioxidant, does not readily react with superoxide or hydrogen peroxide. Rather, this thiol compound can react with protein disulfides as does GSH [see reaction (13)] or act as a precursor for cysteine, which is needed for GSH synthesis. In this manner, NAC may appear to inhibit oxidant-induced signaling via an indirect mechanism. On the other hand, NAC added to the outside of cells may reduce disulfides that are necessary for the function of surface receptors and channels. NAC can also reduce transition metals and thereby promote the production of reactive oxygen species and Fenton chemistry [see reaction (4b)] as well as give rise to a thiyl radical that can initiate other oxidative chemistry:

$$NAC(SH) + Fe^{3+} \rightarrow NAC(S^{\cdot -}) + Fe^{2+} + H^{+} \qquad (9)$$

Although commonly used to indicate the potential involvement of oxidants in signaling, NAC can initiate signaling through its prooxidant chemistry. Another thiol compound, pyrrolidine dithiocarbamate (PDTC), also does not react rapidly with superoxide or hydrogen peroxide but reacts with transition metals. Actually, PDTC is an excellent chelator of copper, and its ability to interfere in signal transduction has been shown to be due to a prooxidant rather than antioxidant reaction, although still used as such. Even antioxidants such as vitamin E that can clearly inhibit lipid peroxidation may have other effects, such as activation of a protein phosphatase that affects signaling by a nonredox mechanism. Another type of reagent, the so-called hydroxyl radical scavenger (DMSO, mannitol, etc.), has been used extensively to inhibit oxidative signaling and to implicate hydroxyl radicals in these processes. Knowing the extreme reactivity of hydroxyl radicals, it is hard to conceive how any compound could have an advantage as a hydroxyl radical scavenger over hundreds of other compounds already present in any biological system, as all organic compounds react with hydroxyl radical with rate constants approaching diffusion limitation. Thus, in solution, for any ''scavenger'' to be 50% effective, it would have to be present at equal or greater concentration than all of those other compounds. The argument might be made that some compound binds to a specific target of oxidation and prevents its oxidation due to Fenton-like chemistry; however, the case must be made then for specificity of binding to that particular site. Thus, the use of antioxidants as tools in studying signal transduction is useful as long as the results are interpreted as potentially indicative of oxidant signaling rather than definitive evidence.

Where Do Oxidants Act to Initiate Signaling?

Oxidation of a critically placed protein cysteine residue appears to be the major chemical alteration caused by oxidants that affects signaling. For many enzymes, the cysteine is essential to activity, and oxidation to a sulfone or sulfinic acid causes inactivation. In general, cysteine residues are unreactive with superoxide or hydrogen peroxide at physiological pH. For cysteine to react with H_2O_2, it must be either in a basic environment, where it becomes a thiyl anion, or in close association with a transition metal. Cysteine in the active site of some enzymes, such as protein tyrosine phosphatases, can be in the anionic state due to the surrounding microenvironment. This allows the cysteine to be susceptible to oxidation even when the protein is in a medium at physiological pH. The metal-catalyzed oxidation of cysteine is similar to the Fenton reaction [reaction (4b)] in which the sulfhydryl reduces the metal. Iron–sulfur clusters in the mitochondrial electron transport chain and aconitase are particularly susceptible to such chemistry. Interestingly, the loss of the iron–sulfur cluster in aconitase converts it to the active form of the iron response protein that affects transcription and translation of enzymes involved in iron metabolism. Nevertheless, the oxidation of cysteines through metal catalysis is not the only process that could produce changes in signaling through cysteine modification. As described above, nitrosation of cysteines is another possible mechanism, although specific examples in signaling are unknown at present.

Better established examples of modification of cysteine residues include those where the cysteines in the signaling proteins are in close proximity (referred to as vicinal thiols). The protein kinase C (PKC) α and β isoforms have clusters of cysteine residues in both the catalytic and regulatory domains. In the regulatory domain, cysteine residues associate with zinc, a nontransition metal, which while not redox active causes the cysteines to be in the anionic state. Addition of physiologically relevant concentrations of H_2O_2 to cells results in the oxidation of the vicinal cysteines in the regulatory domain to form disulfides, while the active site cysteines remain in their reduced state. The formation of cystine(s) in the regulatory domain brings about a conformational change in PKC so that it is active in the absence of calcium and phospholipid, normally required for its activity. This conversion is reversed by reduction of the cystine residue. This reversible activation suggests that PKC may participate in oxidant-initiated signaling. At higher concentrations of H_2O_2, the cysteine in the active site of PKC can be oxidized irreversibly, thereby the result of a damaging reaction.

A reversible oxidation of cysteine residues occurs in the physiological reduction of H_2O_2 by thioredoxin (TRx), both nonenzymatically and enzymatically catalyzed by TRx peroxidases and glutathione peroxidases:

$$TRx(SH)_2 + H_2O_2 \rightarrow TRxS_2 + 2\,H_2O \quad (10)$$

$$2\,GSH + H_2O_2 \rightarrow GSSG + 2\,H_2O \quad (11)$$

where $TRx(SH)_2$ is reduced thioredoxin, $TRxS_2$ is thioredoxin disulfide, GSH is reduced glutathione, and GSSG is glutathione disulfide. The active sites of these proteins are basic microenvironments in which one of the cysteines can be ionized and therefore becomes reactive with H_2O_2. Reaction (10) is catalyzed by thioredoxin peroxidases, and reaction (11) is catalyzed by glutathione peroxidases. Another class of enzymes, the peroxiredoxins, can use thioredoxin, glutathione, or other thiols to reduce hydroperoxides. The active form of some of these enzymes are homodimers in which each monomer contributes a critical cysteine to form a single active site with a vicinal thiol pair. Finally, some isoforms of glutathione-*S*-transferase can catalyze reaction (11) as well. The disulfides forms of thioredoxin and glutathione can then react reversibly with protein sulfhydryls:

$$TRxS_2 + P(SH)_2 \rightleftharpoons TRx(SH)_2 + PS_2 \quad (12)$$

$$GSSG + PSH \rightleftharpoons GSH + PSSG \quad (13)$$

where P symbolizes a protein and PSSG is a mixed disulfide. Reaction (12) can be catalyzed by protein disulfide isomerase (PDI), whereas reaction (13) may be nonenzymatic or possibly catalyzed by PDI. PDI itself has a reaction center that is quite similar to thioredoxin. If the resulting protein disulfide or mixed disulfide has enhanced or depressed enzymatic activity, reactions (12) and (13) may be the chemical steps whereby hydroperoxides transmit their signals in a reversible manner. In plants, the participation of thioredoxin in signaling is established, but in other systems it remains an interesting but unproven hypothesis. The glutathione mixed disulfides have similarly been investigated as signaling intermediates with little more than correlation as supporting evidence. For example, ultraviolet radiation produces reactive oxygen species that cause clustering and thereby activation of receptors in the absence of their natural agonists. These receptors include those that bind tumor necrosis factor α (TNF-α), EGF, and interleukin-1 (IL-1). Could this clustering result from disulfide formation?

Aside from vicinal thiol oxidation, there are several other examples where H_2O_2 has been demonstrated to activate enzymes directly. Cyclooxygenases (also called COX1 and COX2) catalyze the first step in the conversion of arachidonic acid to prostaglandins and related compounds, such as thromboxane. Lipoxygenases are responsible for initiating the production of leukotrienes from arachidonic acid. When cells are stimulated for the production of these arachidonic acid metabolites, these enzymes show a lag in their activity, which can be eliminated by the exogenous addition of very low concentrations of their products, lipid hydroperoxides or hydrogen peroxide. Products of the cyclooxygenase and lipoxygenase pathways are important agonists of receptor-mediated signal transduction affecting many physiological processes. Thus, the H_2O_2-induced activation of these pathways may be one of the key mechanisms in oxidative signaling. Another example of a direct effect of H_2O_2 activation is its effect on guanylate cyclase. Concentrations of H_2O_2 about 10–100 times those found during steady-state metabolism can activate guanylate cyclase by interacting with the same heme site where nitric oxide binds to produce its activating effect on guanylate cyclase. The extent to which this H_2O_2-induced activation participates in normal physiology is unclear.

Intracellular Sources of Oxidants

Superoxide (and hydrogen peroxide) is produced in large amount by phagocytes (i.e., neutrophils and macrophages), not as the result of leaks or metabolic side reactions but through the deliberate induction of an enzymatic process as a defense mechanism for the killing and destruction of invading pathogens. The NADPH oxidase catalyzes the univalent transfer of an electron to oxygen at the expense of NADPH, its preferred substrate [reaction (14)]:

$$2\,O_2 + NADPH \rightarrow 2\,O_2^{\cdot -} + NADP^+ + H^+ \quad (14)$$

The NADPH oxidase is a multicomponent enzyme, which is dormant in resting cells and becomes active on assembly of the cytosolic and membrane components, the ''phox'' (phagocyte oxidase) proteins (Fig. 3). In the membrane, an atypical cytochrome b_{558}, composed of two subunits, $gp91^{phox}$ and $p22^{phox}$, is the binding site for NADPH and flavin adenine dinucleotide (FAD), and it represents the terminal oxidase that transfers the electron to oxygen after assembly and activation. In the cytosol, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are organized in a complex formed by protein–protein interaction through their proline-rich regions and *src* homology domain SH3, whereas the small GTP-binding protein of the Rho family Rac2 (or Rac1) is in a complex with the inhibitor Rho-GDI. On binding of various agonists to their cognate receptors or through membrane perturbation, the cytosolic phox proteins move to the membrane and associate with the flavocytochrome to form the activate oxidase (see Fig. 3 for details). Absence of either of the phox proteins leads to a genetic disease, chronic granulomatous disease (CGD), that is characterized by the inability of the phagocytes to produce superoxide and results in a high propensity to bacterial and fungal infections. All of the known phox proteins are now cloned, and tools have been developed, including CGD knockout mice that lack $gp91^{phox}$ or $p47^{phox}$.

When the concept emerged that reactive oxygen and nitrogen species could serve as signaling molecules in processes such as proliferation and apoptosis, the search began for conditions under which production of these agents occurs in cells other than the phagocytes. This has been hampered by difficulties in accurately measuring low-level production in living cells and in defining the nature of the species involved. Fluorescent dyes that are freely permeable across cell membranes and increase in fluorescence on oxidation, such as 2',7'-dichorofluorescin diacetate (DCF), have been used to measure oxidant production. DCF is oxidized through a reaction involving hydrogen peroxide and a per-

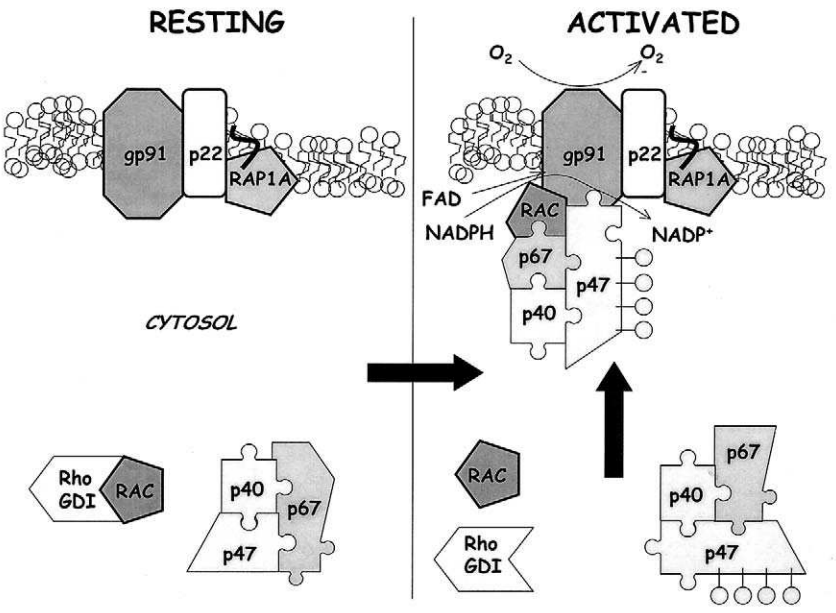


Figure 3 NADPH oxidase. This figure represents the cytosolic ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) and membrane components ($gp91^{phox}$ and $p22^{phox}$) of the phagocyte NADPH oxidase in resting cells and after activation. The cytosolic components are organized in a large complex through protein–protein interaction via SH3 domains and proline-rich regions. In the cytosol, the small GTPase Rac1/2, which is required for oxidase activity, is associated with the inhibitor Rho-GDI. On stimulation, Rho-GDI dissociates and Rac moves to the membrane to bind the flavocytochrome. Stimulation also induces the phosphorylation of $p47^{phox}$ and changes in conformation leading to rearrangement of the complex through new protein–protein interactions. The cytosolic complex then moves to the membrane to bind to the flavocytochrome and Rac. The membrane complex represents the activated NADPH oxidase that catalyzes the reduction of oxygen to superoxide. The organization of the phox proteins in resting or activated cells has been extensively studied using a cell-free system and neutrophils from patients with chronic granulomatous disease (CGD), a genetic disease. Specific mutations in either of the core phox proteins have been identified, and the ability of the other proteins to translocate to the membrane has been studied. Although not shown on the figure, other data indicate that $p67^{phox}$ and the flavocytochrome become phosphorylated during activation.

oxidase, but it is not oxidized by superoxide. Hydroxyl radicals produced via Fenton chemistry [reaction (4b)] and peroxynitrous acid can also oxidize DCF. Thus, DCF oxidation does not discriminate among the reactive species. Nevertheless, using this tool, production of reactive oxygen species was detected in various cell types after stimulation with PDGF or EGF. Furthermore, Rac1, a small GTPase of the Ras superfamily, has been implicated as a regulator of reactive oxygen species (ROS) generation in fibroblasts, using constitutively activated (V12Rac1) or dominant-negative (N17Rac1) forms of Rac1. Production of superoxide under these conditions was inhibited by antioxidants and diphenylene iodonium (DPI), an inhibitor of flavoproteins. Rac is ubiquitously expressed, and it is a required participant in the assembly of the phagocyte NADPH oxidase, prompting the hypothesis that a flavoprotein, similar to that present in phagocytes, may be responsible for the production in nonphagocytic cells. In fact, immunologically related cytosolic oxidase components are found in some types of fibroblasts, endothelial cells, and epithelial cells, among others, and these cells produce low levels of superoxide. The flavocytochrome is an inducible protein and γ-interferon is known to induce its expression. Although not fully explored yet, it s possible that other cytokines may also be effective. Thus, although preliminary at this point, these findings raise interesting possibilities and open new questions. The idea of an NADPH oxidase similar to that of phagocytes is intriguing; however, one should remember that patients with CGD experience only problems in relation with infection. Even wound healing problems are more likely due to the inability to clear an infection than to a proliferative repair problem. If the phagocyte NADPH oxidase were involved in many systems, one would expect more extensive defects. Although only reported in one study, the superoxide production of fibroblasts from a patient with CGD was normal. The role for a flavoprotein in nonphagocytic cells has been inferred in part from inhibition by DPI, which is not specific for the NADPH oxidase; indeed, DPI inhibits all flavoproteins and also reduces mitochondrial production of superoxide. More studies and better tools will be required to determine whether a related but differentially regulated NADPH oxidase is present in nonphagocytic cells. The fact that such an entity exists in plants, and that a $gp91^{phox}$-related protein is expressed in yeast, suggests that the phagocyte NADPH

oxidase may have arisen with evolution from a more ancient and less toxic oxidase, which is still present in nonphagocytic cells.

Oxidants and Signaling Pathways

Much of the rest of this chapter is devoted to examples of signaling pathways in which oxidants have been proposed as participants.

Calcium Signaling

The steady-state intracellular free calcium ion concentration ($[Ca^{2+}]_i$) is 70–100 n*M,* which is 10^4 times lower than that of the plasma and most extracellular fluids. This is maintained by ion pumps, exchange with sodium, and sequestration within organelles (Fig. 4). Membrane receptor-mediated signaling involving elevation of $[Ca^{2+}]_i$ is initiated by the activation of a heterotrimeric G protein coupled to the receptor and activation of phospholipase C (Fig. 2). $[Ca^{2+}]_i$ elevation during signal transduction occurs through both release from the endoplasmic reticulum and opening of channels in the plasma membrane. The latter is signaled through changes in membrane potential, through G proteins coupled to receptors that act directly on channels, and through a response to emptying of the endoplasmic reticulum Ca^{2+} pool, which is still poorly understood. During physiological signaling, opening of plasma membrane Ca^{2+} channels results in small transient changes in $[Ca^{2+}]_i$. An ATPase (a calcium pump) maintains the endoplasmic reticulum pool of calcium through active import of the cation. Release from the pool occurs by activation of an IP_3-sensitive receptor channel. Even in unstimulated cells, calcium is constantly released and taken up by the endoplasmic reticulum. This basal release of Ca^{2+} from the endoplasmic reticulum occurs due to a low steady-state production of IP_3.

Hydroperoxides have profound effects on the cellular control of calcium homeostasis. Exposure to high concentrations of hydroperoxides results in calcium entry through the plasma membrane and substantial elevation of $[Ca^{2+}]_i$, which is usually associated with cell death. On the contrary, addition of relatively low concentrations of hydroperoxides causes transient changes in $[Ca^{2+}]_i$ that resemble the physiological changes occurring in receptor-mediated signaling. The endoplasmic reticulum may contribute to this increase in $[Ca^{2+}]_i$, as an increase in glutathione disulfide observed during oxidative stress correlates with a decrease in the dissociation constant of the IP_3 receptor (and presumably involves formation of a mixed disulfide). Nonetheless, the hydroperoxide-induced increase in $[Ca^{2+}]_i$ can come from sources other than the endoplasmic reticulum and extracellular environment. In the alveolar macrophage, dissociation of annexin VI, a plasma membrane-bound calcium-binding protein, is the apparent hydroperoxide-sensitive source. Mitochondria as a source of calcium has been debated for a long time, but it is now clear that mitochondria only transiently take up calcium released by the proximate endoplasmic reticulum on stimulation. The extent of the involvement of this exchange between the endoplasmic reticulum and mitochondria during oxidative signaling is unknown. Suggestions that nitric oxide can signal calcium release from mitochondria are currently under investigation in several laboratories.

Some of the signaling mechanisms sensitive to the action of oxidants, which regulate $[Ca^{2+}]_i$ or are regulated by changes in $[Ca^{2+}]_i$, include phospholipases, prostaglandins, the constitutive form of nitric oxide synthetase, ion channels, classic forms of protein kinase C (α, β, and γ PKC), and calmodulin-dependent kinases. Although direct effects of hydroperoxides on calmodulin, the calcium-binding pro-

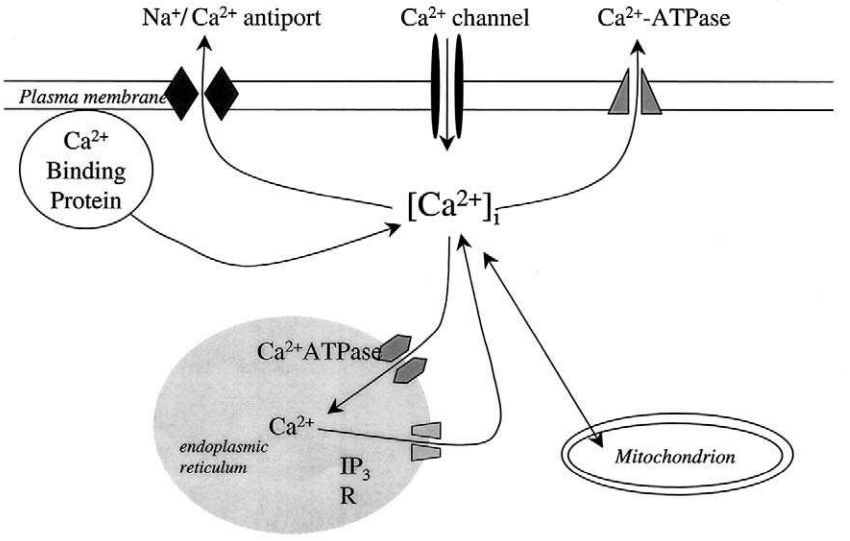


Figure 4 Calcium regulation in signaling. See text for details.

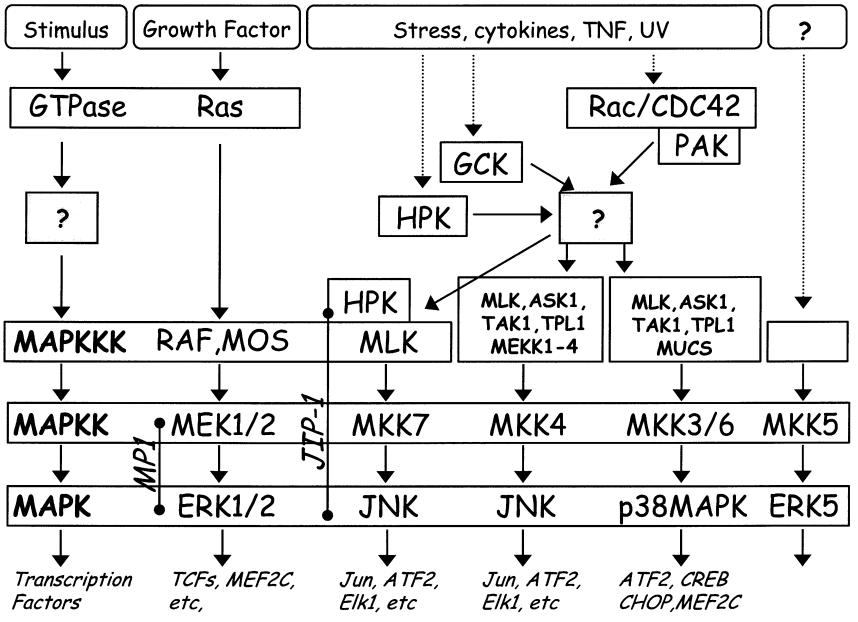


Figure 5 MAP kinase pathways. See text for details and abbreviations.

tein that acts as a nearly ubiquitous mediator of calcium-dependent functions, have not been demonstrated, the sensitivity of calmodulin to changes in $[Ca^{2+}]_i$ makes calmodulin-dependent activities, such as calmodulin-dependent kinases and protein phosphatase 2b, potential indirect targets of hydroperoxides through modulation of $[Ca^{2+}]_i$ (Fig. 2). Phospholipase D activity also appears to be enhanced by an elevation in $[Ca^{2+}]_i$ caused by low concentrations of hydroperoxides.

The MAP Kinase Pathways

Numerous cellular processes, such as cell growth, differentiation, cytoskeletal rearrangement, gene transcription, and apoptosis, are regulated through activation of the mitogen-activated protein kinase (MAPK) pathways (Fig. 5). Various receptors, namely, tyrosine kinase receptors, G-protein linked receptors, and others, activate these pathways, which are well conserved with evolution. Environmental stresses such as oxidant stress, heat shock, osmotic shock, or ionizing radiation can also trigger the activation of one or more MAPK pathways. Each pathway represents separate phosphorylation cascades or modules, at the center of which are the MAPKs, a large family of kinases that require concomitant phosphorylation on Thr and Tyr (TXY) by dual-specificity MAPK kinases or MEKs [MAPK–extracellular signal-regulated kinase (ERK) kinase] or MKKs (MAPKK) for full activation. The MEKs–MKKs, which are themselves activated by Ser/Thr phosphorylation by MAPK kinase kinases [MAP(3)Ks or MEKK], phosphorylate only very specific MAPKs. The MAPKs are proline-directed Ser/Thr kinases, and their known substrates are localized both in the cytosol and in the nucleus, where they translocate on activation. Thus, kinases and transcription factors are phosphorylated by activated MAPKs, leading to alteration in cellular function and gene transcription (see Fig. 6).

The MAPKs are grouped into several subfamilies according to their TXY phosphorylation site. At this time, three such subfamilies defining three separate pathways have been characterized in some detail: ERK1 and ERK2 (TEY), previously known as the p44 and p42 MAPKs, respectively; the c-*jun* N-terminal kinases or JNKs, also known as the stress-activated protein kinases or SAPKs (TPY); and the RK/p38 MAP kinases (TGY) (Fig. 5). The ERKs are preferentially

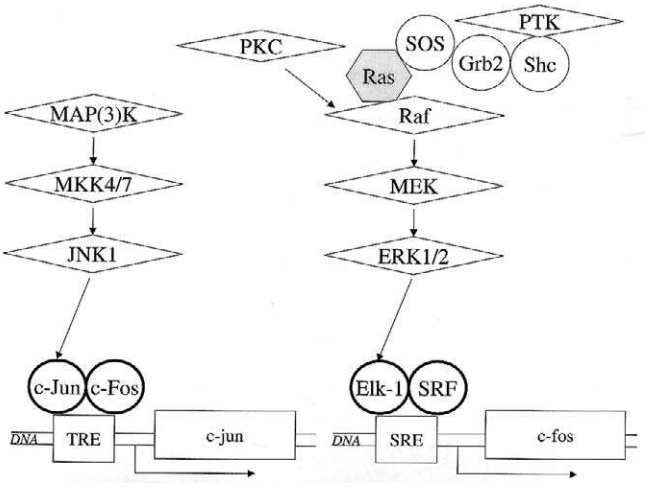


Figure 6 Transcription factors and MAP kinases. The proteins c-Jun and c-Fos (or members of their family) interact to form the AP-1 transcription factor that binds to the TRE *cis*-acting element and increases transcription of AP-1 responsive mRNAs, such as that for c-Jun itself. The proteins Elk-1 and the serum response factor (SRF) bind together with the serum response *cis*-acting element (SRE) to activate transcription of c-*fos*. The signaling pathways are described in the text.

activated by growth factors and are associated with cell growth and differentiation, whereas the JNKs and p38MAPKs are activated by cytokines and environmental stress in a cell-type specific manner and are implicated in apoptosis. MEK1 and MEK2 activate the ERKs, and recent data show that they are in a complex with a scaffold protein, MP-1, that maintains the core enzymes in close proximity. MEK1 and MEK2 are themselves activated by isoforms of Raf-1 or by Mos, forming the ERK module that is downstream of the protooncogene p21 Ras, a small GTPase. The connection between the receptor, Ras, and the ERK module is provided by the adapter Grb2, which is constitutively bound to the GDP exchange factor Sos through its SH3 domain and binds to the phosphorylated receptor either directly or though SHC, another adapter protein, via its Src homology SH2 domain. These protein–protein interactions lead to the activation of Ras by bringing its exchange factor Sos close to the membrane (Fig. 6).

Three JNK isoforms are currently identified, which are activated at least by two MEKs, MKK4 and MKK7. A scaffold protein, JIP-1, which was initially identified as an inhibitor of JNK, associates with JNK, MKK7, MLK, and HPK (see later in this chapter). The p38 MAPK subfamily is also composed of several isoforms (α to δ) and two MEKs, MKK3 and MKK6, are described so far as their activators. Upstream of the MEKs, the JNK and p38 pathways are not as clearly defined as the ERK pathway, and a large amount of cross talk between the two pathways seems to exist, that is, the same MAP(3)K can activate both the JNK and p38 pathways.

The MAP(3)Ks are structurally divergent and include MEKK1,2,3,4, MAP(3)K5/ASK-1, TAK1, MLK2,3, MUK/DLK, and Tlp2/Cot. They activate the MEKs *in vitro* and *in vivo,* with little specificity for the JNK or p38 MAPK pathways, except possibly for MEKK1, MLK2, and MLK3, which demonstrate more specificity for the JNK pathway. Upstream of the MAP(3)Ks, Rac1 and Cdc42Hs, members of the Rho subgroup of the Ras superfamily of GTPases, activate the JNK and p38 pathways *in vivo,* and their direct effectors, a family of p21-activated (PAK) kinases that possess a Cdc42/rac interaction domain (CRIB), activate the JNK and p38 pathways. There is no evidence that the PAKs directly activate the MAP(3)Ks.

The germinal center kinases (GCK), a large family of kinases distantly related to the PAKs but which lack a CRIB domain and do not bind to the Rho GTPases, are also upstream activators of the MAP(3)Ks and include the group I GCK, GCKR, GLK, HPK1, NIK, and group II SOK1, Krs-1, MST1/Krs2, MST3, and LOK. The group I of GCKs manifest selectivity for the JNK pathway over the p38 or ERK pathway. Interestingly, GCK, NIK, and HPK1 can bind MEKK1, and GCK and HPK1 bind MLK3 *in vivo* and *in vitro.*

The link between the events at the cell surface and the upstream activators of the JNK and p38 pathways is not known. There are also a number of ''orphan'' kinases for which the upstream activators are unknown such as ERK3, which has no known activator or substrate, and ERK5, also known as BMK-1 (Big-MAPK-1), which is activated by MEK5.

The role of oxidants in the activation of these pathways is under extensive scrutiny. SOK-1 (ste20-like oxidant stress response kinase 1), a homolog of the yeast Sterile 20 family of protein kinases, and BMK-1 are relatively specifically activated by oxidant stress. In particular, SOK-1 is not activated by growth factors, and its activation does not result in the activation of any of the known MAP kinase cascades, suggesting that this kinase is part of a novel stress response pathway, yet to be identified.

Whereas growth factors do not activate the stress-activated kinases JNKs and p38, there is a strong consensus that exogenous H_2O_2 activates the ERKs in various cell types such as endothelial cells, smooth muscle cells, PC12 cells, and others, with doses as low as 25 μM in some cells. Although the ERK pathway participates in the induction of iNOS and production of NO, so far the data are scarce for the effects of NO on the ERK pathway; however, the JNKs appear to be more sensitive to activation by NO and other related species. Both JNKs and p38 are also activated by H_2O_2, although the extent of activation of each pathway varies with the cell type. The lipid peroxidation product 4-hydroxynonenal (4HNE) strongly activates JNKs and p38 through increased production of H_2O_2. The mechanisms by which the MAPKs are activated are still unclear. Current data show that they are not direct targets for oxidants and that upstream activators such as MEK1, MEK2, Raf, and MKK6 are similarly activated by H_2O_2. An exception might be the recently described direct interaction of JNK1 with the lipid peroxidation product 4 HNE, which was shown to activate JNK without affecting its phosphorylation.

Several candidate molecules are currently under study as the potential upstream mediators of the effects of oxidants on these pathways. Tyrosine kinase inhibitors often inhibit the activation of the MAPKs by oxidants, suggesting activation of tyrosine kinase pathways. Membrane tyrosine kinase receptors for EGF or PDGF are activated by H_2O_2 in the absence of the ligand; however, at least for the EGF receptor, activation by H_2O_2 does not result in ERK activation. The tyrosine kinase c-*src* is implicated in the activation of ERK2 and BMK-1 by H_2O_2. PKC, whose activity is altered by oxidants, phosphorylates and activates Raf-1 and ERKs. Dominant–negative mutants of Ras prevent the activation of the ERK pathway by oxidants. Thus, Ras was proposed as an oxidant sensor, in part because of a critical cysteine residue at position 118 that is regulated in a redox-dependent fashion and affects nucleotide exchange.

Overexpression of the GCK or other kinases upstream of the JNK/p38 pathways result in constitutively active kinases, which indicates either that they are bound to an inhibitor or that they are able to oligomerize. These mechanisms of activation may be at work on treatment with oxidants. In fact, both mechanisms have been demonstrated for the MAP(3)K ASK-1, which activates the MKK4/MKK7-JNK and MKK3/MKK6-p38 signaling cascades. ASK-1 has a cysteine-

rich domain in its N terminus, which is the binding site for TRx under reducing conditions. TRx is highly sensitive to oxidation by ROS, and its oxidation by H_2O_2 would result in its dissociation from ASK-1 and activation of the MKKs, making TRx a physiological inhibitor of ASK-1. The activation of ASK-1 would occur via dimerization of ASK-1, as observed in cells overexpressing ASK-1 and treated either with H_2O_2 or TNF-α. Another mode of upstream activation can be through generation of metabolites that affect the pathways. Arachidonic acid, a product of phospholipase A_2, which is activated by H_2O_2 and other oxidants, activates the ERKs and the ERK module in various cell types. Ceramide is produced on treatment with H_2O_2 via an increase in sphingomyelin hydrolysis by the neutral sphingomyelinase, and ceramide activates the JNKs through activation of the MAP(3)K TAK1, leading to apoptosis.

Finally, another mode of regulation of the MAPKs by oxidants may involve the induction of dual-specificity phosphatases. This large family of phosphatases removes phosphate from both Thr and Tyr residues, and most of the family members are inducible proteins, initially identified as a gene induced by oxidant stress. The mitogen-activated protein kinase phosphatases (MKP) have different specificities for the various MAPKs and different subcellular localization. H_2O_2 and arachidonic acid induce the expression of MKP-1, and agents that increase the release of arachidonic acid stimulate MKP-1 induction and activation of the MAPKs. Thus, redox-sensitive molecules and lipid mediators may play a significant role in the mechanisms of activation by oxidants of the MAPKs.

Tyrosine Phosphorylation Pathways

Tyrosine phosphorylation has emerged as an important means by which cell function is regulated, and many of the effects exerted by oxidants appear to be mediated through deregulation of the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Treatment of various cell types with exogenous oxidants results in an increase in the tyrosine phosphorylation of many proteins, implying that tyrosine kinases and tyrosine phosphatases are targets for the oxidants. This is supported by the fact that these effects are increased by sodium vanadate, which forms peroxyvanadate in the presence of H_2O_2 and is a very potent inhibitor of tyrosine phosphatases. The effect of oxidants is quite cell type specific, depending on the antioxidant capability of the cells and on the basal activity of the kinases and phosphatases. As an example, treatment of neutrophils with H_2O_2 will not result in the accumulation of tyrosine phosphorylated proteins, unless catalase is inhibited or sodium vanadate is present, indicating the powerful antioxidant and tyrosine phosphatase activities in these cells. The doses of exogenous oxidants required to observe any net effect on tyrosine phosphorylation vary greatly from cell to cell, ranging from 1 n*M* to 10 m*M,* and the consequences of treatment with oxidants also vary from proliferation to growth inhibition and apoptosis.

Although numerous PTPs have been identified, there is as yet little understanding of their mechanisms of activation and inactivation. The active site of PTPs contains an essential cysteine, which is sensitive to vanadate and peroxides of vanadate. The PTP catalytic activity involves the formation of a transient phosphoenzyme where the phosphate group is transferred to the cysteine residue. At physiological pH, the cysteine is a thiolate anion. *In vitro* incubation with H_2O_2 inhibits several PTPs, whereas large alkyl hydroperoxides do not, and serine/threonine phosphatases are not affected. Concentrations as low as 45 μ*M* are able to rapidly and completely inactivate the PTP activity *in vitro.* The inhibition of PTPs is reversed by treatment with thiols, indicating that reversible oxidation and reduction of a single catalytic residue regulate the activity of PTPs. The thiolate anion is the target of H_2O_2, which converts it to sulfenic acid (Cys-SOH), preventing the transfer of the phosphate group. Thus, oxidants can modulate the activity of PTPs through a direct and reversible mechanism and the overall redox state of the cells dictates the steady-state levels of active enzymes. Glutathione is the most likely intracellular reduced thiol that could reverse the inactivation of PTPs *in vivo* [reaction (15)]:

$$\text{P}-\text{SOH} + \text{GSH} \rightarrow \text{GSSG} + \text{PSH} \qquad (15)$$

where P−SOH is a protein with a sulfenic acid residue and PSH is the cysteine containing form of the protein.

The activity of tyrosine kinases is also affected by reactive oxygen and nitrogen species. PTKs are grouped by the transmembrane receptor type, such as the receptors for growth factors (EGF, PDGF, NGF) and the nonreceptor PTKs, that is, the Src, Syk, or JAK families. Activation of the receptor PTKs on ligand binding involves dimerization of the receptors and activation of the tyrosine kinase activity, leading to transphosphorylation. The nonreceptor PTKs are involved in the signaling pathways triggered by receptors lacking intrinsic tyrosine kinase activity, such as G protein coupled receptors and cytokine receptors. Oxidants play a role in signaling by the EGF receptor. Stimulation of A431 cells with EGF induces a transient increase in the production of H_2O_2, as measured by fluorescence with DCF. Addition of catalase inhibits the EGF-induced tyrosine phosphorylation of the EGF receptor and of phospholipase C-γ (PLC-γ), and these effects are dependent on the tyrosine kinase activity of the receptor. Exogenous H_2O_2 also induces the tyrosine phosphorylation and activation of the EGF receptor in A549 cells and other cells, resulting in the activation of PLC-γ but not that of other downstream signaling pathways. EGF stimulation of these cells results in growth inhibition, and a comparable inhibition is observed when cells are treated with H_2O_2. This indicates that, in the absence of the ligand, oxidants can modulate cell growth. It is possible that oxidative modification of a reduced cysteine in the EGF receptor may reversibly affect its activation. Of interest, donors of nitric oxide inhibit the transphosphorylation of the EGF receptor in intact cells, and this effect is partially reversed by incubation with dithiothreitol (DTT), indicating the involvement of thiol groups and S-nitrosylation of the receptor.

Ligand binding of the T-cell and B-cell receptors induces an increase in tyrosine phosphorylation that involves the coordinated participation of several nonreceptor tyrosine kinases of the Src and Syk families and of the transmembrane tyrosine phosphatase CD45. Treatment of lymphocytes with H_2O_2 induces the activation of the PTK Syk family (i.e., Syk and ZAP-70) and the downstream signaling pathways, although 5–10 m*M* H_2O_2 is required. *In vitro* treatment with H_2O_2 of the immunoprecipitated Syk or ZAP-70 does not result in their activation; thus, H_2O_2 is likely to act indirectly through modulation of upstream activators. Inhibition of CD45 by oxidants may result in activation of the Syk family of PTKs, as they are activated by tyrosine phosphorylation. On the contrary, proper function of CD45 is required for the positive regulation of the Src kinases. CD45 is implicated in the dephosphorylation of a tyrosine residue that maintains the Src kinases in an inactivate state through intramolecular binding between the SH3 domain and the SH2-kinase linker. Release of this inhibitory conformation leads to partial activation of the Src kinases.

It was first reported that the activity of the Src kinases lck and fyn, and Iyn and yes is affected by H_2O_2 only in the presence of vanadate. However, others reported that lck is actually strongly activated by diamide and H_2O_2 alone. This activation does not require the dephosphorylation of the inhibitory tyrosine residue at the C terminus and may be the result of phosphorylation by another tyrosine kinase. Activation of the nonreceptor PTKs has been observed in cells other than lymphocytes, including in neutrophils. Exogenous addition of GTPγS to electropermeabilized neutrophils induces the production of reactive oxygen species via activation of the NADPH oxidase and increases protein tyrosine phosphorylation. Under these conditions, the tyrosine kinases hck, syk, and btk are activated, whereas lyn and fgr are inhibited. As in lymphocytes, the effects of the reactive oxygen species are indirect, as they do not activate these kinases *in vitro.* In addition, CD45 is also inhibited under the same conditions. Stimulation of intact neutrophils with phorbol esters, which also activates the NADPH oxidase, had a similar inhibitory effect on CD45 activity, although CD45 is also inhibited in CGD neutrophils, albeit to a lesser extent and with different kinetics than in normal neutrophils. This indicates that, although oxidants produced by the NADPH oxidase contribute to the regulation of CD45 activity, additional pathways are at play.

Tyrosine phosphorylation seems to be involved in the activation of phospholipase D (PLD) by oxidants. PLD catalyzes the formation of phosphatidic acid (PA) and choline from phosphatidylcholine. Phosphatidic acid may play a direct role in intracellular signaling and generates second messengers by conversion to 1,2-diacylglycerol by a PA-phosphatase or to lysophosphatidic acid by phospholipase A2. Two separate mammalian genes have been cloned, PLD1 and PLD2, and four regions highly conserved among isoforms were identified, including a weak pleckstrin (PH) homology domain, which may serve as the binding site for phosphatidylinositol 4,5-bisphosphate (Ptdlns $4,5P_2$), an activator of all PLD isoforms. The regulation of agonist-induced PLD activation involves several PKC isoforms, the small GTP-binding proteins of the Rho and ARF (ADP-ribosylation factors) subfamilies, calcium, and tyrosine kinases and phosphatases. PKC is the major activator of PLD, and only the conventional Ca^{2+}-dependent α and β isoforms are effective. PMA activates PLD, and PKC inhibitors inhibit *in vivo* activation; however, activation of PLD by PKC *in vitro* does not require phosphorylation. Thus, the mechanisms of regulation of PLD activity by PKC are still unclear. The involvement of RhoA and ARF was demonstrated, in part, by the use of C3 exoenzyme of *Clostridium botulinum,* which ADP-ribosylates and inhibits the Rho proteins, and brefeldin A, which inhibits ARF activation. Whereas agonist-stimulated activation of PLD by RhoA has been demonstrated, the evidence for that by ARF is limited.

Many growth factors that induce tyrosine phosphorylation also induce PLD activity. Treatment of various cell types with H_2O_2 or other oxidants results in activation of PLD that was sensitive to both tyrosine kinase and tyrosine phosphatase inhibitors but not to PKC inhibitors, indicating a predominant role for tyrosine phosphorylation in PLD activation by oxidants. In these studies, the PLD entity was not characterized other than as an enzymatic activity present in intact cells. It is now known that several PLD isoforms with different localization, substrate specificities, and mode of regulation exist in the same cell. The effects of H_2O_2 treatment on the activation of a rat brain PLD isoform, homologous to human PLD1, have been studied in fibroblasts, using immunoprecipitation with a specific antibody. These data showed that H_2O_2 activates PLD only in the presence of vanadate, resulting in the tyrosine phosphorylation of PLD1 and other associated proteins in the PLD1 immune complexes. The activation of PLD1 was partially inhibited by either PTK or PKC inhibitors, but it was 90% inhibited by combining both inhibitors, contrary to previous reports in other cell types. Thus, further studies aimed at identifying the PLD isoforms affected by H_2O_2 are probably forthcoming.

Thus, there are discrepancies among cell types about the mechanisms by which oxidants exert their effects and whether H_2O_2 can act alone or in concert with vanadate. However, there is mounting evidence that tyrosine phosphorylation is modulated by oxidants through activation of tyrosine kinases and inhibition of tyrosine phosphatases, which involves both direct effects on critical thiols in these enzymes and/or indirect effects by modulation of the upstream activators.

Activation of Transcription Factors

Oxidants activate several transcription factors in eukaryotic and bacterial systems. In eukaryotes, the transcription factors, nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and the antioxidant response element binding protein (ARE) have been investigated (Fig. 7). Others have been described to a lesser extent.

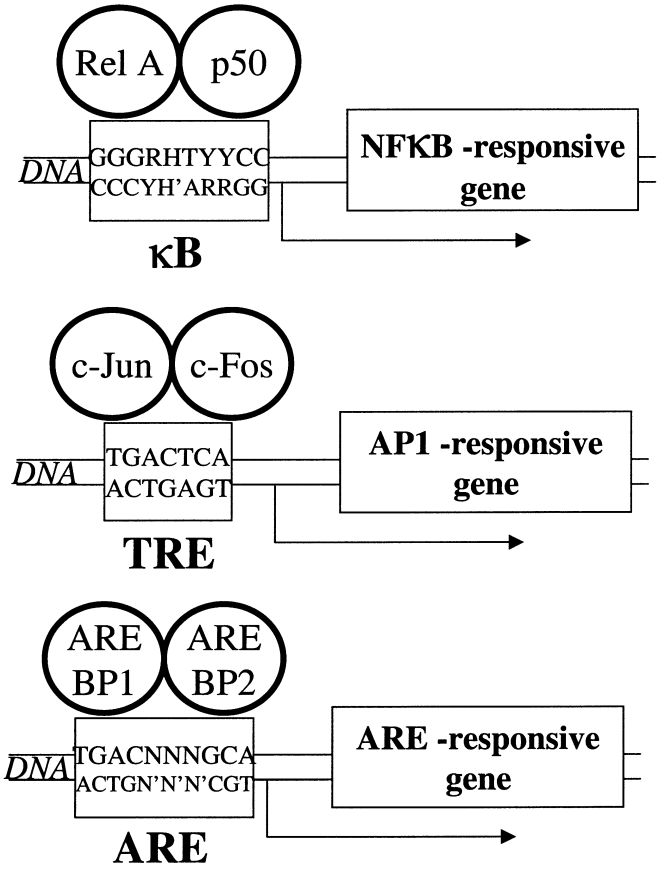


Figure 7 Oxidant-responsive *cis*-acting elements. See text for description of the transcription factors. The consensus sequences are shown.

NF-κB regulates transcription of several genes involved in inflammation, including TNF-α. Ultraviolet radiation, viruses, cytokines (such as TNF-α), phorbol esters, okadaic acid, and hydrogen peroxide can activate NF-κB. Interestingly, most of these stimuli appear to cause cells to produce reactive oxygen species. Nevertheless, separate hydrogen peroxide-dependent and -independent pathways for NF-κB activation can clearly exist within the same cell, and exogenous hydrogen peroxide does not stimulate NF-κB in all cell lines. NF-κB is found predominantly in the cytoplasm as a heterodimer of p65 (Rel A) and p50, complexed to the inhibitory subunit I-κB. On stimulation, IκB dissociates, allowing NF-κB to migrate to the nucleus where it binds to DNA. In general, IκB dissociation involves its phosphorylation on two serines, which marks it for ubiquitinization and degradation, although tyrosine phosphorylation of IκB may also permit its dissociation from NF-κB without subsequent degradation. NF-κB activation by reactive oxygen species is inferred from inhibition by antioxidants, including overexpression of catalase but not superoxide dismutase. The apparent mediator of oxidant-induced activation of NF-κB is hydrogen peroxide, but its primary site of action remains unclear.

The AP-1 transcription factor is often composed of the two well-known protooncogene products c-Fos and c-Jun. These proteins are part of families that include Jun homologs and Fos-related activators (Fra), which form a variety of homo- and heterodimers that bind to DNA. There are two mechanisms whereby AP-1 becomes activated by oxidative stress. The first involves phosphorylation of the Jun proteins and their increased binding to DNA. The second involves the increased synthesis of Fos and Jun, so-called early response gene products. The binding of various combinations of the Fos/Jun family to AP-1 sites can either activate or inhibit transcription. Activation of AP-1 can be achieved through activation of the JNKs. Intriguingly, it appears that 4HNE, a product of lipid peroxidation, can directly activate JNK and increase transcription of 4HNE-responsive genes through Jun dimers, independently of JNK phosphorylation. The Ras–Raf–Erk pathway, which is activated by physiologically relevant generation of reactive oxygen species, results in phosphorylation of Elk-1, a transcription factor, which enhances production of Fos (Fig. 6). In addition, oxidants affect AP-1 mediated transcription by inhibiting the binding of AP-1 to DNA. This is a process that is regulated by the protein Ref-1, which catalyzes the reduction of a critical sulfhydryl involved in DNA binding.

Antioxidant responsive elements were first described in the promoter/enhancer regions of rat GSH-*S*-transferase Ya subunit and NAD(P)H:quinone reductase genes. Although ARE appears to have the ability to regulate transcription in response to oxidative stress, the details are still largely unresolved. Depending on the species and gene, ARE contains imperfect or perfect AP-1 binding consensus sequences. The transcription factor that specifically binds to the ARE has not been conclusively identified. Although c-Fos/c-Jun heterodimers have been ruled out as the activators of transcription by ARE, the interaction of Jun family proteins with members of the Nrf family of transcription factors seems likely.

Conclusions

Oxidants clearly participate in signaling a multitude of cell functions. In terms of their production, metabolic fate, and chemistry, superoxide, hydrogen peroxide, and peroxynitrite have most, if not all, of the essential characteristics of second messengers. The effectors of oxidant signaling include calcium-dependent enzymes, phospholipases, members of the MAP kinase family, protein tyrosine phosphatases, and several transcription factors. At present, we are beginning to determine the sites at which the oxidants act. Thus far, many involve the reversible oxidation of critical protein cysteine residues in the active sites of signaling enzymes. Nonetheless, we have only begun the journey down this stimulating pathway of biological discovery.

Additional Reading

Babior, B. M. (1999). NADPH Oxidase: An update. *Blood* **93,** 1464–1476.
Burdon, R. H. (1995). Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radicals Biol. Med.* **18,** 775–794.

Darley-Usmar, V. M., McAndrew, J., Patel, R., Moellering, D., Lincoln, T. M., Jo, H., Cornwell, T., Digerness, S., and White, C. R. (1997). Nitric oxide, free radicals and cell signalling in cardiovascular disease. *Biochem. Soc. Trans.* **25,** 925–929.

Exton J. H. (1998). Phospholipase D. *Biochim. Biophys. Acta* **1436,** 105–115.

Finkel, T. (1998). Oxygen radicals and signaling. *Curr. Opin. Cell Biol.* **10,** 248–253.

Forman, H. J., and Cadenas, E. (1997). ''Oxidative Stress and Signal Transduction.'' Chapman & Hall, New York.

Hoyal, C. R., J. Giron-Calle, and Forman, H. J. (1998). The alveolar macrophage as a model of calcium signaling in oxidative stress. *J. Toxicol. Environ. Health B Crit. Rev.* **1,** 117–134.

Hunter, T. (1998). The phosphorylation of proteins on tyrosine: Its role in cell growth and disease. *Philos. Trans. R. Soc. London B Biol. Sci.* **353,** 583–605.

Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* **74,** 49–139.

Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radicals Biol. Med.* **22,** 269–285.

Torres, M. (1996). Mitogen-activated protein (MAP) kinases and signal transduction in myeloid cells. *Int. J. Pediatr. Hematol. Oncol.* **3,** 387–405.

Wolin, M. S. (1996). Reactive oxygen species and vascular signal transduction mechanisms. *Microcirculation* **3,** 1–17.

CHAPTER 22

Mechanisms of Antioxidant Defense against Nitric Oxide/Peroxynitrite

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NITRIC OXIDE/PEROXYNITRITE CAN MEDIATE OXIDATION, NITRATION, OR NITROSATION REACTIONS, LEADING TO IMPAIRED FUNCTION, TOXICITY, AND ALTERATIONS IN SIGNALING PATHWAYS. DEFENSE AGAINST SUCH REACTIONS IS IMPORTANT FOR PROTECTION OF NORMAL TISSUE, ESPECIALLY DURING INFLAMMATION. ANTIOXIDANT DEFENSE IS ALSO EXPLOITED BY MICROORGANISMS TO PROTECT THEMSELVES AGAINST OXIDATIVE CHALLENGES FROM THEIR HOST OR THE ENVIRONMENT. BIOLOGICAL ANTIOXIDANT DEFENSE IS ORGANIZED INTO THREE CATEGORIES: PREVENTION, INTERCEPTION, AND REPAIR. PREVENTION IS THE CONTROL OF THE FORMATION OF NITRIC OXIDE AND/OR SUPEROXIDE AND THUS OF PEROXYNITRITE. INTERCEPTION IS BY DIRECT REACTION WITH NITRIC OXIDE, SUPEROXIDE, AND PEROXYNITRITE, LEADING TO NONTOXIC PRODUCTS. REPAIR PROCESSES REMOVE DAMAGE PRODUCTS AND RESTITUTE INTACT BIOMOLECULES. THIS CHAPTER SUMMARIZES THESE GENERAL STRATEGIES AND PRESENTS SOME SPECIFIC EXAMPLES RELATED TO NITRIC OXIDE AND RELATED SPECIES.

Nitric Oxide, Peroxynitrite, and Oxidative Stress

Nitric oxide[1] is a free radical containing an unpaired electron in the highest occupied molecular orbital. Nitric oxide is not a potent prooxidant in its own right; prooxidant effects are mediated predominantly by reaction products of nitric oxide with other molecules. Peroxynitrite is produced by the diffusion-limited reaction of nitric oxide and superoxide anion. Peroxynitrite is stable, but on protonation to peroxynitrous acid (pK_a6.8) it decays to nitrate, with a rate constant of 1.3 s^{-1} at 25°C. Peroxynitrous acid is highly reactive, yielding oxidizing and nitrating species (see Beckman *et al.,* 1990; Koppenol *et al.,* 1992; Beckman, 1996; Koppenol, 1998; Murphy *et al.,* 1998). The combined effects of nitric oxide and peroxynitrite can be oxidation, nitration, as well as nitrosation reactions. Figure 1 shows some products of the reaction of peroxynitrite with biological molecules.

The balance between prooxidants and antioxidants is critical for survival and functioning of aerobic organisms. An imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage, has been called oxidative stress (Sies, 1985, 1986, 2000). Nitric oxide serves as a useful illustration of this point. At the level of the whole organism, the reactive chemistry of nitric oxide/peroxynitrite can be considered beneficial. In addition to nitric oxide maintaining vascular tone, nitric oxide and derived species

[1] The systematic names of nitric oxide (NO·) and nitrosoperoxycarbonate ($ONO_2CO_2^-$) are nitrogen monoxide and 1-carboxylato-2-nitrosodioxidane, respectively. The term peroxynitrite is sometimes used to refer to both the peroxynitrite anion proper ($ONOO^-$) and peroxynitrous acid (ONOOH); the IUPAC names are oxoperoxonitrate(1−) and hydrogen oxoperoxonitrate, respectively.

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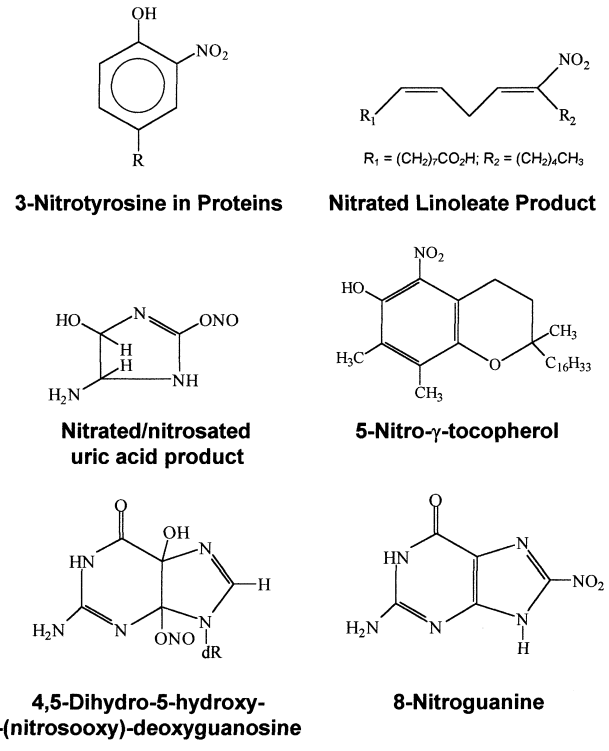


Figure 1 Examples of products formed by nitrating/nitrosating reactions of nitric oxide/peroxynitrite.

(e.g., peroxynitrite) are cytotoxic to bacteria (Zhu *et al.,* 1992) or other invading organisms. Inflammatory cells, such as macrophages and neutrophils, produce large amounts of both nitric oxide and superoxide which, in turn, rapidly form peroxynitrite (Ischiropoulos *et al.,* 1992; Huie and Padmaja, 1993; Kissner *et al.,* 1997). However, excessive production can damage normal tissue. Indeed, the formation of protein 3-nitrotyrosine (Fig. 1), an index of reactive nitrogen species (Ohshima *et al.,* 1990), has been shown in a number of inflammatory conditions, a situation in which nitric oxide/peroxynitrite production is often increased (Ischiropoulos, 1998). Although the reactions mediated by nitric oxide/peroxynitrite include both reactive nitrogen and oxygen species, for simplicity, an imbalance favoring the production of any of these species will be referred to here as oxidative stress.

The physiological and pharmacological strategies for antioxidant defense are organized in three categories: prevention, interception, and repair (see Sies, 1993). This chapter will summarize these general strategies and present specific examples for nitric oxide and related species (Table I; see also Arteel *et al.,* 1999a). Microorganisms have also developed strategies to protect against killing by phagocytes and other cell types; this field of research is expanding rapidly (see Haas and Goebel, 1992; Nunoshiba *et al.,* 1993; De Groote *et al.,* 1997; Farrant *et al.,* 1997; Liochev *et al.,* 1999).

Mechanisms of Defense against Nitric Oxide/Peroxynitrite

Prevention

The first line of defense against reactive species is prevention against their formation by physical or biochemical means. An example of a physical defense can be avoidance, such as plankton submerging from the surface of seawater to lower levels of solar irradiation. Further, organisms design barriers to protect sensitive areas from reactive species. Barriers can be molecules (e.g., melanin in the skin), cells (e.g., intestinal mucosal cells with high turnover rates), or even organs (e.g., the ''first pass effect'' of liver removing ingested toxins from the portal blood).

Biochemical prevention refers to the control of the level of prooxidant or antioxidant proteins as well as small molecules maintained by an organism. For example, the concentration of free metal ions in the mammalian body is kept in check by metal-binding proteins, such as ferritin, transferrin, and metallothionein. As described for nitric oxide and superoxide synthesis (see later in this chapter), enzymes that produce potential prooxidants are generally under tight control, often with redundant mechanisms.

PREVENTION OF NITRIC OXIDE FORMATION

Obviously, prevention of the formation of nitric oxide will lead to a decrease in nitric oxide-derived reactive species. During inflammation, the expression of inducible nitric oxide synthase (NOS II) is often upregulated, concomitant with an increase in NOS activity. Further, the expression of ''constitutive'' nitric oxide synthases (NOS I and III) can also be upregulated by inflammatory cytokines (Förstermann *et al.,* 1998). In this context, suppressing the immune response may decrease nitric oxide formation and have a protective effect. Prevention at this level can include decreasing the immune stimulus (e.g., infection), preventing inflammatory cell recruitment, and/or suppressing activation of inflammatory cells. For example, the anti-inflammatory steroid budesonide has been shown to be protective against nitric oxide/peroxynitrite-mediated reactions and to decrease protein nitrotyrosine formation in human asthmatic patients (Saleh *et al.,* 1998). Even in the presence of a stimulated immune response, nitric oxide synthase inhibitors (e.g., N^G-monomethyl-L-arginine; L-NAME) can block the generation of nitric oxide and are protective in models of tissue damage (Matheis *et al.,* 1992; Cuzzocrea *et al.,* 1998).

PREVENTION OF SUPEROXIDE FORMATION

Because superoxide reacts with nitric oxide to form peroxynitrite, decreasing the formation of superoxide can also be a protective measure against nitric oxide/peroxynitrite-induced oxidative stress. Inhibition of enzymes that generate superoxide, for example, xanthine oxidase or NADPH oxidase, can be protective by preventing peroxynitrite formation (White *et al.,* 1996). Further, impairment of the immune response may also have a beneficial effect in

Table I Defense against Oxidant Effects of Nitric Oxide, Superoxide, and Peroxynitrite

System	Prevention[a]	Interception[b] (direct)	Interception (indirect)	Support and repair[c]
Small molecules				
Anti-inflammatory agents (e.g., budesonide)	√			
Nitric oxide synthase inhibitors (e.g., L-NAME)	√			
Metal chelators	√			
NADPH-oxidase/xanthine oxidase inhibitors	√			
Nitric oxide scavengers (e.g., carboxy-PTIO)		√		
Thiols (e.g., GSH)		√	√	√
Carbon dioxide		√		
Ebselen		√	√	
Selenomethionine		√		
Organotellurium compounds		√		
Metalloporphyrins		√		
Uric acid		√	√	
Tocopherols		√	√	
Carotenoids		√?	√	
Flavonoids			√	
Ascorbate			√	√
Ubiquinol-10			√	
Nitric oxide			√	
Bilirubin			√	
Proteins				
Metallothionein	√			
Heme peroxidases (e.g., myeloperoxidase)		√	√	
Hemoglobin		√		
Selenoproteins (e.g., glutathione peroxidase)		√	√	√
Albumin		√		
Superoxide dismutase		√		
Catalase		√	√	
''Antioxidant network''				√
GSSG reductase				√
NADPH supply				√
Repair systems (e.g., DNA repair)				√

[a] Prevention suppresses the formation of nitric oxide and/or superoxide, and thereby peroxynitrite (see text).

[b] Interception refers to small molecules and proteins that react directly with nitric oxide, superoxide, and/or peroxynitrite (direct), or prooxidants derived from these species (indirect) (see text).

[c] Compounds involved in support and repair play a role in maintaining the antioxidative status of the cell and in repair of proteins and small molecules, once damaged by oxidative stress (see text).

decreasing superoxide production. It has been established that superoxide, directly or indirectly, can cause induction of the defensive SoxRS regulon in *Escherichia coli* (Liochev *et al.,* 1999).

PREVENTION OF PEROXYNITRITE FORMATION

Unlike the formation of nitric oxide and superoxide, peroxynitrite formation is an enzyme-independent process. Prevention of peroxynitrite formation thus focuses on preventing the production of nitric oxide and/or superoxide, as described above, or on intercepting nitric oxide and superoxide (see later in this chapter).

Interception

The main point of this level of defense is to intercept a damaging species, once formed, so as to exclude it from further activity. Further, interception often leads to transfer

of the prooxidant away from more sensitive compartments of the cell. For determining the detoxifying capacity of a direct reaction of a given compound with a prooxidant, it is useful to consider the rate constant of this reaction and the achievable concentrations (see Table II). For homogeneous systems, multiplication of the concentration of a given compound with the corresponding second-order rate constant for the reaction with the prooxidant yields the rate of its disappearance. This first-approximation approach was previously used for considering the reaction of peroxynitrite with CO_2, hemoglobin, and peroxidases (Lymar and Hurst, 1996; Squadrito and Pryor, 1998; Arteel *et al.,* 1999a).

In addition to the rate with which a putative antioxidant interception reaction occurs, it is also useful to consider whether this reaction can be catalytically maintained. For example, melatonin has been shown to react with peroxynitrite (Gilad *et al.,* 1997); however, this reaction leads to a loss of the parent molecule via oxidation reactions (Zhang *et al.,* 1998), requiring *de novo* synthesis of more melatonin by the organism. Further, given the low concentrations in the body (with the exception of the pineal gland), it is unlikely that the reaction of melatonin with peroxynitrite is a predominant reaction pathway. It is likely that the protective effect of melatonin observed in *in vivo* models of inflammation (Cuzzocrea *et al.,* 1997) is related to its indirect antioxidant effects [e.g., stimulation of superoxide dismutase (SOD) production and inhibition of nitric oxide production; for review, see Reiter *et al.,* 1998] and not direct interception.

Direct Reactions: Small Molecules

Scavengers of Nitric Oxide Exogenous scavengers of nitric oxide (e.g., 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; carboxy-PTIO) react with nitric oxide. This effect may prevent excessive concentrations of nitric oxide during inflammation and subsequent formation of more potent prooxidants such as peroxynitrite. Inactivators of nitric oxide have been shown to be protective in animal models of inflammation, decreasing nitrotyrosine formation and the severity of tissue damage (Hooper *et al.,* 1997).

Carbon Dioxide Considering the concentration of carbon dioxide *in vivo* (~1 m*M*) and the rate constant of its reaction with peroxynitrite (Table II; Lymar and Hurst, 1995; Denicola *et al.,* 1996), the formation of the adduct nitrosoperoxycarbonate ($ONO_2CO_2^-$) is most likely a major route of peroxynitrite reactivity *in vivo*. However, whether the carbon dioxide/peroxynitrite reaction is a detoxication pathway *in vivo* is unclear (Lymar and Hurst, 1996). For example,

Table II Interception of Peroxynitrite by Small Molecules and Proteins

Addition	Rate constant[a] ($M^{-1}s^{-1}$)	*In vivo* concentration (*M*)	Disappearance of peroxynitrite (s^{-1})
Spontaneous decay	—	—	0.4
Small molecules			
Carbon dioxide	3.0×10^4 (Denicola *et al.,* 1996)	1×10^{-3}	30
Glutathione	5.8×10^2 (Lee *et al.,* 1997)	1×10^{-2}[b]	5.8
Ascorbate	5.0×10^1 (Bartlett *et al.,* 1995)	1×10^{-2}[c]	0.5
Ebselen	2.0×10^6 (Masumoto *et al.,* 1996)	—	—
Metalloporphyrins	2×10^6 (Lee *et al.,* 1997, 1998a)	—	—
Proteins			
Myeloperoxidase	4.8×10^6 (Floris *et al.,* 1993)	5×10^{-4}[d]	2400
Hemoglobin	2.5×10^4 (Alayash *et al.,* 1998)	5×10^{-3}[e]	125
Glutathione peroxidase[f]	8.0×10^6 (Briviba *et al.,* 1998a)	2×10^{-6}[g]	16
Albumin	5.6×10^3 (Radi *et al.,* 1991a)	6×10^{-4}[h]	3.4

[a] Second-order rate constants are for pH 7.4, 25°C, with the exception of myeloperoxidase, which was at 12°C, and hemoglobin and albumin, which were at 37°C.

[b] Concentration in hepatocytes.

[c] Concentration in neutrophils.

[d] Concentration in neutrophils. The concentration is calculated from the amount of myeloperoxidase per neutrophil (~1.5×10^{-16} mol) and neutrophil volume (~300 fl).

[e] Concentration in erythrocytes.

[f] Rate constant for the reduced form of glutathione peroxidase. The rate constant for oxidized glutathione peroxidase is 7.4×10^5 M^{-1} s^{-1} at pH 7.4, 25°C (Briviba *et al.,* 1998a).

[g] Concentration in hepatocytes. The concentration is calculated from the amount of selenium in liver (~2.6 mg $liter^{-1}$) (Behne and Wolters, 1983), assuming 23% to be present as GPx (Burk and Gregory, 1982).

[h] Concentration in serum.

carbon dioxide enhances protein tyrosine nitration by peroxynitrite (Gow *et al.,* 1996). Further, whereas carbon dioxide decreases two-electron oxidation of thiols by peroxynitrite (Denicola *et al.,* 1996), one-electron oxidation reactions are enhanced (Scorza and Minetti, 1998). The latter pathway would lead to thiyl radical formation and subsequent radical chain reactions. Thus, it may be appropriate to call carbon dioxide a modifier of peroxynitrite reactions rather than a detoxifier.

Thiols and Uric Acid The formation of nitrosothiols by nitric oxide may be an important way to extend the lifetime of nitric oxide *in vivo* (Ignarro *et al.,* 1981; Marshall and Kontos, 1990; Stamler *et al.,* 1992a,b). This reaction may be considered as an interception in the context that nitrosothiol formation may allow nitric oxide to diffuse away from the site of synthesis, often colocalized with superoxide formation (Ischiropoulos *et al.,* 1992). This could thereby prevent the formation of peroxynitrite. S-Nitrosylated thiols may also cause oxidative modifications of proteins independent of the release of nitric oxide (Ji *et al.,* 1999). The interaction of peroxynitrite with free thiols in proteins (see later in this chapter) or in glutathione is also rapid enough (Table II; Radi *et al.,* 1991a) in view of their *in vivo* concentrations (millimolar range) to make this reaction significant (Koppenol *et al.,* 1992). Although the bulk of the reaction is a two-electron oxidation by peroxynitrite, at least a portion reacts via a one-electron pathway, leading to a sulfur-centered radical (Quijano *et al.,* 1997). Uric acid also reacts with peroxynitrite to form a vasoactive nitrated/nitrosated product (Fig. 1) and could thereby be a detoxication pathway of peroxynitrite (Skinner *et al.,* 1998).

Organoselenium Compounds: Ebselen Selenium-containing amino acids and the organoselenium compound ebselen (Fig. 2) rapidly react with peroxynitrite (Briviba *et al.,* 1996; Masumoto and Sies, 1996; Sies and Masumoto, 1997). The rate constant of the reaction of ebselen with peroxynitrite is $2.0 \times 10^6\ M^{-1}\ s^{-1}$ (25°C, pH 7.4; Masumoto *et al.,* 1996), and ebselen protects against DNA damage caused by peroxynitrite more effectively than their sulfur analogs (Roussyn *et al.,* 1996). The reaction scheme depicted in Fig. 3 applies generally to ebselen or to selenocysteine in free or protein-bound form, such as in glutathione peroxidase (see later in this chapter). Peroxynitrite is reduced to nitrite by these compounds; the resulting selenoxide is subsequently reduced by GSH, establishing a catalytic cycle so that the defense can be maintained in a peroxynitrite reductase reaction. Organotellurium compounds also protect against oxidation and nitration reactions caused by peroxynitrite (Briviba *et al.,* 1998b, 1999; Jacob *et al.,* 2000); bis(4-aminophenyl) telluride protects against peroxynitrite-mediated oxidation of dihydrorhodamine 123 more efficiently than its selenium analog or ebselen (Briviba *et al.,* 1998b).

Clinically, ebselen has been found to be protective in patients with delayed neurological deficits and aneurysmal subarachnoid hemorrhage (Saito *et al.,* 1998), in acute

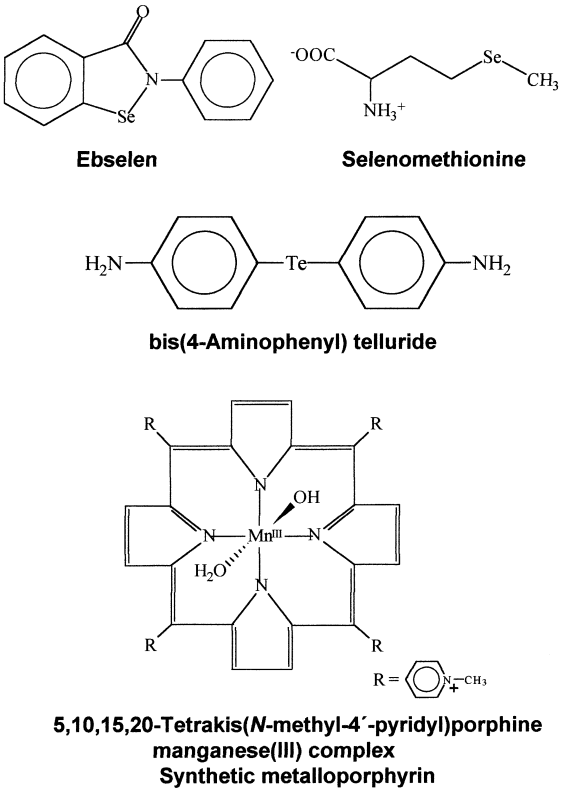


Figure 2 Structures of some low-molecular-weight compounds with high second-order rate constants for the reaction with peroxynitrite, including organoselenium, organotellurium, and synthetic metal porphyrin compounds (see Table II).

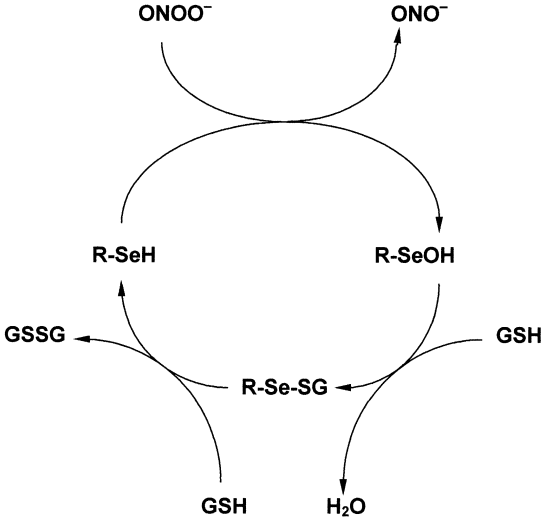


Figure 3 Proposed catalytic mechanism of selenoperoxidases in the reduction of peroxynitrite to nitrite (or peroxynitrous acid to nitrous acid). The mechanism is based on that established for glutathione peroxidases and the mimic, ebselen, which use ROOH and ROH as substrate and product, respectively (see Masumoto and Sies, 1996; Sies *et al.,* 1997). Selenomethionine can also react with peroxynitrite, with the resultant methionine selenoxide being reduced by glutathione in a catalytic cycle (Assmann *et al.,* 1998).

ischemic stroke (Yamaguchi *et al.,* 1998), and in acute middle cerebral artery occlusion (Ogawa *et al.,* 1999). The clinical development of ebselen has been reviewed (Parnham and Sies, 2000). Because increased nitrotyrosine levels were found to be associated with such neurological disorders, these protective effects of ebselen could be due in part to peroxynitrite defense.

Metalloporphyrins Synthetic metalloporphyrins (Fig. 2) may defend against prooxidant effects of nitric oxide/peroxynitrite at several levels. First, they are superoxide dismutase mimics, and thereby may decrease the amount of peroxynitrite formed. They also react with peroxynitrite at second-order rate constants similar to that of ebselen (Table II; Stern *et al.,* 1996; Lee *et al.,* 1997). The metal in the porphyrin shown in Fig. 2 can be manganese(III) or iron(III). Both compounds catalyze the isomerization of peroxynitrite to nitrate. It is proposed that peroxynitrite attacks the metal, and the manganese(IV) can be reduced by thiols or ascorbate (Lee *et al.,* 1997, 1998a); on the other hand, the respective iron(IV) compound catalyzes its own reduction (Stern *et al.,* 1996; Lee *et al.,* 1998b). Synthetic porphyrins have been shown to be cytoprotective against peroxynitrite *in vitro* (Misko *et al.,* 1998) and to alleviate some of the toxic effects of endotoxic shock in rats (Zingarelli *et al.,* 1997). It should be mentioned that nitrogen dioxide can be released during the reaction process (Lee *et al.,* 1997, 1998b), and that these compounds may enhance nitration reactions of peroxynitrite (Ferrer-Sueta *et al.,* 1997), so that toxicity might become an issue.

DIRECT REACTIONS: PROTEINS

Heme Peroxidases The mammalian heme peroxidases (e.g., myeloperoxidase; Floris *et al.,* 1993) also react with peroxynitrite (Table II). Although the concentration of myeloperoxidase in blood is low relative to more abundant biomolecules that react with peroxynitrite (e.g., CO_2; Squadrito and Pryor, 1998), concentrations are much higher in inflammatory cells (e.g., neutrophils; Table II). Further, localized extracellular concentrations near inflammatory cells during the respiratory burst may be high enough to react with peroxynitrite. In plants, the reaction between horseradish peroxidase and peroxynitrite may also be significant; the rate constant is $3.2 \times 10^6\ M^{-1}\ s^{-1}$ at pH 6.8, 25°C (Floris *et al.,* 1993). The reaction of horseradish peroxidase can be catalytically maintained using chlorogenic acid as a reductant (Grace *et al.,* 1998). However, both myeloperoxidase and horseradish peroxidase increase formation of nitrotyrosine by peroxynitrite (Sampson *et al.,* 1996).

Hemoglobin Oxyhemoglobin reacts with nitric oxide to methemoglobin and nitrate via a short-lived intermediate peroxynitrite complex (Herold, 1998), and it may play an important role in regulating concentrations of nitric oxide in blood. The biology and chemistry of the interaction of NO and hemoglobin are reviewed in more detail elsewhere (see Stamler and McMahon, Chapter 15, this volume). Hemoglobin also reacts rapidly with peroxynitrite and is suitable for efficient protection of erythrocytes. However, hemoglobin is not able to fully protect the plasma membrane of erythrocytes; when peroxynitrite was generated outside the cells, hemolysis was found to occur (Kondo *et al.,* 1997).

Selenoproteins The selenocysteine-containing glutathione peroxidase (GPx) can act as a peroxynitrite reductase, preventing oxidation and nitration reactions caused by peroxynitrite (Sies *et al.,* 1997). Glutathione peroxidase reduces peroxynitrite to nitrite using GSH in a catalytic reaction at a stoichiometry of 1:2, the same as that of hydroperoxide reduction, that is, the classic GPx reaction (Fig. 3), and similar to that described above for ebselen, and also for selenomethionine. Increases in nitrite during exposure to peroxynitrite were observed with GPx (Sies *et al.,* 1997), indicating two-electron reduction of peroxynitrite; however, the nitrite yield was less than complete (~50%). The second-order rate constant for the reaction of glutathione peroxidase (tetrameric) with peroxynitrite is $8.0 \times 10^6\ M^{-1}\ s^{-1}$ (Briviba *et al.,* 1998a). Although there is no net loss of GPx activity when GPx is maintained in the reduced state by supplying reductants (Sies *et al.,* 1997; Briviba *et al.,* 1998a), GPx is inactivated in the absence of GSH (Padmaja *et al.,* 1998) or on exposure to nitric oxide donors (Asahi *et al.,* 1997). These data suggest that inside the cell, GPx outcompetes thiols for the direct reaction with peroxynitrite (Table II). It was also shown that increasing the level of selenoproteins (e.g., GPx 14-fold) by selenium supplementation attenuated mitogen-activated protein kinase (p38, JNK1/2, and ERK1/2) activation by peroxynitrite in WB-F344 rat liver cells (Schieke *et al.,* 1999). Selenium supplementation also prevents decreases in gap junctional intracellular communication caused by peroxynitrite (Sharov *et al.,* 1999). Thus, the reaction of GPx with peroxynitrite is considered a biologically efficient detoxication pathway *in vivo.*

Selenoprotein P in human plasma also protects against peroxynitrite (Arteel *et al.,* 1998), suggesting that it may serve as a protectant against peroxynitrite in human blood. The heparin-binding domains of selenoprotein P enable surface coating of cellular membranes (e.g., endothelial cells; Wilson and Tappel, 1993; Burk *et al.,* 1997). This coating may serve as a protective barrier against peroxynitrite. Selenoprotein P was also shown to serve a function as an extracellular phospholipid hydroperoxide glutathione peroxidase using thiols as reductants (Saito *et al.,* 1999); this function may also make selenoprotein P important in removal of potential free radical products of nitric oxide/peroxynitrite (see later in this chapter).

The selenoprotein thioredoxin reductase can function in the reduction of peroxynitrite by selenocysteine or ebselen (Arteel *et al.,* 1999b). Although the thioredoxin system (NADPH, thioredoxin reductase, and thioredoxin) has been shown to directly reduce lipid hydroperoxides (Mitsui *et al.,* 1992; Björnstedt *et al.,* 1995), the reaction rate is relatively

slow. However, the ability of the thioredoxin system to reduce more potent antioxidants may be biologically significant. For example, ascorbate (Mendiratta *et al.,* 1998), glutathione peroxidases (Björnstedt *et al.,* 1994), and thioredoxin peroxidases (Cha and Kim, 1995) are reduced by the thioredoxin system. In this context, the thioredoxin system can contribute to antioxidant defense and play a specific role in peroxynitrite defense.

Selenomethionine (Fig. 2) is oxidized to the selenoxide by peroxynitrite with a second-order rate constant approximately 100-fold higher than that for the reaction of methionine with peroxynitrite (Padmaja *et al.,* 1996). Methionine selenoxide is effectively and rapidly reduced to selenomethionine by glutathione, permitting a catalytic reaction by selenomethionyl residues in proteins (Assmann *et al.,* 1998). In contrast, methionine sulfoxide is not reduced by glutathione; the enzyme methionine sulfoxide reductase is necessary for the reduction of methionine sulfoxide to methionine (Levine *et al.,* 1996). Because selenomethionine can occur in proteins such as hemoglobin (Beilstein and Whanger, 1986), these residues may play a defensive role against peroxynitrite. However, work by Hondal *et al.* (1999) failed to show protection against peroxynitrite by selenomethionine residues in albumin and IgG.

Albumin The reaction product of nitric oxide and the reactive cysteine in albumin is highly stable and may increase the diffusion distance of nitric oxide *in vivo* (see earlier in this chapter; Stamler *et al.,* 1992a,b). Further, the high concentration of albumin in blood (millimolar) makes its reaction with peroxynitrite significant (Table II; Radi *et al.,* 1991a). Whereas the reaction of peroxynitrite with albumin is largely due to the one free cysteine residue (Radi *et al.,* 1991a), human albumin also contains six tyrosine residues adjacent to glutamate residues, which are preferentially nitrated by peroxynitrite (Beckman, 1996). Indeed, oxidized (Scorza and Minetti, 1998) and nitrated (e.g., Arteel *et al.,* 1998) products of albumin are observable when peroxynitrite is added to plasma.

Superoxide Dismutase Superoxide dismutase decreases superoxide levels (McCord and Fridovich, 1969) and thereby can prevent peroxynitrite formation (White *et al.,* 1994). Expression of superoxide dismutase can be induced by proinflammatory cytokines (Wong and Goeddel, 1988), possibly conferring protection to normal tissue during inflammation. Furthermore, superoxide dismutase reacts with peroxynitrite, catalyzing the nitration of tyrosine residues (MacMillan-Crow *et al.,* 1996, 1998). However, this reaction inactivates Mn-SOD (MacMillan-Crow *et al.,* 1998) and has been linked to renal allograft failure in humans (MacMillan-Crow *et al.,* 1996); this reaction therefore may well not be a detoxification pathway.

In contrast to Mn-SOD, nitration of Cu,Zn-SOD by peroxynitrite does not inactivate the protein (Smith *et al.,* 1992). The glycosylated extracellular Cu,Zn-containing SOD (ecSOD) binds glycosaminoglycans (Karlsson *et al.,* 1988) and coats endothelial cells (Karlsson and Marklund, 1989). This mechanism may also be a defensive barrier in endothelial cells, analogous to that discussed earlier for selenoprotein P. The expression of ecSOD is upregulated in atherosclerotic plaques, and it is colocalized with iNOS and modified lipids (Luoma *et al.,* 1998). Further, Cu,Zn-SOD has been shown to reversibly convert nitric oxide to nitroxyl anion (NO^-) (Murphy and Sies, 1991). Analogous to nitrosothiol formation (see earlier in this chapter), this reaction may allow nitric oxide to diffuse away from the site of synthesis, contributing to the prevention of peroxynitrite formation.

INDIRECT REACTIONS

In addition to direct interception of prooxidants, biological molecules can react with products of oxidative stress and thereby play a role in defense. Further, there are proteins and molecules that have supportive roles in maintaining the antioxidative status of the cell, forming a veritable antioxidant network.

Glutathione and Ascorbate In addition to reacting directly with nitric oxide and peroxynitrite, glutathione (see earlier in this chapter) is a potent intracellular reductant and helps to maintain catalytic antioxidant reactions (e.g., coupled with glutathione peroxidase as discussed above; see Fig. 3). For a general discussion on the role of glutathione in cellular functions, see Sies (1999). Ascorbate has a low second-order rate constant for the reaction with peroxynitrite (Table II; Bartlett *et al.,* 1995) and is relatively unreactive with nitric oxide (Jackson *et al.,* 1998). Even with millimolar concentrations in cells, it is therefore unlikely that ascorbate plays a significant role in direct reactions with peroxynitrite or nitric oxide. However, in reducing chain-breaking antioxidants, such as tocopherol (Leung *et al.,* 1981; Scarpa *et al.,* 1984; Niki, 1987; Wefers and Sies, 1988), oxidized metalloporphyrin compounds (Lee *et al.,* 1997, 1998a), or heme-containing proteins attacked by nitric oxide/peroxynitrite, ascorbate can play an important role in antioxidant defense against nitric oxide/peroxynitrite.

Flavonoids, Carotenoids, and Tocopherols Flavonoids are natural products found in plants, and these polyphenols are ingested with the diet. Flavonoids are general free radical scavengers, and they chelate transition metals (Morel *et al.,* 1993; Jovanovic *et al.,* 1994; De Groot and Rauen, 1998); both functions may indirectly assist in defense against oxidative stress caused by nitric oxide/peroxynitrite. Flavonoids also react with nitric oxide (van Acker *et al.,* 1995), superoxide (Robak and Gryglewski, 1988; Girard *et al.,* 1995), and protect against oxidation and nitration reactions caused by peroxynitrite (Haenen *et al.,* 1997; Pannala *et al.,* 1997; Arteel and Sies, 1999). It is likely that these compounds react with nitrating and oxidizing intermediate species formed during peroxynitrite decay, and not with peroxynitrite

proper, similar to simple phenolic compounds (Ramezanian *et al.,* 1996). However, due to their protective effects, they can thereby can play a role in nitric oxide/peroxynitrite protection.

Carotenoids (e.g., β-carotene) are also potent general antioxidants (Stahl and Sies, 1994), reacting with nitric oxide and superoxide (Zhao *et al.,* 1998), and they protect against reactions of peroxynitrite and nitrogen dioxide (Kikugawa *et al.,* 1997; Zhao *et al.,* 1998). In addition to their free radical chain-breaking abilities, tocopherols (e.g., γ-tocopherol) react with nitric oxide (De Groot and Sies, 1993) and protect against peroxynitrite and other reactive nitrogen species (Hogg *et al.,* 1993; Christen *et al.,* 1997; Fig. 1), and they may assist in protecting lipid membranes from attack by nitric oxide/peroxynitrite. As β-carotene and γ-tocopherol are chemically modified by the reaction with peroxynitrite (Kikugawa *et al.,* 1997; Christen *et al.,* 1997), the question arises whether there is a possibility for regeneration of these micronutrients, which occurs only at very low concentrations in cells and tissues.

General Free Radical Scavengers As mentioned previously, the reaction of peroxynitrite with biological molecules (e.g., thiols) can lead to the formation of free radicals and initiate radical chain reactions. Therefore, free radical scavengers not specific to nitric oxide, superoxide, or peroxynitrite (e.g., ubiquinol-10, and bilirubin; Table 1) may also play a role in defense against damage due to peroxynitrite. α-Tocopherol is an effective chain-breaking antioxidant and may help limit lipid peroxidation due to oxidative stress caused by nitric oxide/peroxynitrite. It is of interest that whereas products of nitric oxide may stimulate lipid peroxidation (Radi *et al.,* 1991b), nitric oxide itself can also serve as a radical chain-breaking antioxidant during lipid peroxidation (Rubbo *et al.,* 1994; Gutierrez *et al.,* 1996).

Proteins Proteins can also play a role in indirectly decreasing the prooxidant effects of nitric oxide/peroxynitrite. For example, hydroperoxides and lipid hydroperoxides are often products formed during oxidative stress, for example, during excessive nitric oxide/peroxynitrite production (Radi *et al.,* 1991b). In this context, peroxidases and catalase can play a major role in defense (Chance *et al.,* 1979). Given that heme peroxidases and selenoperoxidases react directly with peroxynitrite, these enzymes play a dual role in antioxidant defense.

Another important ancillary protein is GSSG reductase, which maintains the glutathione pool in the reduced state at the expense of NADPH. In addition to the reactions mentioned previously, the thioredoxin system also helps maintain cellular thiol pools in the reduced state at the expense of NADPH (Björnstedt *et al.,* 1997). By extension, proteins responsible for maintaining cellular energy status and NADPH supply are also important. An illustration of this latter point is the fact that cells depleted of their glycogen reserves are more prone to oxidant insults than glycogen-replete cells (Smith *et al.,* 1987). As mentioned previously, proteins involved in chelating free metals constitute an indirect antioxidant defense, especially considering the fact that reactions of nitric oxide and peroxynitrite can be catalyzed by metals. Lastly, transport systems responsible for removing reactive compounds from the cells are also important, for example, the glutathione *S*-transferases and the transport systems for glutathione *S*-conjugates (Sies and Ketterer, 1988).

Repair

Protection from the effects of oxidative stress can be by repair of damage once it has occurred or by protection from further damage (e.g., 8-oxo-7,8-dihydroguanine). As prevention and interception processes are not completely effective, damage products could continuously form in low yields during excessive nitric oxide/peroxynitrite generation and may accumulate. Damaged biomolecules include the following: DNA, occurring as modified bases (Yermilov *et al.,* 1995; Douki and Cadet, 1996; Douki *et al.,* 1996; Spencer *et al.,* 1996; Fig. 1) or in the form of single- or double-strand breaks (Szabó and Ohshima, 1997) leading to increased mutagenicity (Juedes and Wogan, 1996), similar in pattern to that observed with singlet oxygen (Ribeiro *et al.,* 1992); membranes, occurring as phospholipid oxidation and nitration products (Fig. 1; O'Donnell *et al.,* 1999); and proteins, occurring as oxidized and nitrated amino acid side chains. Correspondingly, there are biological systems that are capable of providing the functions of restitution or replenishment. 3-Nitrotyrosine denitrase activity has been identified in rat tissues (Kamisaki *et al.,* 1998), enabling cells to remove this damage from proteins.

Biomolecules damaged by nitric oxide/peroxynitrite may not be easily repaired. Under these conditions, the degradation of damaged molecules may be enhanced. For example, modification of lipoproteins by nitric oxide/peroxynitrite leads to recognition and uptake by macrophages, increasing their turnover rate relative to unmodified lipoproteins (Graham *et al.,* 1993; da Silva and Abdalla, 1998). The degradation of proteins such as aconitase by proteasomes is enhanced on treatment with peroxynitrite (Grune *et al.,* 1998). The emerging and active field of research on repair of nitric oxide/peroxynitrite-induced damage is not reviewed in detail here.

Concluding Remarks

Antioxidant defense against nitric oxide/peroxynitrite occurs by multiple strategies and at various levels. Further, the compartmental nature of cell systems may make one type of defense more prevalent than others in different areas of the cell. Understanding of general principles of antioxidant defense, as well as mechanisms of defense specific against certain reactive species, can be used as a predictive tool for pharmacologic measures that may be useful in models of overproduction of nitric oxide/peroxynitrite.

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References

Alayash, A. I., Ryan, B. A., and Cashon, R. E. (1998). Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins. *Arch. Biochem. Biophys.* **349,** 75–73.

Arteel, G. E., and Sies, H. (1999). Protection against peroxynitrite by cocoa polyphenol oligomers. *FEBS Lett.* **462,** 167–170.

Arteel, G. E., Mostert, V., Oubrahim, H., Briviba, K., Abel, J., and Sies, H. (1998). Protection by selenoprotein P in human plasma against peroxynitrite-mediated oxidation and nitration. *Biol. Chem.* **379,** 1201–1205.

Arteel, G. E., Briviba, K., and Sies, H. (1999a). Protection against peroxynitrite. *FEBS Lett.* **445,** 226–230.

Arteel, G. E., Briviba, K., and Sies, H. (1999b). Function of thioredoxin reductase as a peroxynitrite reductase using selenocystine or ebselen. *Chem. Res. Toxicol.* **12,** 264–269.

Asahi, M., Fujii, J., Takao, T., Kuzuya, T., Hori, M., Shimonishi, Y., and Taniguchi, N. (1997). The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor. *J. Biol. Chem.* **272,** 19152–19157.

Assmann, A., Briviba, K., and Sies, H. (1998). Reduction of methionine selenoxide to selenomethionine by glutathione. *Arch. Biochem. Biophys.* **349,** 201–203.

Bartlett, D., Church, D. F., Bounds, P. L., and Koppenol, W. H. (1995). The kinetics of the oxidation of L-ascorbic acid by peroxynitrite. *Free Radicals Biol. Med.* **18,** 85–92.

Beckman, J. S. (1996). The physiological and pathophysiological chemistry of nitric oxide. *In* ''Nitric Oxide: Principles and Actions'' (J. Lancaster, ed.), pp. 1–82. Academic Press, San Diego.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Behne, D., and Wolters, W. (1983). Distribution of selenium and glutathione peroxidase in the rat. *J. Nutr.* **113,** 456–461.

Beilstein, M. A., and Whanger, P. D. (1986). Deposition of dietary organic and inorganic selenium in rat erythrocyte proteins. *J. Nutr.* **116,** 1701–1710.

Björnstedt, M., Xue, J., Huang, W., Åkesson, B., and Holmgren, A. (1994). The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *J. Biol. Chem.* **269,** 29382–29384.

Björnstedt, M., Hamberg, M., Kumar, S., Xue, J., and Holmgren, A. (1995). Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. *J. Biol. Chem.* **270,** 11761–11764.

Björnstedt, M., Kumar, S., Björkhem, L., Spyou, G., and Holmgren, A. (1997). Selenium and the thioredoxin and glutaredoxin systems. *Biomed. Environ. Sci.* **10,** 271–279.

Briviba, K., Roussyn, I., Sharov, V. S., and Sies, H. (1996). Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. *Biochem. J.* **319,** 13–15.

Briviba, K., Kissner, R., Koppenol, W. H., and Sies, H. (1998a). Kinetic study of the reaction of gluathione peroxidase with peroxynitrite. *Chem. Res. Toxicol.* **11,** 1398–1401.

Briviba, K., Tamler, R., Klotz, L.-O., Engman, L., Cotgreave, I. A., and Sies, H. (1998b). Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. *Biochem. Pharmacol.* **55,** 817–823.

Briviba, K., Klotz, L.-O., and Sies, H. (1999). Defenses against peroxynitrite. *Methods Enzymol.* **301,** 301–310.

Burk, R. F., and Gregory, P. E. (1982). Some characteristics of ^{75}Se-P found in rat liver and plasma, and comparison of it with selenoglutathione peroxidase. *Arch. Biochem. Biophys.* **213,** 73–80.

Burk, R. F., Hill, K. E., Boeglin, M. E., Ebner, F. F., and Chittum, H. S. (1997). Selenoprotein P associates with endothelial cells in rat tissues. *Histochem. Cell Biol.* **108,** 11–15.

Cha, M.-K., and Kim, I.-H. (1995). Thioredoxin-linked peroxidase activity from human red blood cell: Evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. *Biochem. Biophys. Res. Commun.* **217,** 900–907.

Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59,** 527–605.

Christen, S., Woodall, A. A., Shigenaga, M. K., Southwell-Keely, P. T., Duncan, M. W., and Ames, B. N. (1997). gamma-Tocopherol traps mutagenic electrophiles such as NO(x) and complements alpha-tocopherol: Physiological implications. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3217–3222.

Cuzzocrea, S., Zingarelli, B., Gilad, E., Hake, P., Salzman, A. L., and Szabó, C. (1997). Protective effect of melatonin in carrageenan-induced models of local inflammation: Relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging ability. *J. Pineal Res.* **23,** 106–116.

Cuzzocrea, S., Zingarelli, B., Hake, P., Salzman, A. L., and Szabó, C. (1998). Antiinflammatory effects of mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, in carrageenan-induced models of inflammation. *Free Radicals Biol. Med.* **24,** 450–459.

da Silva, E. L., and Abdalla, D. S. P. (1998). Increased metabolism of peroxynitrite-modified [^{125}I]β-VLDL by monocytes from hypercholesterolemic rabbits. *Biochem. Mol. Biol. Int.* **46,** 343–353.

De Groot, H., and Rauen, U. (1998). Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam. Clin. Pharmacol.* **12,** 249–255.

De Groot, H., and Sies, H. (1993). Loss of α-tocopherol upon exposure to nitric oxide or the sydnonimine SIN-1. *FEBS Lett.* **315,** 139–142.

De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y., and Fang, F. C. (1997). Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 13977–14001.

Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* **333,** 49–58.

Douki, T., and Cadet, J. (1996). Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radical Res.* **24,** 369–380.

Douki, T., Cadet, J., and Ames, B. N. (1996). An adduct between peroxynitrite and 2'-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2'-deoxyguanosine. *Chem. Res. Toxicol.* **9,** 3–7.

Farrant, J. L., Sansone, A., Canvin, J. R., Pallen, M. J., Langford, P. R., Wallis, T. S., Dougan, G., and Kroll, J. S. (1997). Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Mol. Microbiol.* **25,** 785–796.

Ferrer-Sueta, G., Ruiz-Ramirez, L., and Radi, R. (1997). Ternary copper complexes and manganese(III) tetrakis(4-benzoic acid) porphyrin catalyze peroxynitrite-dependent nitration of aromatics. *Chem. Res. Toxicol.* **10,** 1338–1344.

Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993). Interaction of myeloperoxidase with peroxynitrite. A comparison with lactoperoxidase, horseradish peroxidase and catalase. *Eur. J. Biochem.* **215,** 767–775.

Förstermann, U., Boissel, J.-P., and Kleinert, H. (1998). Expressional control of the "constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J.* **12,** 773–790.

Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A. L., and Szabó, C. (1997). Melatonin is a scavenger of peroxynitrite. *Life Sci.* **60,** PL169–PL174.

Girard, P., Sercombe, R., Sercombe, C., Le Lem, G., Seylaz, J., and Potier, P. (1995). A new synthetic flavonoid protects endothelium-derived relaxing factor-induced relaxation in rabbit arteries *in vitro:* Evidence for superoxide scavenging. *Biochem. Pharmacol.* **49,** 1533–1539.

Gow, A., Duran, D., Thom, S. R., and Ischiropoulos, H. (1996). Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch. Biochem. Biophys.* **333,** 42–48.

Grace, S. C., Salgo, M. G., and Pryor, W. A. (1998). Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA. *FEBS Lett.* **426,** 24–28.

Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V., and Moncada, S. (1993). Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **330,** 181–185.

Grune, T., Blasig, I. E., Sitte, N., Roloff, B., Haseloff, R., and Davies, K. J. A. (1998). Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J. Biol. Chem.* **273,** 10857–10862.

Gutierrez, H. H., Nieves, B., Chumley, P., Rivera, A., and Freeman, B. A. (1996). Nitric oxide regulation of superoxide-dependent lung injury: Oxidant-protective actions of endogenously produced and exogenously administered nitric oxide. *Free Radicals Biol. Med.* **21,** 43–52.

Haas, A., and Goebel, W. (1992). Microbial strategies to prevent oxygen-dependent killing by phagocytes. *Free Radical Res. Commun.* **16,** 137–157.

Haenen, G. R. M. M., Paquay, J. B. G., Korthouwer, R. E. M., and Bast, A. (1997). Peroxynitrite scavenging by flavonoids. *Biochem. Biophys. Res. Commun.* **236,** 591–593.

Herold, S. (1998). Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin. *FEBS Lett.* **439,** 85–88.

Hogg, N., Darley-Usmar, V. M., Wilson, M. T., and Moncada, S. (1993). The oxidation of alpha-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Lett.* **326,** 199–203.

Hondal, R. J., Motley, A. K., Hill, K. E., and Burk, R. F. (1999). Failure of selenomethionine residues in albumin and immunoglobulin G to protect against peroxynitrite. *Arch. Biochem. Biophys.* **371,** 29–34.

Hooper, D. C., Bagasra, O., Marini, J. C., Zborek, A., Ohnishi, S. T., Kean, R., Champion, J. M., Sarker, A. B., Bobroski, L., Farber, J. L., Akaike, T., Maeda, H., and Koprowski, H. (1997). Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: Implications for the treatment of multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2528–2533.

Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radical Res. Commun.* **18,** 195–199.

Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J., and Gruetter, C. A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of *S*-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.* **218,** 739–749.

Ischiropoulos, H. (1998). Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356,** 1–11.

Ischiropoulos, H., Zhu, L., and Beckman, J. S. (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* **298,** 446–451.

Jackson, T. S., Xu, A., Vita, J. A., and Keaney, J. F. (1998). Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ. Res.* **83,** 916–922.

Jacob, C., Arteel, G. E., Kanda, T., Engman, L., and Sies, H. (2000). Water-soluble organotellurium compounds: Catalytic protection against peroxynitrite and release of zinc from metallothionein. *Chem. Res. Toxicol.* **13,** 3–9.

Ji, Y., Akerboom, T. P., Sies, H., and Thomas, J. A. (1999). S-Nitrosylation and S-glutathiolation of protein sulfhydryls by *S*-nitrosoglutathione. *Arch. Biochem. Biophys.* **362,** 67–78.

Jovanovic, S. V., Steenken, S., Tosic, M., Marjanovic, B., and Simic, M. G. (1994). Flavonoids as antioxidants. *J. Am. Chem. Soc.* **116,** 4846–4851.

Juedes, M. J., and Wogan, G. N. (1996). Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat. Res.* **349,** 51–61.

Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 11584–11589.

Karlsson, K., and Marklund, S. L. (1989). Binding of human extracellular-superoxide dismutase C to cultured cell lines and to blood cells. *Lab. Invest.* **60,** 659–666.

Karlsson, K., Lindahl, U., and Marklund, S. L. (1988). Binding of human extracellular superoxide dismutase C to sulphated glycosaminoglycans. *Biochem. J.* **256,** 29–33.

Kikugawa, K., Hiramoto, K., Tomiyama, S., and Asano, Y. (1997). β-Carotene effectively scavenges toxic nitrogen oxides: Nitrogen dioxide and peroxynitrous acid. *FEBS Lett.* **404,** 175–178.

Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem. Res. Toxicol.* **10,** 1285–1292.

Kondo, H., Takahashi, M., and Niki, E. (1997). Peroxynitrite-induced hemolysis of human erythrocytes and its inhibition by antioxidants. *FEBS Lett.* **413,** 236–238.

Koppenol, W. H. (1998). The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radicals Biol. Med.* **25,** 385–391.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992). Peroxynitrite: A cloaked oxidant from superoxide and nitric oxide. *Chem. Res. Toxicol.* **5,** 834–842.

Lee, J. L., Hunt, J. A., and Groves, J. T. (1997). Rapid decomposition of peroxynitrite by manganese poryphyrin-antioxidant redox couples. *Bioorg. Med. Chem. Lett.* **7,** 2913–2918.

Lee, J. L., Hunt, J. A., and Groves, J. T. (1998a). Mechanisms of iron porphyrin reactions with peroxynitrite. *J. Am. Chem. Soc.* **120,** 7493–7501.

Lee, J. L., Hunt, J. A., and Groves, J. T. (1998b). Manganese porphyrins as redox-coupled peroxynitrite reductases. *J. Am. Chem. Soc.* **120,** 6053–6061.

Leung, H. W., Vang, M. J., and Mavis, R. D. (1981). The cooperative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. *Biochim. Biophys. Acta* **664,** 266–272.

Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15036–15040.

Liochev, S. I., Benov, L., Touati, D., and Fridovich, I. (1999). Induction of the *soxRS* regulon of *Escherichia coli* by superoxide. *J. Biol. Chem.* **274,** 9479–9481.

Luoma, J. S., Stralin, P., Marklund, S. L., Hiltunen, T. P., Sarkioja, T., and Yla-Herttuala, S. (1998). Expression of extracellular SOD and iNOS in macrophages and smooth muscle cells in human and rabbit atherosclerotic lesions: Colocalization with epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. *Arterioscler. Thromb. Vasc. Biol.* **18,** 157–167.

Lymar, S. V., and Hurst, J. K. (1995). Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J. Am. Chem. Soc.* **117,** 8867–8868.

Lymar, S. V., and Hurst, J. K. (1996). Carbon dioxide: Physiological catalyst for peroxynitrite-mediated cellular damage or cellular protectant? *Chem. Res. Toxicol.* **9,** 845–850.

MacMillan-Crow, L. A., Crow, J. P., Kerby, J. D., Beckman, J. S., and Thompson, J. A. (1996). Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11853–11858.

MacMillan-Crow, L. A., Crow, J. P., and Thompson, J. A. (1998). Peroxynitrite-mediated inactivation of manganese superoxide dismutase in-

volves nitration and oxidation of critical tyrosine residues. *Biochemistry* **37,** 1613–1622.

McCord, J. M., and Fridovich, I. (1969). Superoxide dismutase: An enzymatic function of erythrocuprein (hemocuprein). *J. Biol. Chem.* **244,** 6049–6055.

Marshall, J. J., and Kontos, H. A. (1990). Endothelium-derived relaxing factors. A perspective from *in vivo* data. *Hypertension* **16,** 371–386.

Masumoto, H., and Sies, H. (1996). The reaction of ebselen with peroxynitrite. *Chem. Res. Toxicol.* **9,** 262–267.

Masumoto, H., Kissner, R., Koppenol, W. H., and Sies, H. (1996). Kinetic study of the reaction of ebselen with peroxynitrite. *FEBS Lett.* **398,** 179–182.

Matheis, G., Sherman, M. P., Buckberg, G. D., Haybron, D. M., Young, H. H., and Ignarro, L. J. (1992). Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am. J. Physiol.* **262,** H616–H620.

Mendiratta, S., Qu, Z.-C., and May, J. M. (1998). Enzyme-dependent ascorbate recycling in human erythrocytes: Role of thioredoxin reductase. *Free Radicals Biol. Med.* **25,** 221–228.

Misko, T. P., Highkin, M. K., Veenhuizen, A. W., Manning, P. T., Stern, M. K., Currie, M. G., and Salvemini, D. (1998). Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. *J. Biol. Chem.* **273,** 15646–15653.

Mitsui, A., Hirakawa, T., and Yodoi, J. (1992). Reactive oxygen-reducing and protein-refolding activities of adult T cell leukemia-derived factor/human thioredoxin. *Biochem. Biophys. Res. Commun.* **186,** 1220–1226.

Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P., and Cillard, J. (1993). Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem. Pharmacol.* **45,** 13–19.

Murphy, M. E., and Sies, H. (1991). Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10860–10864.

Murphy, M. P., Packer, M. A., Scarlett, J. L., and Martin, S. W. (1998). Peroxynitrite: A biologically significant oxidant. *Gen. Pharmacol.* **31,** 179–186.

Niki, E. (1987). Interaction of ascorbate and alpha-tocopherol. *Ann. N.Y. Acad. Sci.* **498,** 186–199.

Nunoshiba, T., deRojas-Walker, T., Wishnok, J. S., Tannenbaum, S. R., and Demple, B. (1993). Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9993–9997.

O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. (1999). Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12,** 83–92.

Ogawa, A., Yoshimoto, T., Kikuchi, H., Sano, K., Saito, I., Yamaguchi, T., and Yasuhara, H. (1999). Ebselen in acute middle artery occlusion: A placebo-controlled, double-blind clinical trial. *Cerebrovasc. Dis.* **9,** 112–118.

Ohshima, H., Friesen, M., Brouet, I., and Bartsch, H. (1990). Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem. Toxicol.* **28,** 647–652.

Padmaja, S., Squadrito, G. L., Lemercier, J.-N., Cueto, R., and Pryor, W. A. (1996). Rapid oxidation of DL-selenomethionine by peroxynitrite. *Free Radicals Biol. Med.* **21,** 317–322.

Padmaja, S., Squadrito, G. L., and Pryor, W. A. (1998). Inactivation of glutathione peroxidase by peroxynitrite. *Arch. Biochem. Biophys.* **349,** 1–6.

Pannala, A. S., Rice-Evans, C. A., Halliwell, B., and Singh, S. (1997). Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem. Biophys. Res. Commun.* **232,** 164–168.

Parnham, M., and Sies, H. (2000). Ebselen: Prospective therapy for cerebral ischaemia. *Exp. Opin. Invest. Drugs* **9,** 607–619.

Quijano, C., Alvarez, B., Gatti, R. M., Augusto, O., and Radi, R. (1997). Pathways of peroxynitrite oxidation of thiol groups. *Biochem. J.* **322,** 167–173.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991a). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991b). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288,** 481–487.

Ramezanian, M. S., Padmaja, S., and Koppenol, W. (1996). Nitration and hydroxylation of phenolic compounds by peroxynitrite. *Chem. Res. Toxicol.* **9,** 232–240.

Reiter, R. J., Guerro, J. M., Garcia, J. J., and Acuna-Castroviejo, D. (1998). Reactive oxygen intermediates, molecular damage, and aging. Relation to melatonin. *Ann. N.Y. Acad. Sci.* **854,** 410–424.

Ribeiro, D. T., Madzak, C., Sarasin, A., Di Mascio, P., Sies, H., and Menck, C. F. M. (1992). Singlet oxygen induced DNA damage and mutagenicity in a single-stranded SV40-based shuttle vector. *Photochem. Photobiol.* **55,** 39–45.

Robak, J., and Gryglewski, R. J. (1988). Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **37,** 837–841.

Roussyn, I., Briviba, K., Masumoto, H., and Sies, H. (1996). Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. *Arch. Biochem. Biophys.* **330,** 216–218.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **269,** 26066–26075.

Saito, I., Asano, T., Sano, K., Takakura, K., Abe, H., Yoshimoto, T., Kikuchi, H., Ohta, T., and Ishibashi, S. (1998). Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage. *Neurosurgery* **42,** 269–278.

Saito, Y., Hayashi, T., Tanaka, A., Watanabe, Y., Suzuki, M., Saito, E., and Takahashi, K. (1999). Selenoprotein P in human plasma as an extracellular phospholipid hydroperoxide glutathione peroxidase. Isolation and enzymatic characterization of human selenoprotein P. *J. Biol. Chem.* **274,** 2866–2871.

Saleh, D., Ernst, P., Lim, S., Barnes, P. J., and Giaid, A. (1998). Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: Effect of inhaled glucocorticoid. *FASEB J.* **12,** 929–937.

Sampson, J. B., Rosen, H., and Beckman, J. S. (1996). Peroxynitrite-dependent tyrosine nitration catalyzed by superoxide dismutase, myeloperoxidase, and horseradish peroxidase. *Methods Enzymol.* **269,** 210–218.

Scarpa, M., Rigo, A., Maiorino, M., Ursini, F., and Gregolin, C. (1984). Formation of alpha-tocopherol radical and recycling of alpha-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes. An electron paramagnetic resonance study. *Biochim. Biophys. Acta* **801,** 215–219.

Schieke, S. M., Briviba, K., Klotz, L.-O., and Sies, H. (1999). Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: Attenuation by selenium supplementation. *FEBS Lett.* **448,** 301–303.

Scorza, G., and Minetti, M. (1998). One-electron oxidation pathway of thiols by peroxynitrite in biological fluids: Bicarbonate and ascorbate promote the formation of albumin disulphide dimers in human blood plasma. *Biochem. J.* **329,** 405–413.

Sharov, V. S., Briviba, K., and Sies, H. (1999). Peroxynitrite diminishes gap junctional communication: Protection by selenite supplementation. *IUBMB Life* **48,** 379–384.

Sies, H. (1985). Oxidative stress: Introductory remarks. *In* ''Oxidative Stress'' (H. Sies, ed.), pp. 1–8. Academic Press, London.

Sies, H. (1986). Biochemistry of oxidative stress. *Angew. Chem. Int. Ed. Engl.* **25,** 1058–1071.

Sies, H. (1993). Strategies of antioxidant defense. *Eur. J. Biochem.* **215,** 213–219.

Sies, H. (1999). Glutathione and its role in cellular functions. *Free Radicals Biol. Med.* **27,** 916–921.

Sies, H. (2000). What is oxidative stress? *In* ''Oxidative Stress and Vascular Diseases'' (J. F. Keaney, ed.), pp. 1–8. Kluwer Academic Publ., Boston.

Sies, H., and Ketterer, B., eds. (1988). ''Glutathione Conjugation. Mechanisms and Biological Significance.'' Academic Press, London.

Sies, H., and Masumoto, H. (1997). Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. *Adv. Pharmacol.* **38,** 229–246.

Sies, H., Sharov, V. S., Klotz, L.-O., and Briviba, K. (1997). Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *J. Biol. Chem.* **272,** 27812–27817.

Skinner, K. A., White, C. R., Patel, R., Tan, S., Barnes, S., Kirk, M., Darley-Usmar, V., and Parks, D. A. (1998). Nitrosation of uric acid by peroxynitrite: Formation of a vasoactive nitric oxide donor. *J. Biol. Chem.* **273,** 24491–24497.

Smith, C. D., Carson, M., van der Woerd, M., Chen, J., Ischiropoulos, H., and Beckman, J. S. (1992). Crystal structure of peroxynitrite-modified bovine Cu,Zn superoxide dismutase. *Arch. Biochem. Biophys.* **299,** 350–355.

Smith, P. F., Alberts, D. W., and Rush, G. F. (1987). Menadione-induced oxidative stress in hepatocytes isolated from fed and fasted rats: the role of NADPH-regenerating pathways. *Toxicol. Appl. Pharmacol.* **89,** 190–201.

Spencer, J. P. E., Wong, J., Jenner, A., Aruoma, O. I., Cross, C. E., and Halliwell, B. (1996). Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem. Res. Toxicol.* **9,** 1152–1158.

Squadrito, G. L., and Pryor, W. A. (1998). The nature of reactive species in systems that produce peroxynitrite. *Chem. Res. Toxicol.* **11,** 718–719.

Stahl, W., and Sies, H. (1994). Role of carotenoids in antioxidant defense. *In* ''Vitamin A in Health and Disease'' (R. Blomhoff, ed.), pp. 275–287. Dekker, New York.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalozo, J. (1992a). S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stamler, J. S., Singel, D. J., and Loscalozo, J. (1992b). Biochemistry of nitric oxide and its redox-activated forms. *Science* **258,** 1898–1902.

Stern, M. K., Jensen, M. P., and Kramer, K. (1996). Peroxynitrite decomposition catalysts. *J. Am. Chem. Soc.* **118,** 8735–8736.

Szabó, C., and Ohshima, H. (1997). DNA damage induced by peroxynitrite: Subsequent biological effects. *Nitric Oxide* **1,** 373–385.

van Acker, S. A. B. E., Tromp, M. N. J. L., Haenen, G. R. M. M., van der Vijgh, W. J. F., and Bast, A. (1995). Flavonoids as scavengers of nitric oxide radical. *Biochem. Biophys. Res. Commun.* **214,** 755–759.

Wefers, H., and Sies, H. (1988). The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. *Eur. J. Biochem.* **174,** 353–357.

White, C. R., Brock, T. A., Chang, L. Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., and Freeman, B. A. (1994). Superoxide and peroxynitrite in atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1044–1048.

White, C. R., Darley-Usmar, V., Berrington, W. R., McAdams, M., Gore, J. Z., Thompson, J. A., Parks, D. A., Tarpey, M. M., and Freeman, B. A. (1996). Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8745–8749.

Wilson, D. S., and Tappel, A. L. (1993). Binding of plasma selenoprotein P to cell membranes. *J. Inorg. Biochem.* **51,** 707–714.

Wong, G. H., and Goeddel, D. V. (1988). Induction of manganous superoxide dismutase by tumor necrosis factor: Possible protective mechanism. *Science* **242,** 941–944.

Yamaguchi, T., Sano, K., Takakura, K., Saito, I., Shinohara, Y., Asano, T., and Yasuhara, H. (1998). Ebselen in acute ischemic stroke: A placebo-controlled, double-blind clinical trial. Ebselen Study Group. *Stroke* **29,** 12–17.

Yermilov, V., Rubio, J., and Ohshima, H. (1995). Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett.* **376,** 207–210.

Zhang, H., Squadrito, G. L., and Pryor, W. A. (1998). The reaction of melatonin with peroxynitrite: Formation of melatonin radical cation and absence of stable nitrated products. *Biochem. Biophys. Res. Commun.* **251,** 83–87.

Zhao, W., Han, Y., Zhao, B., Hirota, S., Hou, J., and Xin, W. (1998). Effect of carotenoids on the repiratory burst of rat peritoneal macrophages. *Biochim. Biophys. Acta* **1381,** 77–88.

Zhu, L., Gunn, C., and Beckman, J. S. (1992). Bactericidal activity of peroxynitrite. *Arch. Biochem. Biophys.* **298,** 452–457.

Zingarelli, B., Day, B. J., Crapo, J. D., Salzman, A. L., and Szabó, C. (1997). The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock. *Br. J. Pharmacol.* **120,** 259–267.

CHAPTER 23

Regulation of Oxygen Metabolism by Nitric Oxide

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A CLASSIC PHYSIOLOGICAL PARADIGM WAS THE STABILITY OF MOLECULAR OXYGEN (O_2) UPTAKE UP TO VERY LOW HYPOXIC O_2 TENSIONS. THE NOTION WAS SUPPORTED BY THE VERY LOW K_m FOR O_2 OF MITOCHONDRIAL CYTOCHROME OXIDASE. RECENTLY, NITRIC OXIDE WAS SHOWN TO REVERSIBLY INHIBIT CYTOCHROME OXIDASE AFTER BINDING TO THE ENZYME WITH EVEN GREATER AFFINITY THAN O_2; HENCE, O_2 UPTAKE BECAME DEPENDENT ON THE O_2:NO RATIO. TO DEFINE A REGULATORY MECHANISM, IT WAS FURTHER REQUIRED THAT A FAST DISSOCIATION OF NO–CYTOCHROME COMPLEX FOLLOWING REMOTION OF NO COULD OCCUR. ACCORDINGLY, WE OBSERVED THAT NO ELICITS A STOICHIOMETRICAL PRODUCTION OF SUPEROXIDE ANION IN MITOCHONDRIA BY REACTING WITH MEMBRANE-BOUND UBIQUINOL; CONSEQUENTLY, NO LARGELY DECAYS AND INHIBITION OF CYTOCHROME OXIDASE IS RELEASED THROUGH THE OXIDATIVE ROUTE OF FORMATION OF PEROXYNITRITE, THE PRODUCT OF THE REACTION OF NO AND SUPEROXIDE RADICAL. THE OVERALL MECHANISM IS LIKELY A METABOLIC PATHWAY COMMON TO MANY PHYSIOLOGICAL PURPOSES EMERGING FROM THE EXISTENCE OF DIFFERENT NITRIC OXIDE SYNTHASE ISOFORMS.

Introduction: The Regulation of Mitochondrial Respiration

During evolution, first bacteria and then eukaryotic cells and multicellular organisms utilized molecular oxygen (O_2). Most of O_2 was taken up first by membranes and then by mitochondria, small intracellular organelles characterized by a high degree of metabolic and genetic autonomy. The reduction of molecular oxygen to water in mitochondria is coupled to the phosphorylation of ADP to ATP, the useful form of energy for cells.

The mitochondrial steady-state O_2 concentration in highly evolved mammals is sustained by a convenient O_2 gradient provided by convective mechanisms such as the cardiorespiratory system. On the basis of capillarity, cell shape and size, and O_2 diffusion, the intracellular pO_2 may differ considerably from one to another tissue (Gayeski and Honig, 1991). The affinity of mitochondrial respiration for O_2 is known to be very high, but its actual value, and consequently the role of intracellular pO_2 in the regulation of mitochondrial function, is a matter of controversy. The reaction of reduced cytochrome oxidase, the oxygen acceptor and terminal enzyme of the mitochondrial respiratory chain, with O_2 is very fast ($k = 10^8\ M^{-1}\ s^{-1}$, Greenwood and Gibson, 1967). The rate of electron transfer to cytochrome oxidase by the electron transfer chain is the key factor to define the operational O_2 concentration for a half-maximal rate of O_2 uptake, $K_{0.5}$, sometimes called K_{mO_2} (Boveris *et al.,* 1999). The intracellular oxygen concentration in mammalian tissues, which is in the 5–25 μM O_2 range, is close to and

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partially overlaps the critical concentration, which is in the 2–6 μM O_2 range, that limits the rate of mitochondrial respiration (Costa *et al.,* 1997).

The tissue pO_2 necessary to maintain high rates of mitochondrial respiration is a subject of interest, mainly within the framework of controversies about the control of mitochondrial respiration by O_2, and about whether mitochondrial function is O_2 limited in the tissues under physiological conditions. It has been claimed that maximal rates of respiration could be maintained at very low O_2 levels (Gayeski and Honig, 1991; Wittenberg and Wittenberg, 1989), with the relevant $K_{0.5}$ values being between 0.02 and 0.3 μM O_2 in experiments with isolated mitochondria (de Groot *et al.,* 1985; Sugano *et al.,* 1974). On the basis of this high affinity of mitochondrial respiration for O_2, the rates at which mitochondria perform oxidative phosphorylation were supposed to be rather independent of prevailing pO_2. The oxidative phosphorylation rates drop only below 0.8 μM O_2 (Weibel and Hoppeler, 1991); therefore, O_2 control of respiration was considered to be important only under severe hypoxia. However, more recent evidence obtained by high-resolution respirometry indicates $K_{0.5}$ values of 0.25–0.30 μM O_2 for state 4 mitochondria and 1.45–1.69 μM O_2 for state 3 mitochondria (Boveris *et al.,* 1999; Costa *et al.,* 1997). The relatively high value of $K_{0.5}$ for state 3 mitochondria is close enough to the physiological O_2 concentration to assume a critical dependence of mitochondrial respiration on tissue pO_2, understanding that the critical pO_2, by strict application of Michaelis–Menten kinetics, is the one that will limit mitochondrial O_2 uptake below 80% of the maximal rate.

The Paradigm of "All or Nothing" for Oxygen Uptake and Energy Supply

Since the pioneering work of Chance and others (Chance and Williams, 1955, 1956; Estabrook, 1967; Lardy and Wellman, 1952), it has been thought that the main regulation of O_2 uptake was provided by ADP availability. In the absence of ADP, but with excess substrate and O_2, isolated mitochondria take up O_2 at a relatively slow rate. The mitochondrial metabolic state, named resting state or state 4, depends on the proton permeability and energy leaks of the inner membrane of the isolated mitochondria. The physiological relevance of O_2 uptake, which may be close to useless, in this metabolic state for cell physiology is under debate. After addition of ADP, and in the presence of excess substrate and O_2, there is a rapid transition to the maximal physiological rate of respiration and ATP synthesis. In the active metabolic state or state 3, O_2 uptake increased by 5- to 10-fold, and ADP is converted to ATP at maximal speed by reacting with P_i in the phosphorylating ATPase system. State 3 respiration is understood to represent the physiological O_2 uptake that yields energy useful for cell function. The ratio of the rates of O_2 uptake in state 3 and/or state 4 respiration, or respiratory control ratios, as well as the P/O (the ratio of moles of ADP phosphorylated to total O consumed) are sensitive parameters to assess the coupling between the redox and the energy-conserving reactions in isolated mitochondria.

The interaction of ADP with mitochondrial ATPase or a high [ATP]/[ADP] ratio represents a feedback signal (Senior, 1988) consistent with a "simple and elegant model of respiratory control" (Heineman and Balaban, 1990). The net respiratory rate (state 3 minus state 4 oxygen uptake) after a pulse of ADP stimulation follows a hyperbolic relationship with ADP concentration, with $[ADP]_{0.5}$, the ADP concentration to induce a half-maximal respiratory rate, being 30 and 52 μM for liver and heart mitochondria, respectively (Boveris *et al.,* 1999). The initial concept for kinetic control of respiration by ADP availability (Chance and Williams, 1956; LaNoue *et al.,* 1986) has been reconsidered on the basis of taking as rate-limiting steps the ADP translocation from cytosol to mitochondria or the reduction of electron transfer intermediates in equilibrium with the external phosphorylation potential ([ADP] + $[P_i]$/[ATP]). Moreover, ^{31}P nuclear magnetic resonance (NMR) spectrometry failed to demonstrate significant changes in [ADP] or $[P_i]$ in the working heart (Clarke and Willis, 1987; Hassinen *et al.,* 1981), where it was determined that ADP concentration would have to increase by fivefold to sustain a threefold increase in myocardial respiration from resting to active respiration.

In addition, the redox state of the couple NADH/NAD as the regulatory coenzyme of mitochondrial dehydrogenases and cytosolic $[Ca^{2+}]$, in its double role as a universal dehydrogenase activator and as a cation able to discharge mitochondrial membrane potential, has been considered to play different roles in the regulation of O_2 metabolism. Nevertheless, the intramitochondrial concentration of reduction equivalents always appear very high when derived from dehydrogenase activity, but a dependence *in vivo* of cellular and mitochondrial O_2 uptake on substrate availability and mitochondrial redox state seems likely. In the "all or nothing" paradigm, mitochondrial O_2 uptake and the coupled ATP-producing reactions operate at maximal efficiency and rates over a wide range of O_2 concentrations that certainly include physiological conditions. Both O_2 uptake and energy production cease almost simultaneously in reaching anoxia or state 5. Considering the all or nothing paradigm, the rate of O_2 uptake of perfused rat organs (2.4 μmol O_2/min/g liver and 3.6 μmol O_2/min/g heart), and the tissue O_2 concentrations (about 20 μM in the liver and 6 μM in the heart), it can be estimated that the transition between normoxia and anoxia will be a rapid and abrupt phenomenon with a time course of 0.5 s in the liver and 0.1 s in the heart. Then, the absence of cellular and mitochondrial regulatory mechanisms for O_2 uptake will leave organelles and cells under an absolute dependence on tissue O_2 supply without a response time to adjust for sudden anoxia.

The Renaissance of Inorganic Nitric Oxide as a Compound of Great Biological Significance

Since 1987 (Ignarro *et al.,* 1987; Palmer *et al.,* 1987), studies on nitric oxide (NO) have characterized this small,

amphipathic nitrogen radical as an important physiological and pharmacological mediator (Bredt and Snyder, 1994; Lowenstein *et al.,* 1994). It is noteworthy that bacteria, which share a close genomic homology with mitochondria, include the synthesis and degradation of NO in the electron transfer process of nitrification–denitrification. Accordingly, the presence of NO oxidoreductases in species such as *Paracoccus denitrificans* was confirmed by the release of NO after exposure of bacteria to nitrites (Goretski *et al.,* 1990). A few years ago, a provocative question was whether mitochondrial electron transfer membranes had some relic pathways keeping the biochemical machinery involving utilization or production of NO. The answer to this question appears to be yes, after the identification of mitochondrial NO synthase (mtNOS) (Ghafourifar and Richter, 1997; Giulivi *et al.,* 1998, Tatoyan and Giulivi, 1998).

The Modulation of Cytochrome Oxidase Activity and Mitochondrial Oxygen Uptake by NO: The O_2–NO Competition

Different groups of investigators have recognized that NO inhibits mitochondrial respiration reversibly and dose dependently (Cleeter *et al.,* 1994; Brown and Cooper, 1994; Okada *et al.,* 1996; Poderoso *et al.,* 1996). This effect was observed in organelles from different tissues containing cyanide-sensitive cytochrome oxidase, whereas it was absent in cyanide-insensitive plant oxidases (Millar and Day, 1996). Formerly, the cytotoxic effects of activated macrophages on tumor cells or bacteria (Adams and Hamilton, 1984; Steinman and North, 1986) were thought to be due to inhibitory NO effects on iron–sulfur compounds and to iron loss at complexes I–II of the respiratory chain. Nevertheless, although NO is now considered a multisite inhibitor of electron transfer, the most impressive effects are related to the inhibition of cytochrome oxidase (complex IV) and to additional reactions involving the intermediates of complex III.

Nitric oxide, with estimated steady-state concentrations in mammalian tissues in the 0.02–0.1 μM range (Knowles *et al.,* 1990, Poderoso *et al.,* 1998), has been recognized as a high-affinity inhibitor of cytochrome oxidase, acting in competition with oxygen (Boveris *et al.,* 1999; Cleeter *et al.,* 1994; Koivisto *et al.,* 1997). The NO concentrations that produce a half-inhibition of cytochrome oxidase activity or O_2 uptake in isolated mitochondria are low, in the range of 80–200 nM. The effect was described in mitochondria and submitochondrial particles from rat gastrocnemius muscle (Cleeter *et al.,* 1994), rat liver (Takehara *et al.,* 1995), rat heart (Borutait'e and Brown, 1996; Cassina and Radi, 1996; Poderoso *et al.,* 1996), and brown fat (Koivisto *et al.,* 1997), in tissue preparations such as rat brain synaptosomes (Brown and Cooper, 1994), in skeletal muscle slices (Shen *et al.,* 1995), and in the isolated rat heart (Poderoso *et al.,* 1998). In addition, the observed increase in whole-body O_2 uptake in the conscious dog after the administration of NOS inhibitors (Shen *et al.,* 1994) is consistent with the NO inhibition of respiration observed in tissues and subcellular preparations.

The effects of NO supplementation on the cytochrome oxidase activity of rat heart submitochondrial particles, O_2 uptake of rat liver mitochondria, and O_2 uptake of perfused rat heart are shown in Fig. 1. Very low NO concentrations, in the 0.10–0.15 μM range, are capable of inhibiting cytochrome oxidase or O_2 uptake in mitochondrial preparations by 50%. The addition of NO to mitochondria produces an inhibition of O_2 uptake and a decrease in membrane potential, the latter detected by rhodamine fluorescence (Takehara *et al.,* 1995). The half-maximal effect of NO on membrane potential is observed in liver mitochondria at about 0.25 μM NO, and it is clearly a less sensitive indicator of NO effects than O_2 uptake as mentioned before.

The inhibition of O_2 uptake is also observed in perfused organs, where the steady-state intracellular NO concentration reached on infusion depends on the NO concentration in the infusion fluid, perfusate flow, NO tissue uptake, and

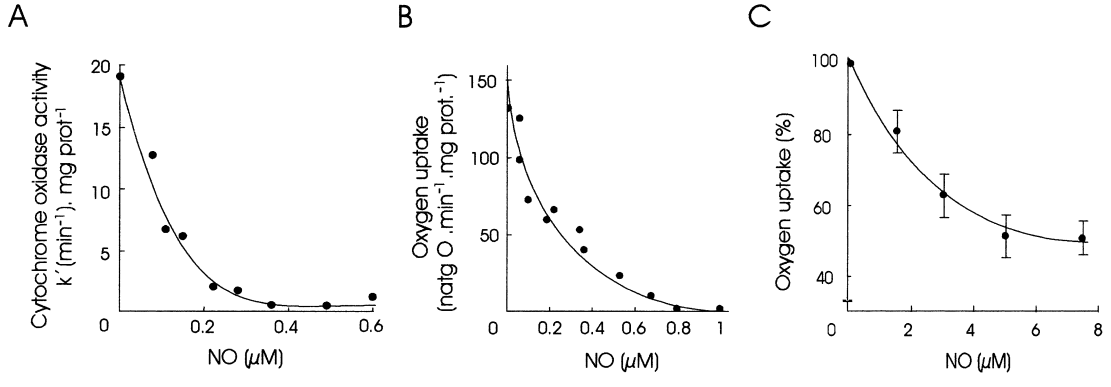


Figure 1 Inhibitory effects of nitric oxide on cytochrome oxidase activity of rat heart submitochondrial particles (A), on the state 3 oxygen uptake of rat liver mitochondria (B), and on the oxygen uptake of isolated and perfused beating rat heart (C). In C, the NO concentration in the solution infused into the rat heart coronary circulation is given in the abscissa. When NO was infused at a rate of 2 μM/min the calculated tissue NO concentration was 0.2 μM. Hence, NO-dependent 50% inhibition of oxygen uptake was comparable to the data in A and B.

NO binding to cellular and mitochondrial components such as myoglobin (Mb) and cytochrome oxidase. These two hemoproteins react with NO with great affinity, with nearly diffusion-limited rate constants ($k \approx 10^8$–$10^9\ M^{-1}\ s^{-1}$; Shen *et al.,* 1995). The presence of Mb significantly shortens the half-life of NO in the perfused heart and decreases NO steady-state concentration (Konorev *et al.,* 1996). In the NO-perfused heart, the tissue metmyoglobin (metMb) levels depend on NO infusion levels as shown by tissue spectral studies (Poderoso *et al.,* 1998). It is likely that, under physiological conditions, the main part of NO generated by endothelial NOS reacts with MbO_2, and a remnant NO diffuses to mitochondria. The diffusion process is analogous to that of oxygen, where O_2–Mb and O_2–cytochrome oxidase are the two complexes involved in O_2 transport to mitochondria (Kennedy and Jones, 1986). The reactivity of MbO_2 with NO and the metmyoglobin reductase activity of mucle tissue keep, by means of a sort of buffer action, the cellular and mitochondrial concentration of NO in a low, nontoxic range with a minimal effect on cytochrome oxidase activity. In this setting, muscle steady-state NO concentrations can be considered as equal to the NO concentration measured by diffusion equilibrium in the perfusate or in the expanded intracellular space, namely, 20 n*M* NO in rat diaphragm (Boczkowski *et al.,* 1999) and 100 n*M* in perfused heart after bradykinin stimulation (Poderoso *et al.,* 1998). These NO concentrations are similar to those that effectively inhibit cytochrome oxidase (Fig. 1).

The biological effect of a given NO concentration will depend on the simultaneous O_2 concentration; lowering the pO_2 increases and extends the effects of NO (Takehara *et al.,* 1996; Cassina and Radi, 1996). A series of reports indicated a competition between O_2 and NO for cytochrome oxidase reaction centers (Boveris *et al.,* 1999; Brown, 1995; Cassina and Radi, 1996; Zhang *et al.,* 1996). The inhibition of O_2 uptake by NO in isolated rat liver and heart mitochondria is consistent with an affinity of NO for cytochrome oxidase that is 150 times higher than the corresponding O_2 affinity (Boveris *et al.,* 1999). Similar O_2/NO competition ratios of 400–500 have been reported for rat brain synaptosomes (Brown and Cooper, 1994) and ratios of 500–1000 for brown fat mitochondria (Koivisto *et al.,* 1997). This point is of physiological interest because O_2 concentrations in myocardial cells have been reported as low as 3 to 8 μM O_2 (Gayeski and Honig, 1991), a condition in which a NO steady-state concentration of 50 n*M* would effectively compete with O_2, producing a cytochrome oxidase inhibition of about 30%. Detailed studies on the interactions of NO with the binuclear center of cytochrome oxidase are available (Torres *et al.,* 1995). In addition, some authors proposed that cytochrome oxidase may reduce NO to N_2O (Zhao *et al.,* 1995), whereas others were unable to observe such reducing activity (Stubauer *et al.,* 1998).

The inhibitory effects of NO on cytochrome oxidase activity and oxygen metabolism have been considered as a regulatory and adaptative physiological mechanism in response to changes in O_2 availability (Poderoso *et al.,* 1996, 1998). The effects have also been implicated in inflammatory, neurodegenerative, or ischemic diseases (Brown, 1997). To some extent, the two roles could be just two views of the same reversible effects of NO on cytochrome oxidase and of the role of NO in the generation of peroxynitrite ($ONOO^-$). Both effects arise from the ability of NO to react with superoxide anion (O_2^-), producing $ONOO^-$. The utilization of NO by the reaction of NO with O_2^- determines the intramitochondrial NO steady-state concentrations and the O_2:NO ratio that regulates cytochrome oxidase activity. Production of the powerful oxidant $ONOO^-$ is easily linked to mitochondrial and cellular toxic effects (Xie *et al.,* 1998; Boczkowski *et al.,* 1999). In this way, pathophysiology or toxicity depends on the amplification of a normal control pathway based on the intramitochondrial NO steady-state concentrations.

The Mitochondrial Production of Oxygen Active Species

Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are reactive oxygen species normally produced as by-products of mitochondrial respiration and as products of cytosolic and peroxisomal enzyme activities involving the univalent and bivalent reduction of oxygen. The discovery of superoxide dismutase (SOD) by McCord and Fridovich (1969) and its ubiquitous distribution in aerobic tissues indicated that O_2^- was a normal metabolite in mammalian cells (Fridovich, 1978). Moreover, it was observed that both O_2^- and H_2O_2 are produced in mitochondria and in the cytosol in specific ways. Accordingly, Mn-SOD and Cu,Zn-SOD are strictly localized in the mitochondrial and cytosolic compartments, respectively. The concentration of O_2^- is similar in mitochondria and in bacteria ($\sim 10^{-10}\ M$) (Boveris and Cadenas, 1997; Imlay and Fridovich, 1991), and it is higher than in the cytosol, where it is about $10^{-11}\ M$. The production of noncharged H_2O_2 by stoichiometric dismutation of O_2^- occurs in both compartments catalyzed by both SOD types with diffusion-limited rate reactions ($k = 1.9$–$2.3 \times 10^9\ M^{-1}\ s^{-1}$). Hydrogen peroxide can diffuse freely outside mitochondria and is partially utilized by glutathione peroxidase, and by peroxisomal catalase.

The production of O_2^- in mitochondria is a process that encompasses electron transfer and enzyme activities inherent to mitochondrial function and is considered a normal consequence of aerobic metabolism. In fact, diversion of electrons to reduce O_2 by one-electron transfer can be considered as a thermodynamically favored and kinetically unfavored process of the redox steady states of the components of the mitochondrial respiratory chain. Some intermediates such as ubisemiquinone and the flavin semiquinone of NADH dehydrogenase are kinetically able to undergo collisional and noncatalyzed reactions with one-electron transfer to the O_2 molecule (Boveris and Cadenas, 1997). In the past, before the utilization of NO, the mitochondrial production of O_2^-

was measured in submitochondrial particles devoid of superoxide dismutase and supplemented with nonphysiological compounds, such as antimycin or myxothiazol (Cadenas and Boveris, 1980).

The Mitochondrial Production of Superoxide Radical Is Stimulated by Nitric Oxide

The exposure of mitochondria or submitochondrial particles to NO initiates or markedly increases the mitochondrial production of both O_2^- and H_2O_2 (Poderoso *et al.,* 1996; Packer *et al.,* 1996). In the presence of 0.2–1 μ*M* NO, mitochondria isolated from almost all rat tissues have NO-dependent H_2O_2 production rates of 0.05 to 0.2 nmol/min/mg protein.

The dependence of the rate of H_2O_2 production in rat liver mitochondria on NO concentration is shown in Fig. 2A; addition of NO has a marked effect that saturates the mitochondrial respiratory chain at about 0.7 μ*M* NO, also reported as an inhibition of succinate–cytochrome *c* reductase activity (Poderoso *et al.,* 1996). The observed effect appears to express the reactions of (a) ubisemiquinone formation by the direct nonenzymatic reaction of UQH_2 with NO and $ONOO^-$ (Poderoso *et al.,* 1999a), (b) ubisemiquinone autoxidation to yield O_2^-, and (c) formation of H_2O_2 from O_2^-.

The amperometric determination of NO in a suspension of submitochondrial particles supplemented with the NO donor DETA-NO results in a model of NO steady-state levels in the mitochondrial matrix. The continuous release of NO by the donor provides a constant, quasi-equilibrium NO concentration that mimics the maintenance of an intramitochondrial NO steady-state concentration by the continuous NO production from mtNOS (Giulivi, 1998). Considering the inside-out topology of the submitochondrial particles in which the M side of the mitochondrial inner membrane is exposed to the reaction medium and the electrode, the conditions of the experiment in Fig. 2B afford a model of the intramitochondrial matrix space. With such view, the NO electrode is monitoring an expanded mitochondrial matrix. The NO-generating system was able to keep a constant steady-state level of about 0.5 μ*M* NO. The addition of succinate initiated active production of O_2^- in the already NO-supplemented mitochondrial membranes that decreased, through $ONOO^-$ formation, the steady-state concentration of NO to about 0.2 μ*M.* Addition of superoxide dismutase, which nearly eliminates O_2^- from the reaction mixture, restored the NO level to the initial values. Thus, the O_2^- and NO produced by the inner mitochondrial membrane are vectorially released into the matrix, where the steady-state levels of both are determined by the very fast reaction of $ONOO^-$ formation. This fast reaction is a classic free radical termination reaction in which two species with unpaired electrons react at diffusion-controlled rates ($k = 1.9 \times 10^{10}\ M^{-1}\ s^{-1}$) (Koppenol, 1998). The increased production of mitochondrial H_2O_2 under NO influence was also observed in the isolated beating rat heart, where H_2O_2 was released in the cardiac effluent depending on the infused NO concentration (Poeroso *et al.,* 1998).

The mechanistic basis for the NO-induced formation of intramitochondrial O_2^- and $ONOO^-$ include three cooperative actions: (a) NO inhibits the electron transfer chain in complex III at the cytochromes b–c_1 region (Poderoso *et al.,* 1996, 1998), increasing the levels of ubisemiquinone which on autoxidation reduces O_2 to O_2^-; (b) NO inhibits cytochrome oxidase and increases the reduction levels of the components of the electron transfer chain, including ubiquinol and ubisemiquinone, on the substrate side of cytochrome oxidase; and (c) NO reacts directly with ubiquinol to produce nitroxyl anion (NO^-) and ubisemiquinone (Poderoso *et al.,* 1999a,1999b). The occurrence of the latter reaction is illustrated in Fig. 3 with the use of ubiquinol-0 (UQ_0H_2). The rate of NO utilization after addition of ubiquinol, the formation of the semiquinone intermediate, and the spectral changes indicating the oxidation of the quinol to quinone by NO are shown in Fig. 3A–C, respectively. The one-electron transfer reaction of ubiquinol to NO has a rate

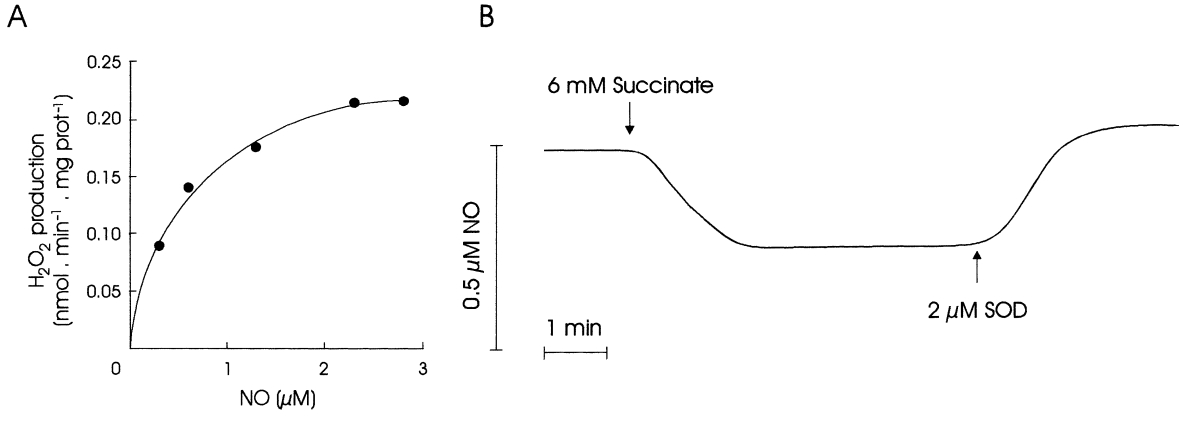


Figure 2 Nitric oxide increases H_2O_2 production in liver submitochondrial particles in the presence of succinate (A). The addition of succinate to liver submitochondrial particles decreases the NO steady-state concentration of the reaction medium that was released by 0.5 m*M* DETA-NO, and the effect is completely reversed by 2 μ*M* SOD (B).

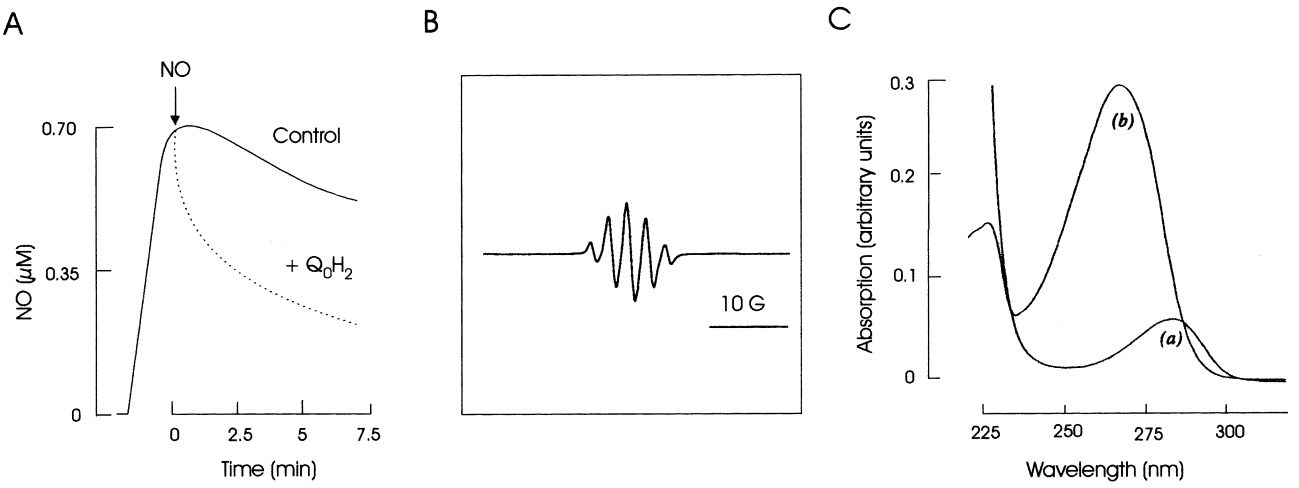


Figure 3 The NO-induced cohort of reactions leading to increased mitochondrial production of O_2^- and its dismutation product H_2O_2 are initiated by the reaction of NO with membrane ubiquinol, as shown in model reactions. A sensitive NO electrode shows the increased decay rate of a 0.7 μ*M* NO pulse after addition of 10 μ*M* UQ_0H_2 in anaerobiosis *in vitro,* at pH 5.5 (A). The reaction produces a ubisemiquinone intermediate detected by electron paramagnetic resonance (EPR) (amplitude 1 G, 50 kHz) (B). The ubisemiquinone on autoxidation forms O_2^-, and finally, as followed by UV spectral changes (C), the consecutive reactions oxidize UQH_2 (a) to UQ (b). Modified from *Free Radical Biology and Medicine* vol. 26, Poderoso *et al.,* "The reaction of nitric oxide with ubiquinol," p. 925; copyright 1999, with permission from Elsevier Science.

constant of 10^3–10^4 (Poderoso *et al.,* 1999a,1999b); nitroxyl anion (NO^-) is a reactive intermediate that forms nitrous oxide (N_2O) as a stable end product. It has been reported that the reaction of NO with mitochondrial cytochrome *c* also produces NO^- (Sharpe and Cooper, 1998). Hence, the reactions of NO with components or products of the mitochondrial respiratory chain are, as in bacteria, reductive and yield NO^-, or they are oxidative and yield $ONOO^-$.

The Rate of Mitochondrial NO Utilization

The rate of mitochondrial NO utilization can be determined through the amperometric monitoring of NO pulses added to mitochondria and submitochondrial particles (Poderoso *et al.,* 1999b). In this way, the time course of NO decay after the addition of 0.4 μ*M* NO pulses to rat liver submitochondrial particlesunder different experimental conditions were monitored to characterize the routes and rates of mitochondrial NO metabolism (Fig. 4A). In anaerobic conditions, NO was utilized at a rate of about 0.12 nmol/min/mg protein. It is apparent that under the assayed conditions, some reduced components of the mitochondrial respiratory chain, likely ubiquinol, cytochrome *c,* and cytochrome oxidase, are able to reduce NO to NO^-. In aerobic conditions, the NO pulses were utilized following first-order processes with rates of NO metabolism that linearly depended on NO concentrations in the range of 0.025–0.40 μ*M* NO. The rate of NO utilization corresponding to a maximal concentration of 0.2 μ*M* NO, in the physiological NO range, and in the presence of succinate as mitochondrial reductant, was 1.0 nmol NO/min/mg protein (Fig. 4B). Submitochondrial particles supplemented with 2 μ*M* ubiquinone (UQ_0) to expand the ubiquinol pool showed an increased rate of NO utiliza-

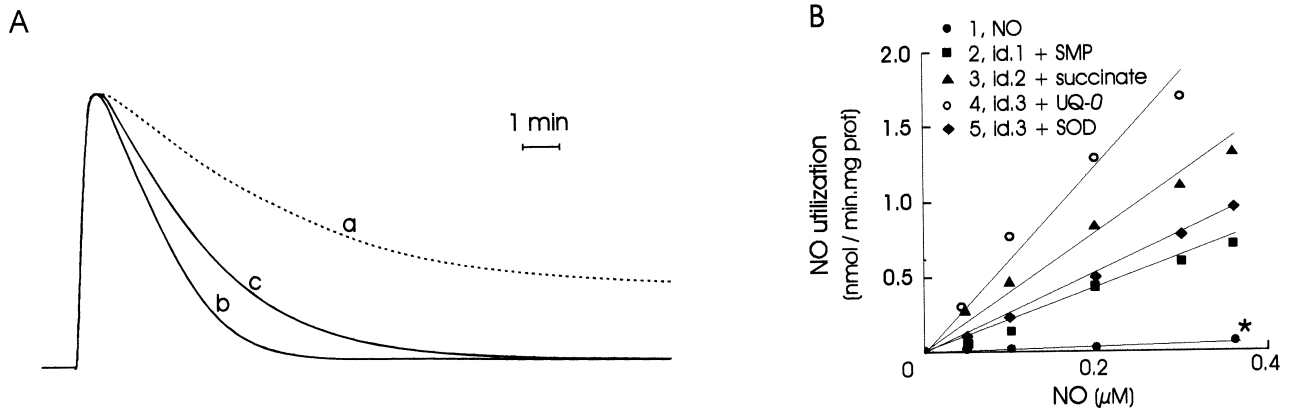


Figure 4 NO decays in the presence of submitochondrial particles and 6 m*M* succinate in anaerobiosis (trace a), in aerobiosis at 220 μ*M* O_2 (trace b), and in aerobiosis at 220 μ*M* O_2 plus 2 μ*M* SOD (trace c) (A). The NO decay rates at physiological NO concentration (0–0.4 μ*M*) follow first-order kinetics for NO (B). *, units are in μ*M* min^{-1}. Adapted from (Poderoso *et al.,* 1999b), *Journal of Biological Chemistry,* with permission.

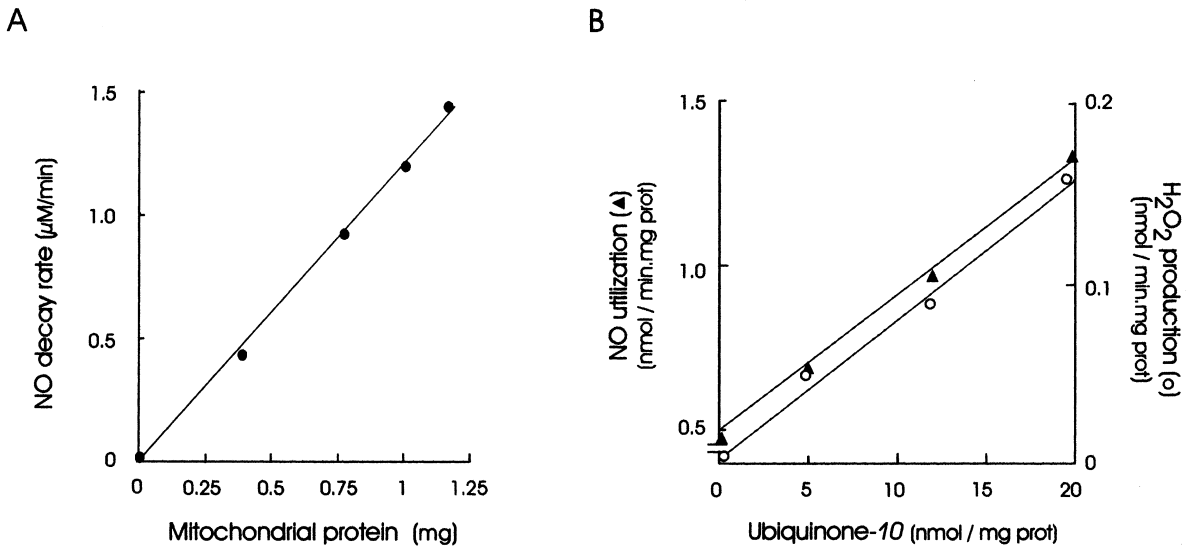


Figure 5 The NO decay rate in liver mitochondria is linearly dependent on mitochondrial protein content (A). Both H_2O_2 production and NO decay rates depend on reduced ubiquinone incorporated into liver submitochondrial particles, previously depleted by extraction with cyclopentane and lyophilized, in the presence of 6 m*M* succinate and 2 μ*M* myxothiazol (B). Adapted from (Poderoso *et al.,* 1999b), *Journal of Biological Chemistry,* with permission.

tion, 1.3 nmol NO/min/mg protein, that was inhibitable by the addition of superoxide dismutase (Poderoso *et al.,* 1999b). As previously described (Richter, 1997; Boczkowski *et al.,* 1999), these effects are consistent with the reaction of NO and O_2^- to yield $ONOO^-$ as the main route of NO utilization by mitochondria under aerobic conditions.

Ubiquinone Supplementation, Superoxide Production, NO Decay, and Cytochrome Oxidase Inhibition

Both the soluble and the membrane-bound quinones that are reduced by the mitochondrial respiratory chain produce, by autoxidation, O_2^-, the stoichiometric precursor of H_2O_2 (Cadenas *et al.,* 1977). Acetone-extracted and ubiquinone-supplemented submitochondrial particles showed rates of H_2O_2 production and of NO utilization that are linearly dependent on the content of membrane-bound and substrate-reducible ubiquinone-10 (UQ_{10}) (Fig. 5), as was previously described for H_2O_2 production (Boveris and Chance, 1973; Boveris *et al.,* 1976). Similarly, rat liver submitochondrial particles supplemented with exogenous soluble ubiquinones, such as UQ_0 and UQ_2, showed up to 10 times higher H_2O_2 production (Poderoso *et al.,* 1999b). In this setting, NO decay rates appeared well correlated with the rates of O_2^- generation in the ubiquinol-supplemented mitochondrial preparations. These results acquire more relevance when it is considered that the experiments were done at constant $[O_2]$, that oxygen uptake was unaffected by ubiquinone depletion (except at the lowest levels), and that other components of the electron transfer system were not essentially modified. The rate constant for the ubiquinol/NO reaction is about $0.5 \times 10^4\ M^{-1}\ s^{-1}$ at pH 6.0, with ubiquinol-10 (UQH_2) (Poderoso *et al.,* 1999a), and $2.1 \times 10^3\ M^{-1}\ s^{-1}$ at pH 7.4, with UQ_9H_2 in the rat liver mitochondrial membranes (Poderoso *et al.,* 1999b).

The cytochrome oxidase activity of rat liver submitochondrial particles supplemented with NO exhibited a lag phase or inhibition time before enzyme activity starts. The NO inhibition time of cytochrome oxidase activity was strongly decreased either by addition of soluble ubiquinones to the reaction medium or by supplementation with membrane-bound ubiquinone in acetone-extracted mitochondrial membranes; a decrease to one-half of the inhibition time was observed at about 15 nmol/mg protein of UQ_{10} (Fig. 6).

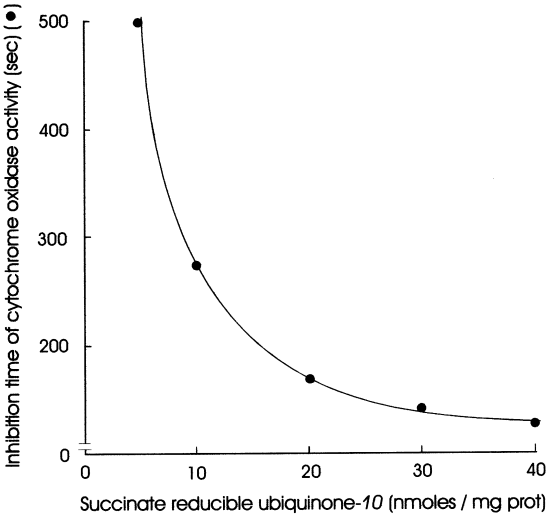


Figure 6 The time of inhibition of cytochrome oxidase activity by NO is decreased by increasing the succinate reducible ubiquinone (UQ_{10}) in cyclopentane-extracted and UQ_{10}-supplemented rat liver mitochondrial membranes. Adapted from (Poderoso *et al.,* 1999b), *Journal of Biological Chemistry,* with permission.

The Dual Effect of NO on Mitochondrial Hydrogen Peroxide Production

A biphasic effect is observed when the dependence of the rate of mitochondrial H_2O_2 production on NO concentration is examined in the 0–20 μ*M* NO range (Fig. 7). In the first phase, in the 0–3 μ*M* range, a marked increase in the rate of H_2O_2 was observed, as described before and shown in Fig. 2A. At higher NO concentrations, in the 3–20 μ*M* range, a second phase was observed in which there was a decrease of H_2O_2 production on increasing NO concentration. The biphasic nature of the NO effect on mitochondrial production of O_2^- and H_2O_2 was also recognized when submitochondrial particles added with succinate and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were analyzed by electron paramagnetic resonance (EPR). At 5 μ*M* NO, the formation of a DMPO–H_2O_2 adduct was detected by a characteristic six-line spectrum that was inhibited by catalase; however, at 20 μ*M* NO the adduct was not formed (Fig. 7). In the second phase, at high NO levels, formation of $ONOO^-$ precluded the reaction of O_2^- with DMPO, which evolves to DMPO–H_2O_2 after the formation of a DMPO–O_2^- adduct.

Peroxynitrite-Dependent Production of Superoxide Radical

The addition of relatively low concentrations of 0.25–2 μ*M* $ONOO^-$ to rat heart submitochondrial particles supplemented with myxothiazol and succinate elicited production of O_2^-, which was consistent with an oxidation of the membrane-bound ubiquinol to the corresponding ubisemiquinone and its subsequent autoxidation, yielding O_2^- (Fig. 8). The process yield was about 0.5 O_2^- produced per $ONOO^-$ added. An additional indication of the reactivity of $ONOO^-$ toward ubiquinol was indirectly observed when the steady-state concentration of NO was amperometrically followed in submitochondrial particles in the presence of a DETA–NO system that continuously generates NO (as shown in Fig. 2B). Uric acid, which in these conditions behaves as an specific $ONOO^-$ scavenger, prevents almost totally the succinate effect, indicating a role of $ONOO^-$ as reactant in O_2^- production. The reaction of ubiquinol with $ONOO^-$ ($k = 4.8 \times 10^2\ M^{-1}\ s^{-1}$) eliminates a powerful oxidant of the mitochondrial matrix and completes the metabolic pathway of mitochondrial NO. This reaction constitutes an extra source of O_2^- to eliminate NO. On the other hand, at relatively high NO concentrations, the continuous formation and exposure of mitochondrial matrix components to $ONOO^-$ will produce protein nitration and nitrosylation, leading to alterations that lead to enzyme inactivation and alterations in energy-linked functions (Lizasoain *et al.,* 1996; Szabó *et al.,* 1996; Xie *et al.,* 1998).

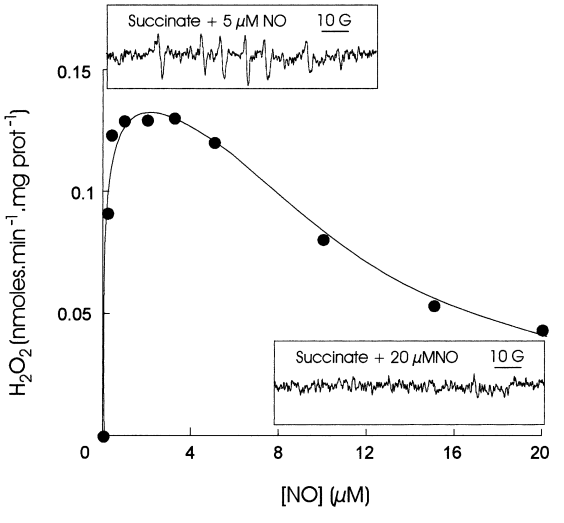


Figure 7 Dual effect of NO on mitochondrial production of H_2O_2 in mitochondria in the presence of succinate. The production of hydrogen peroxide increases up to a plateau at about 5 μ*M* NO and afterward progressively decreases due to formation of $ONOO^-$. After addition of succinate and DMPO, the DMPO–H_2O_2 adducts are detected by EPR only at the lower NO concentrations (5 μ*M* NO) and not at higher NO concentrations (20 μ*M* NO).

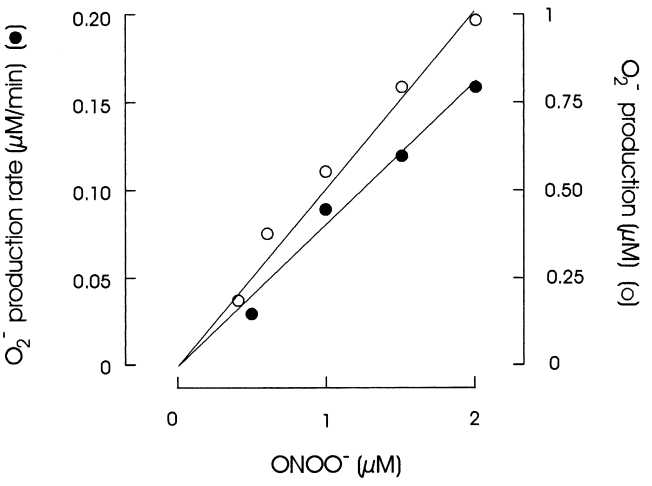


Figure 8 Peroxynitrite induces the production of O_2^- in heart mitochondrial membranes.

The NO-Induced Mitochondrial Production of Superoxide Anion Regulates NO Metabolism, Cytochrome Oxidase Activity, and Oxygen Uptake

The rich biochemistry of NO has a key role in the regulation of cell oxygen uptake and energy production. This highly diffusible small molecule has dual effects on tissue respiration. On the one hand, NO generated by endothelial NOS produces dilation of the small blood vessels; by lengthening the equilibration time between oxygenated hemoglobin (HbO_2) in the blood and tissue pO_2, this NO generation favors oxygen delivery and uptake by the tissues. On the

other hand, it reversibly inhibits cytochrome oxidase activity and cell oxygen uptake, which allows O_2 molecules to further diffuse in the tissue and decreases the steepness of the oxygen gradient in the tissue near the area of the normoxia/anoxia transition. The degree of cytochrome oxidase inhibition by NO depends on the simultaneous pO_2, a fact that enhances the physiological NO effects at low pO_2. Apparently, NO and O_2 compete for a binding site at the binuclear center formed by cytochrome a_3^{2+} and Cu_B^+ of cytochrome oxidase. Then, the inhibition of mitochondrial respiration by NO can be expressed as a function of the ratio $[O_2]/[NO]$. As was discussed previously, values of 150, 400–500, and 500–1000 (Boveris *et al.,* 1999) have been reported for this ratio to produce half-maximal inhibition of mitochondrial respiration.

Nitric oxide also inhibits mitochondrial electron transfer at the ubiquinol–cytochrome *b* region of the respiratory chain, blocking succinate–cytochrome *c* reductase activity with increased reduction of cytochrome *b* (Poderoso *et al.,* 1996) and increased rates of O_2^- production in submitochondrial particles and of H_2O_2 in mitochondria. The interaction of NO with the NO-reactive component of the ubiquinol–cytochrome *b* area of the mitochondrial respiratory chain is also reversible but is not affected by the $[O_2]$:[NO] ratio. The NO-dependent mitochondrial production of O_2^- provides a regulatory mechanism to remove NO and the reversible inhibition of cytochrome oxidase. An increasing NO concentration will sequentially inhibit cytochrome oxidase activity and electron transfer in the ubiquinone–cytochrome *b* region of the mitochondrial respiratory chain. The increased O_2^- production and steady-state concentration will in turn set a feedback mechanism by clearing NO through the Beckman reaction ($O_2^- + NO \rightarrow ONOO^-$) (Beckman, 1990). In that sense, as was previously described, the use of ubiquinone-supplemented mitochondrial preparations provides clear evidence that the increased rates of O_2^- production are associated with faster removal of the NO-induced cytochrome oxidase inhibition.

The active NO synthase located in the inner membrane of rat liver mitochondria (mtNOS) is able to produce NO at rates of 2–3 nmol/min/mg protein, rates that are about two times higher than the inner membrane production of O_2^- (Giulivi *et al.,* 1998). The enzyme uses L-arginine (K_m 5–7 μM) and is activated by Ca^{2+}; the activity is better measured in submitochondrial particles, toluene-permeabilized mitochondria, or broken mitochondria. It has been shown that the addition of the NOS inhibitor N^G-L-monomethyl-L-arginine to respiring mitochondria increases oxygen uptake by 10–15%, which indicates that NO produced by mtNOS is able to regulate mitochondrial O_2 uptake. The normal mitochondrial NO steady-state concentration can be estimated to be about 30 n*M;* it has been calculated as 50 n*M* in rat liver on the basis of maximal mtNOS activity (Giulivi, 1998), measured as 20 n*M* NO in isolated rat diaphragm (Boczwaski *et al.,* 1999) and measured as 100 n*M* in perfused rat heart stimulated by bradykinin (Poderoso *et al.,* 1998). The sequential effects of bradykinin on myocardial O_2 uptake, NO release, and production of oxygen active species are self-explanatory with respect to the regulatory mechanisms of oxygen metabolism (Fig. 9).

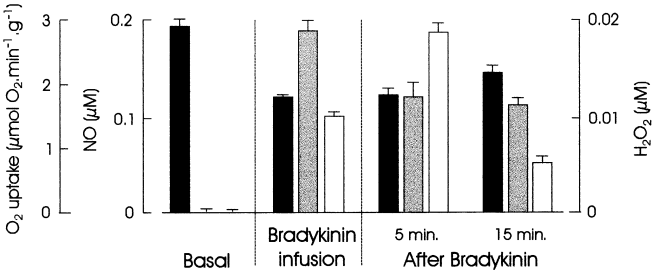


Figure 9 Oxygen uptake (black bars), NO release (gray bars), and H_2O_2 release (white bars) in normal and bradykinin-stimulated isolated perfused rat heart.

An understanding of the biological signaling by which NO regulates mitochondrial oxygen uptake and energy supply requires a knowledge of the metabolic routes of mitochondrial NO (Fig. 10). For the following analysis only mitochondrial NO utilization was considered, disregarding the sink effect of the fast reactions of NO with muscle cytosolic myoglobin and blood hemoglobin that certainly will have an effect on mitochondrial and cellular NO steady-state concentrations.

The selective permeability of the inner mitochondrial membrane makes the mitochondrial matrix space a differentiated intracellular compartment. The key features involving NO and O_2^- metabolism are the impermeability of the inner membrane to O_2^- and H^+, the relative impermeability of the same membrane to $ONOO^-$, and the presence of Mn-SOD at a level that is about five times lower than in the cytosol. The reactions of O_2^- with NO [reaction (I), $k = 1.9 \times 10^{10}$ M^{-1} s^{-1}] and with Mn-SOD [reaction (II), $k_2 = 2.4 \times 10^9$ M^{-1} s^{-1}] are apparently the only ones that occur in the mitochondrial matrix at rates that effectively contribute to O_2^- utilization.

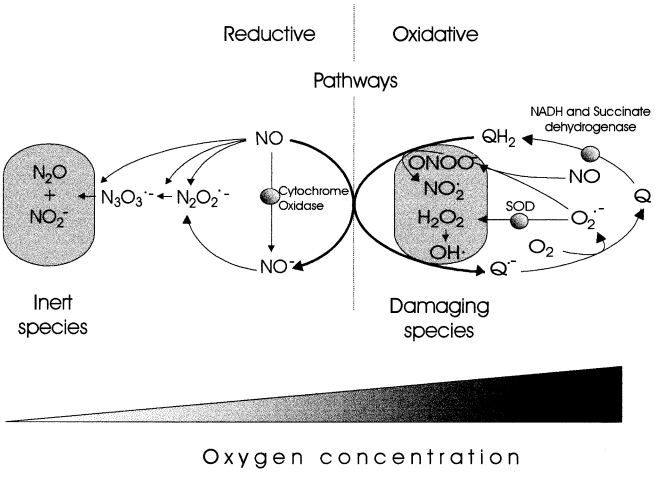


Figure 10 Oxidative and reductive NO reactions in mitochondria that contribute to keep NO steady-state levels and the regulation of oxygen metabolism.

$$NO + O_2^- \rightarrow ONOO^- \quad \text{(I)}$$

$$O_2^- + SOD^+ \text{ (or } SOD^{2+}) \rightarrow H_2O_2 \text{ (or } O_2) + SOD^{2+} \text{ (or } SOD^+) \quad \text{(II)}$$

Alternative chemical reactions [reactions (I) and (II)] yield differential equations [Eq. (1) and (2)], where [SOD] indicates total SOD concentration ($[SOD^+] + [SOD^{2+}]$). The combined expression in equation (3) is obtained after considering the common factor $[O_2^-]$, and it indicates the ratio of production of $ONOO^-$ and H_2O_2 via the two alternative reactions of O_2^- utilization.

$$-d[NO]/dt = -d[O_2^-]/dt = d[ONOO^-]/dt = k_1[NO][O_2] \quad (1)$$

$$-d[O_2^-]/dt = 0.5d[H_2O_2]/dt = k_2[O_2^-][SOD] \quad (2)$$

$$(d[ONOO^-]/dt)/(d[H_2O_2]/dt) = k_1[NO]/0.5k_2[O_2^-][SOD] \quad (3)$$

It can be calculated that for physiological conditions, considering intramitochondrial Mn-SOD as $\cong 3$ μM (Boveris and Cadenas, 1997) and intramitochondrial [NO] as 50 n*M,* that the intramitochondrial production of $ONOO^-$ will account for 15% of O_2^- utilization, with the remaining 85% yielding H_2O_2 as final product (Poderoso *et al.,* 1999b). Under conditions of inducible NOS (iNOS) activation, intramitochondrial NO may reach 100 n*M* (Poderoso *et al.,* 1998), and consequently $ONOO^-$ formation may account for as much as 28% of O_2^- utilization. Finally, in the setting of induction of iNOS, cell and intramitochondrial NO may reach 0.5 μM NO with very high production rates of $ONOO^-$ that could account for more than 50% of O_2^- utilization. Besides the oxidative pathway of reaction (I), intramitochondrial NO will be metabolized through reductive one-electron transfer reactions from cytochrome oxidase [reaction (III)] and from UQH_2 [reaction (IV)].

$$NO + \text{Cyt } a_3^{2+} \rightarrow \text{Cyt } a_3^{2+}\text{–NO} \rightarrow \text{Cyt } a_3^{3+} + NO^- \quad \text{(III)}$$

$$NO + UQH_2 \rightarrow NO^- + UQH + H^+ \quad \text{(IV)}$$

The rate of reaction (I) under physiological conditions will be, according to equation (1) and after taking $[O_2^-]$ as 10^{-10} *M,* about 90 n*M* s^{-1}. The rate of reaction (III) is, according to our data on the metabolism of NO pulses by rat liver submitochondrial particles in the presence of cyanide, about 0.1 nmol NO/min/mg protein. The process can be understood as a secondary pathway of cytochrome oxidase activity with a slow first-order decay of Cyt a_3^{2+}–NO ($k_6 = 0.13$ s^{-1}) (Giuffre *et al.,* 1996). With 18 μM cytochrome oxidase in the liver and assuming that 10% of the enzyme is in the form of the oxidase–NO complex, the rate of NO utilization will be about 6 n*M* s^{-1}. However, the mechanism of this reaction, postulated on the basis of the inhibition by cyanide of NO utilization (Zhao *et al.,* 1995), has not been confirmed in other studies with purified cytochrome oxidase (Stubauer *et al.,* 1998), but a reduction of NO by cytochrome *c,* the reductant of cytochrome oxidase, was reported (Sharpe and Cooper, 1998). The rate of reaction (IV) can be, after considering UQH_2 content in the rat liver and a value of k_7 of 2.1×10^3 M^{-1} s^{-1}, about 30 n*M* s^{-1}.

In summary, the considered rates seem to indicate that in rat liver mitochondria, NO is metabolized about 70% through the oxidative formation of $ONOO^-$, about 5% through the reduction by cytochrome oxidase and cytochrome *c,* and about 20–30% through reduction by ubiquinol, in the latter two cases yielding NO^-. The rate of NO oxidation by O_2 in the tissues to yield NO_2 is considered negligible, considering the quadratic dependence on $[O_2]$ and the low tissue pO_2. At 25 μM O_2 in the liver, utilization of 1 μM NO by this reaction will take 35 hours. Peroxynitrite, a strong oxidant with $E^\circ = 1.13$ V, appears to be produced in the mitochondrial matrix at the rate of 90 n*M* s^{-1}, as discussed before. The reaction of $ONOO^-$ with ubiquinol yields ubisemiquinone [reaction (V); $k = 4.8 \times 10^2$ M^{-1} s^{-1}), which produces O_2^- by autoxidation [reaction (VI); $k = 8 \times 10^3$ M^{-1} s^{-1}) and subsequently, by dismutation, H_2O_2. The whole series of reactions transforms the powerful oxidant $ONOO^-$ into the less reactive and diffusible H_2O_2.

$$ONOO^- + UQH_2 \rightarrow NO_2 + UQH + HO \quad \text{[V]}$$

$$UQH + O_2 \rightarrow UQ + O_2^- + H^+ \quad \text{[VI]}$$

Two biological roles are played by the two reduced forms of ubiquinone: the fully reduced ubiquinol acts as an antioxidant clearing $ONOO^-$ [reaction (V)], and the univalently reduced ubisemiquinone acts as a prooxidant by the generation of O_2^- radical [reaction (VI)].

The fine regulation of the steady-state concentration of NO in the mitochondrial matrix, accomplished by its reactions with O_2^-, cytochrome oxidase, and ubiquinol, does regulate cytochrome oxidase activity. Knowledge of the intracellular or intramitochondrial signaling that activates mtNOS is essential to fully understand the overall process.

The Different NOS Isoforms and the Utility of Regulating Mitochondrial O_2 Uptake

On the basis of the previous considerations, it is reasonable to speculate about the role of the different NOS isoforms and the NO regulation of mitochondrial and cellular O_2 uptake (Fig. 11). The widespread distribution of NOS I in the nervous system and skeletal muscle (Kobzik *et al.,* 1994) may lead, through an inhibition of mitochondrial O_2 uptake and a reduction of ATP availability for the Na^+,K^+−ATPase membrane pumps, to transient decreases in the resting potential of the synaptic terminals and neural fibers. The physiological action will be a facilitation of neural excitability and synaptic transmission, with effects on muscle contractility.

The regulation of O_2 metabolism in connection with transient decreases in tissue O_2 availability during circulatory changes is consistent with activity of endothelial NOS. Ischemia, hypoxia, ''shear'' stress, and microcirculatory and rheological alterations should stimulate eNOS activity and

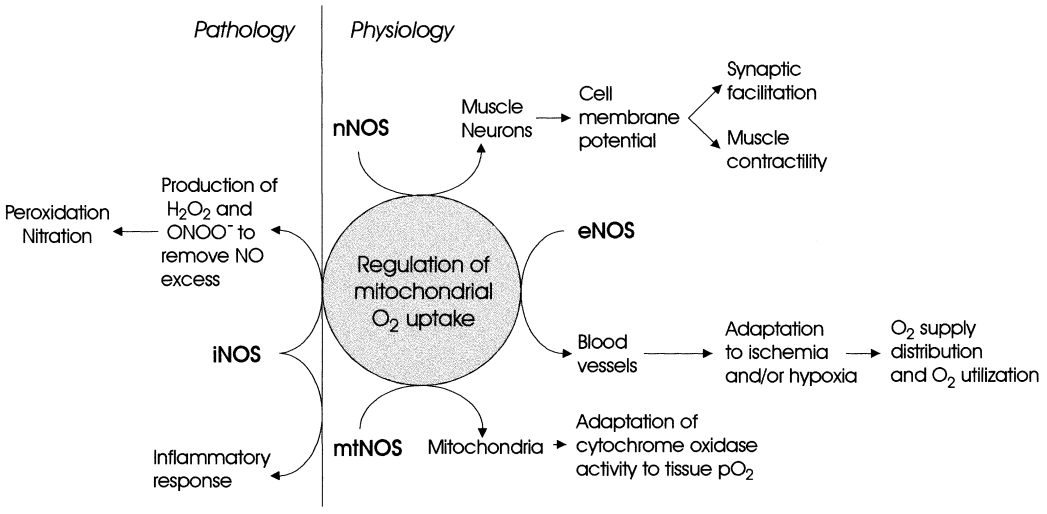


Figure 11 General and tissue-specific effects of the NO inhibition of oxygen uptake related to NOS isoforms in physiological and pathological settings.

release of NO (K_m of eNOS for O_2 is low, about 16 μM), which will diffuse into adjacent cells to inhibit cytochrome oxidase. On the basis of O_2/NO competition, cytochrome oxidase activity and O_2 uptake adapted to the O_2 supply, which allows O_2 to diffuse further along its gradient and lowers the steepness of the pO_2 gradient in the normoxic–anoxic transition (Fig. 12). A more general metabolic action should be expected from mtNOS (NOS IV) (Giulivi, 1998), which is likely related to continuous regulation of the specific activity of cytochrome oxidase to tissue pO_2. In this way, if arginine is available, mitochondrial O_2 uptake should not follow the "all or nothing" paradigm, but it should be exactly adjusted to O_2 concentrations. Evidence provided by Koivisto *et al.* (1997) and by Giulivi (1998) indicates that the rate of mitochondrial O_2 uptake depends on NO concentration in the range of physiological pO_2. Knowledge of the O_2 dependence of mitochondrial NO production, in other words, the K_m for O_2 of mtNOS, is essential to the understanding of the NO regulation of mitochondrial respiration. The pathological dependence of O_2 uptake on O_2 availability in sepsis and septic shock (Bihari *et al.,* 1987; Danek *et al.,* 1980; Poderoso *et al.,* 1994) may be due to increased NO

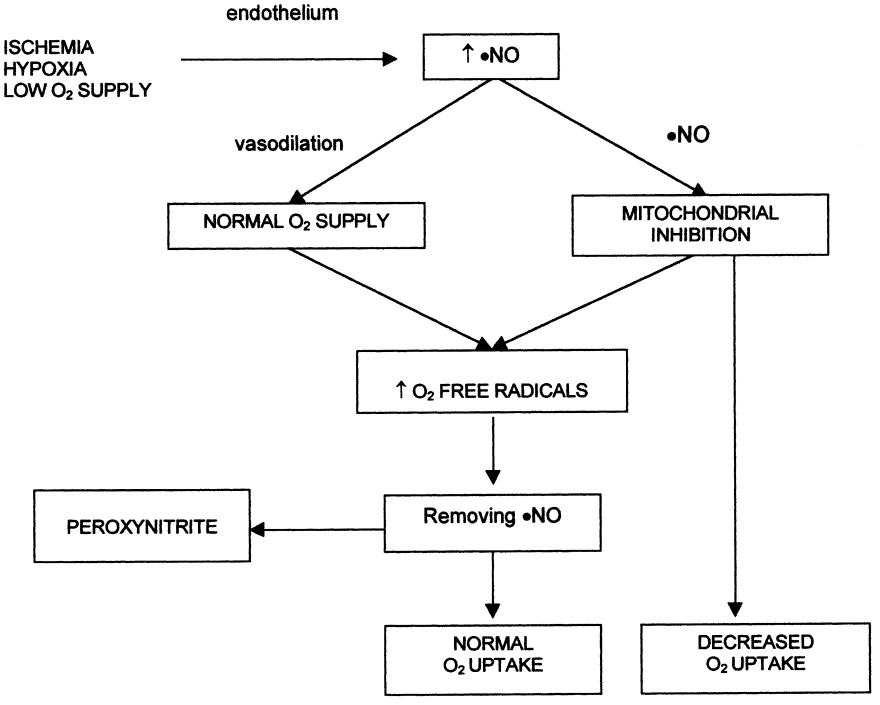


Figure 12 The interplay of nitric oxide and O_2 free radicals in the modulation of O_2 uptake. Reproduced from (Poderoso *et al.,* 199b), *Archives of Biochemistry and Biophysics,* with permission.

steady-state concentrations in the whole body owing to iNOS expression in macrophages and other immunocompetent NO-producing cells, a fact that dangerously amplifies the underling regulatory mechanism.

Summary

Oxygen metabolism has been considered to follow a paradigm in which oxygen uptake does not change with respect to oxygen supply, up to critical hypoxic conditions. This behavior was thought to be dependent on the great affinity of mitochondrial cytochrome oxidase for oxygen. However, the discovery of nitric oxide led to the observation that this radical may modulate oxygen uptake by reacting with cytochrome oxidase with an even greater affinity than oxygen. Considering that, in terms of steady-state concentrations, there is an O_2–NO competition, cytochrome oxidase activity and oxygen uptake in the presence of NO should be dependent on, rather than independent of, O_2 levels. In this way, the modulation of mitochondrial NO concentration should be critical with respect to the regulation of oxygen metabolism.

The routes for NO metabolism include oxidative and reductive pathways. Accordingly, mitochondrial superoxide anion is produced by the oxidation of membrane ubiquinol by NO. In this way, and under physiological conditions, most of the NO decay and, consequently, the inhibitory effects of NO on cytochrome oxidase are modulated by the NO/O_2^- reaction leading to the formation of peroxynitrite. From this perspective, active oxygen species should not be merely toxic end products of oxygen metabolism; they can also modulate NO levels and O_2 uptake. In contrast, a pathological amplification of this process becomes potentially dangerous because of increasing peroxynitrite steady-state concentrations. The regulatory NO effect on oxygen metabolism may be related to specific and general purposes in connection with the presence of different NOS isoforms, including the mitochondrial one.

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References

Adams, D. O., and Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annu. Rev. Immunol.* **2,** 283–318.

Beckman, J. S. (1990). Ischemic injury mediator. *Nature* **345,** 27–28.

Bihari, D., Smithies, M., and Gimson, A. (1987). The effects of vasodilatation with prostacyclin on oxygen delivery and uptake in critically ill patients. *N. Engl. J. Med.* **317,** 397–403.

Boczkowski, J., Lisdero, C. L., Lanone, S., Carreras, M. C., Boveris, A., Aubier, M., and Poderoso, J. J. (1999). Endogenous peroxynitrite mediates mitochondrial dysfunction in rat endotoxemia. *FASEB J.* **13,** 1637–1647.

Borutait'e, V., and Brown, G. C. (1996). Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. *J. Biochem.* (*Tokyo*) **315,** 295–299.

Boveris, A., and Cadenas, E. (1997). Cellular sources and steady-state levels of reactive oxygen species. *In* ''Oxygen, Gene Expression, and Cellular Function'' (L. B. Clerch and D. J. Massaro, eds.), pp. 1–25. Marcel, New York, Basel, and Hong Kong.

Boveris, A., and Chance, B. (1973). The mitochondrial production of hydrogen peroxide. *Biochem. J.* **134,** 707–716.

Boveris, A., Cadenas, E., and Stoppani, A. O. M. (1976). Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem. J.* **156,** 435–444.

Boveris, A. A., Costa, L., Cadenas, E., and Poderoso, J. J. (1999). Regulation of mitochondrial respiration by adenosine diphosphate, oxygen, and nitric oxide. *Methods Enzymol.* **301,** 188–198.

Bredt, D. S., and Snyder, S. H. (1994). Nitric oxide: A physiologic messenger molecule. *Annu. Rev. Biochem.* **63,** 175.

Brown, G. C. (1995). Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett.* **369,** 136–139.

Brown, G. C. (1997). Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: Implications for inflammatory, neurodegenerative and ischaemic pathologies. *Mol. Cell. Biochem.* **174,** 189–192.

Brown, G. C., and Cooper, C. E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett.* **356,** 295–298.

Cadenas, E., and Boveris, A. (1980). Enhancement of hydrogen formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem. J.* **188,** 31–37.

Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. M. (1977). Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef heart mitochondria. *Arch. Biochem. Biophys.* **180,** 248–257.

Cassina, A., and Radi, R. (1996). Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* **328,** 309–316.

Chance, B., and Williams, G. R. (1955). Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. Biol. Chem.* **217,** 409–427.

Chance, B., and Williams, C. M. (1956). The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* **17,** 65–134.

Clarke, K., and Willis, R. J. (1987). Energy metabolism and contractile function in rat heart during graded, isovolumic perfusion using P nuclear magnetic resonance spectroscopy. *J. Mol. Cell. Cardiol.* **19,** 1153–1160.

Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. V. (1994). Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. *FEBS Lett.* **345,** 50–54.

Costa, L., Mendez, G., and Boveris, A. (1997). Oxygen dependence of mitochondrial function measured by high-resolution respirometry in long-term hypoxic rats. *Am. J. Physiol.* **273,** 852–858.

Danek, S., Lynch, J., and Weg, J. (1980). The dependence of oxygen uptake on oxygen delivery in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **122,** 387–395.

De Groot, H., Noll, T., and Sies, H. (1985). Oxygen dependence and subcellular partitioning of hepatic menadione-mediated oxygen uptake. Studies with isolated hepatocytes, mitochondria, and microsomes in an oxystat system. *Arch. Biochem. Biophys.* **243,** 556–562.

Estabrook, R. W. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol.* **10,** 41–57.

Fridovich, I. (1978). The biology of oxygen radicals. *Science* **201,** 875–880.

Gayeski, T. E. J., and Honig, C. R. (1991). Intracellular PO_2 in individual cardiac myocytes in dogs, cats, rabbits, ferrets, and rats. *Am. J. Physiol.* **260,** 522–31.

Ghafourifar, P., and Richter, C. (1997). Nitric oxide synthase activity in mitochondria. *FEBS Lett.* **418,** 291–296.

Giulivi, C. (1998). Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. *Biochem. J.* **332,** 673–679.

Giulivi, C., Poderoso, J. J., and Boveris, A. (1998). Production of nitric oxide by mitochondria. *J. Biol. Chem.* **273,** 11038–11043.

Goretski, J., Zafiriou, O. C., and Hollocher, T. C. (1990). Steady-state nitric oxide concentrations during denitrification. *J. Biol. Chem.* **265,** 11535–11538.

Greenwood, C., and Gibson, G. (1967). The reaction of reduced cytochrome *c* oxidase with oxygen. *J. Biol. Chem.* **242,** 1782–1787.

Hassinen, I. E., Hiltunen, J. K., and Takala, T. E. S. (1981). Reflectance spectrophotometric monitoring of the isolated perfused heart as a method of measuring the oxidation–reduction state of cytochromes and oxygenation of myoglobin. *Cardiovasc. Res.* **15,** 86–91.

Heineman, F. W., and Balaban, R. S. (1990). Control of mitochondrial respiration in the heart in vivo. *Annu. Rev. Physiol.* **52,** 523–42.

Ignarro, L., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–69.

Imlay, J. A., and Fridovich, I. (1991). Assay of metabolic superoxide production in *Escherichia coli. J. Biol. Chem.* **266,** 6957–6965.

Kennedy, F. G., and Jones, D. P. (1986). Oxygen dependence of mitochondrial function in isolated rat cardiac myocytes. *Am. J. Physiol.* **250,** 374–383.

Knowles, R. G., Merrett, M., Salter, M., and Moncada, S. (1990). Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.* **270,** 833–836.

Kobzik, L., Reid, M. B., Bredt, D. S., and Stamler, J. S. (1994). Nitric oxide in skeletal muscle. *Nature* **372,** 546–548.

Koivisto, A., Matthias, A., Bronnkov, G., and Nedergaard, J. (1997). Kinetics of the inhibition of mitochondrial respiration by NO. *FEBS Lett.* **417,** 75–80.

Konorev, E. A., Joseph, J., and Kalyanaranam, B. (1996). *S*-nitrosoglutathione induces formation of nitrosyl myoglobin in isolated hearts during cardioplegic ischemia. An electron spin resonance study. *FEBS Lett.* **378,** 111–114.

Koppenol, W. H. (1998). The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radicals Biol. Med.* **25,** 385–391.

LaNoue, K. F., Jefferies, F. M., and Radda, G. K. (1986). Kinetic control of mitochondrial ATP synthesis. *Biochemistry* **25,** 7667–75.

Lardy, H. A., and Wellman, H. (1952). Oxidative phosphorylations: Role of inorganic phosphate as an acceptor system in control of metabolic rate. *J. Biol. Chem.* **195,** 215–224.

Lizasoain, I., Moro, M. A., Knowels, R. G., Darley-Usmar, V., and Moncada, S. (1996). Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *J. Biochem.* (*Tokyo*) **314,** 877–80.

Lowenstein, C. J., Dinerman, J. L., and Snyder, S. H. (1994). Nitric oxide: A physiologic messenger. *Am. Intern. Med.* **120,** 227.

McCord, J. M., and Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244,** 6049–6055.

Millar, A. H., and Day, D. A. (1996). Nitric oxide inhibits the cytochome oxidase but not the alternative oxidase of plant mitochondria. *FEBS Lett.* **398,** 155–158.

Okada, S., Takehara, Y., Yabuki, M., Yoshioka, T., Yasuda, T., Inoue, M., and Utsumi, K. (1996). Nitric oxide, a physiological modulator of mitochondrial function. *Physiol. Chem. Phys. Med.* **28,** 69–82.

Packer, M. A., Porteous, C. M., and Murphy, M. P. (1996). Superoxide production by mitochondria in the presence of nitric oxide forms peroxynitrite. *Biochem. Mol. Biol. Int.* **40,** 527–534.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Poderoso, J. J., Fernandez, S., Carreras, M. C., Tchercanski, D., Acevedo, C., Rubio, M., Peralta, J., and Boveris, A. (1994). Liver oxygen uptake dependence and mitochondrial function in septic rats. *Circ. Shock* **44,** 175–182.

Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobó, N., Schöpfer, F., and Boveris, A. (1996). Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch. Biochem. Biophys.* **328,** 85–92.

Poderoso, J. J., Peralta, J., Lisdero, C., Carreras, M. C., Radisic, M., Schöpfer, F., Cadenas, E., and Boveris, A. (1998). Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *Am. J. Physiol.* **274,** 112–119.

Poderoso, J. J., Carreras, M. C., Schöpfer, F., Lisdero, C., Riobó, N., Giulivi, C., Boveris, A. D., Boveris, A. A., and Cadenas, E. (1999a). The reaction of nitric oxide with ubiquinol. *Free Radicals Biol. Med.* **26,** 925–935.

Poderoso, J. J., Lisdero, C., Schöpfer, F., Riobó, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999b). The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol. *J. Biol. Chem.* **274,** 37709–37716.

Richter, C. (1997). Reactive oxygen and nitrogen species regulate mitochondrial Ca^{2+} homeostasis and respiration. *Biosci. Rep.* **17,** 53–66.

Senior, A. E. (1988). ATP synthesis by oxidative phosphorylation. *Physiol. Rev.* **68,** 177–231.

Sharpe, M. A., and Cooper, C. E. (1998). Reactions of nitric oxide with mitochondrial cytochrome *c:* A novel mechanism for the formation of nitroxyl anion and peroxynitrite. *J. Biochem.* **332,** 9–19.

Shen, W., Xu, X., Ochoa, M., Zhao, G., Wolin, M., and Hintze, T. H. (1994). Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. *Circ. Res.* **75,** 1086–1095.

Shen, W. Q., Hintze, T. H., and Wolin, M. S. (1995). Nitric oxide. An important signaling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation* **92,** 3505–3512.

Steinman, R. M., and North, R. J. (1986). ''Mechanisms of Host Resistance to Infective Agents, Tumors and Allografts.'' The Rockerfeller Univ. Press, New York.

Stubauer, G., Giuffré, A., Brunori, M., and Sarti, P. (1998). Cytochrome *c* oxidase does not catalyze the anaerobic reduction of NO. *Biochem. Biophys. Res. Commun.* **245,** 459–465.

Sugano, T., Oshino, N., and Chance, B. (1974). Mitochondrial function under hypoxic conditions. The steady states of cytochrome *c* reduction and of energy metabolism. *Biochim. Biophys. Acta* **347,** 340–358.

Szabó, C., Day, B. J., and Salzman, A. L. (1996). Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Lett.* **381,** 82–86.

Takehara, Y., Kanno, T., Yoshioka, T., Inoue, M., and Utsumi, K. (1995). Oxygen-dependent regulation of mitochondrial energy metabolism by nitric oxide. *Arch. Biochem. Biophys.* **323,** 27–32.

Takehara, Y., Nakahara, H., Inai, Y., Yabuki, M., Hamazaki, K., Yoshioka, T., Inoue, M., Horton, A. A., and Utsumi, K. (1996). Oxygen-dependent reversible inhibition of mitochondrial respiration by nitric oxide. *Cell Struct. Funct.* **21,** 251–258.

Tatoyan, A., and Giulivi, C. (1998). Purification and characterization of a nitric oxide synthase from rat liver mitochondria. *J. Biol. Chem.* **273,** 11044–11048.

Torres, J., Darley-Usmar, V., and Wilson, M. (1995). Inhibition of cytochrome oxidase turnover by nitric oxide: Mechanism and implications for the control of respiration. *Biochem. J.* **312,** 169–173.

Weibel, E. R., and Hoppeler, H. (1991). Respiratory system adaptation to hipoxia: Lung to mitochondria. *In* ''Response and Adaptation to Hypoxia'' (S. Lahiri, N. S. Cherniack, and R. S. Fitzgerald, eds.), pp. 3–13. New York, Oxford.

Wittenberg, B. A., and Wittenberg, J. B. (1989). Transport of oxygen in muscle. *Annu. Rev. Physiol.* **51,** 857–878.

Xie, Y. W., Kaminski, M., and Wolin, M. S. (1998). Inhibition of rat cardiac muscle contraction and mitochondrial respiration by endogenous per-

oxynitrite formation during posthypoxic reoxygenation. *Circ. Res.* **82,** 891–97.

Zhang, X., Xie, Y., Nasjletti, X., Xu, X., Wolin, M. S., and Hintze, T. H. (1996). ACE inhibitors promote nitric oxide accumulation to modulate myocardial oxygen consumption. *Circulation* **95,** 176–182.

Zhao, X. J., Sampath, V., and Caughey, W. S. (1995). Cytochrome *c* oxidase catalysis of the reduction of nitric oxide to nitrous oxide. *Biochem. Biophys. Res. Commun.* **212,** 1054–1060.

CHAPTER 24

Structure–Function Relationships in NO-Sensitive Guanylyl Cyclase

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ALTHOUGH RESEARCH IN THE NITRIC OXIDE (NO) FIELD HAS EXPANDED CONSIDERABLY, OUR KNOWLEDGE ABOUT THE NO RECEPTOR, SOLUBLE GUANYLYL CYCLASE, HAS GROWN ONLY SLOWLY. DESPITE THE IDENTIFICATION OF FOUR SUBUNITS (α_1, α_2, β_1, β_2) AT THE DNA LEVEL, SO FAR ONLY TWO HETERODIMERIC ISOFORMS OF THE ENZYME ($\alpha_1\beta_1$, $\alpha_2\beta_1$) HAVE BEEN SHOWN TO OCCUR AT THE PROTEIN LEVEL. HOWEVER, NO FUNCTIONAL DIFFERENCES AMONG THE ISOFORMS HAVE BEEN FOUND, AND THE PHYSIOLOGICAL RELEVANCE OF TWO SIMILARLY REGULATED ISOFORMS REMAINS UNCLEAR.

THE CLOSE STRUCTURAL RELATIONSHIP OF SOLUBLE GUANYLYL CYCLASE TO THE MEMBRANE-BOUND GUANYLYL CYCLASES AND ADENYLYL CYCLASES, INITIALLY SUGGESTED BY THE HIGH DEGREE OF HOMOLOGY, HAS BEEN SUPPORTED BY FUNCTIONAL DATA. THE CRYSTAL STRUCTURE OF THE CATALYTIC CENTER OF ADENYLYL CYCLASES PROVIDES A MODEL OF THE CATALYTIC CENTER OF SOLUBLE GUANYLYL CYCLASE.

MORE PRECISE UNDERSTANDING OF THE REGULATION OF SOLUBLE GUANYLYL CYCLASE BY NO, ESPECIALLY CONCERNING THE ACCEPTOR SITE FOR NO (I.E., THE PROSTHETIC HEME GROUP), AND THE MECHANISM OF ACTIVATION HAVE BEEN OBTAINED. A NEW COMPOUND, YC-1, UNVEILED A NOVEL MECHANISM OF SENSITIZATION OF SOLUBLE GUANYLYL CYCLASE TOWARD NO AND CO, WHICH MAY HAVE BROAD PHARMACOLOGICAL AND EVEN PHYSIOLOGICAL IMPLICATIONS. ENDOGENOUS LIGANDS AT THE YC-1 BINDING SITE MAY EXIST, OR POST-TRANSLATIONAL MODIFICATIONS AT THIS YC-1 EFFECTOR SITE MAY OCCUR, THAT BY ADJUSTING THE NO SENSITIVITY OF THE ENZYME, ARE CAPABLE OF MODULATING NO/CGMP SIGNALING.

Introduction

Soluble guanylyl cyclase (sGC) acts as the receptor molecule for nitric oxide (NO), thereby mediating most effects of NO in its function as a signaling molecule (Fig. 1). The enzyme contains a prosthetic heme group which serves as a receptor for NO; after binding of NO to the heme group of the enzyme, the catalytic activity, that is, the conversion of guanosine triphosphate (GTP) to guanosine cyclic monophosphate (cGMP), is stimulated up to 200-fold. Cyclic GMP, as an intracellular messenger, can act on three different effector molecules, namely, cGMP-dependent protein kinases, cGMP-gated ion channels, and cGMP-regulated phosphodiesterases. Hence, the effects of cGMP depend on the expression of a specific cGMP receptor protein in a given cell type. For example, cGMP-dependent kinases have been

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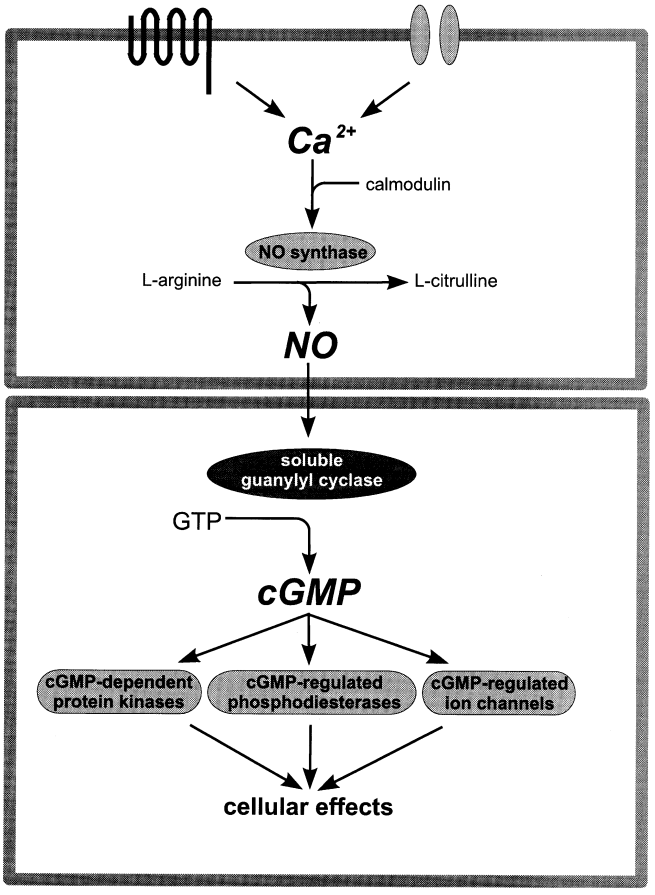


Figure 1 The NO–cGMP signaling cascade. Neuronal and endothelial NO synthases are regulated by the intracellular calcium concentration, with a rise of the calcium concentration leading to activation and NO formation. NO can diffuse into neighboring cells and stimulate soluble guanylyl cyclase, the enzyme that catalyzes the conversion of GTP to cGMP. Cyclic GMP alters the activity of the cGMP receptor molecules (cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, cGMP-regulated ion channels), finally leading to changes in cellular function.

reported to decrease the intracellular calcium concentration, whereas opening of cGMP-gated cation channels is known to increase the intracellular calcium concentration. Important NO/cGMP-mediated cellular effects include smooth muscle relaxation, modulation of synaptic transmission, and inhibition of platelet aggregation (for review, see Schmidt and Walter, 1994, and Moncada and Higgs, 1995).

Besides the soluble, that is, cytosolic GCs, membrane-bound forms of guanylyl cyclases (mGCs) exist (Fig. 2). Because these GCs are not stimulated by NO, they will not be extensively discussed in this chapter (for review, see Wedel and Garbers, 1997). Membrane-bound GCs belong to the group of receptor-linked enzymes with one membrane-spanning domain. The N-terminal extracellular parts of the different isoforms are quite diverse and form the ligand-binding domains, whereas the highly conserved intracellular C-terminal domains possess the catalytic activity. Several isoforms of mGCs (GC-A to GC-F) have been identified. Owing to the diversity of the ligand-binding domains, various isoforms of mGCs (GC-A, GC-B, GC-C) are stimulated by different peptide hormones; GC-A, for example, is stimulated by the natriuretic peptides ANP and BNP, whereas GC-B exhibits the highest affinity for the C-type natriuretic peptide. Physiologically, GC-C is activated by the intestinal peptide hormone guanylin, whereas under pathophysiological conditions stimulation of GC-C by the heat-stable enterotoxin of *Escherichia coli* results in diarrhea. As expected, the catalytic domains of all cGMP-forming isoforms, namely, soluble and membrane-bound guanylyl cyclases, are closely related and, in addition, show similarity to the catalytic domains of the cAMP-forming adenylyl cyclases (see Fig. 2).

This chapter gives a short overview on NO-sensitive sGC with special emphasis on structural features of the enzyme. This chapter first introduces the isoforms, the general structure, and the functional domains of the subunits. In the second part of the chapter, the different stimulators of sGC are presented, and their structural implications are discussed.

Structure of Soluble Guanylyl Cyclase

Soluble Guanylyl Cyclase Isoforms and General Properties

Soluble GC has been purified by several groups. All isolation procedures yielded an enzyme with two subunits termed α_1 and β_1 with molecular masses of 73 and 70 kDa, respectively (Gerzer *et al.,* 1981b; Humbert *et al.,* 1990; Stone and Marletta, 1994). The purified enzyme was shown to contain heme as a prosthetic group (Gerzer *et al.,* 1981a) and was stimulated up to 200-fold by NO. Specific activities of 25 to 40 μmol cGMP min^{-1} mg^{-1} have been reported under NO-stimulated conditions (Stone and Marletta, 1995; Russwurm *et al.,* 1998). Another isoform ($\alpha_2\beta_1$) of sGC has been shown to occur in placenta. This isoform very likely exists in other tissues as well; so far, it is not clear why the different purification procedures never yielded this isoform. Although there are considerable differences between the α subunits, the $\alpha_2\beta_1$ heterodimer purified from Sf9 cells exhibited the same specific activity and level of stimulation by NO as the $\alpha_1\beta_1$ heterodimer. In addition, both isoforms showed comparable behavior toward the new modulators YC-1 and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ). Both sGC isoforms, similar to other nucleotide-converting enzymes, require divalent metal ions as cofactors for catalysis. Besides Mg^{2+}, the enzymes can utilize Mn^{2+}, with catalytic activities under nonstimulated conditions being about fourfold higher in the presence of Mn^{2+}. However, NO-stimulated enzyme catalytic rates are lower in the presence of Mn^{2+} than in the presence of Mg^{2+}. Under nonstimulated conditions, both sGC isoforms exhibit K_m values of approximately 100 and 10 μ*M* for MgGTP and MnGTP as substrate, respectively. Under NO-stimulated conditions, the K_m values are approximately 10 μ*M* in the presence of either metal ion.

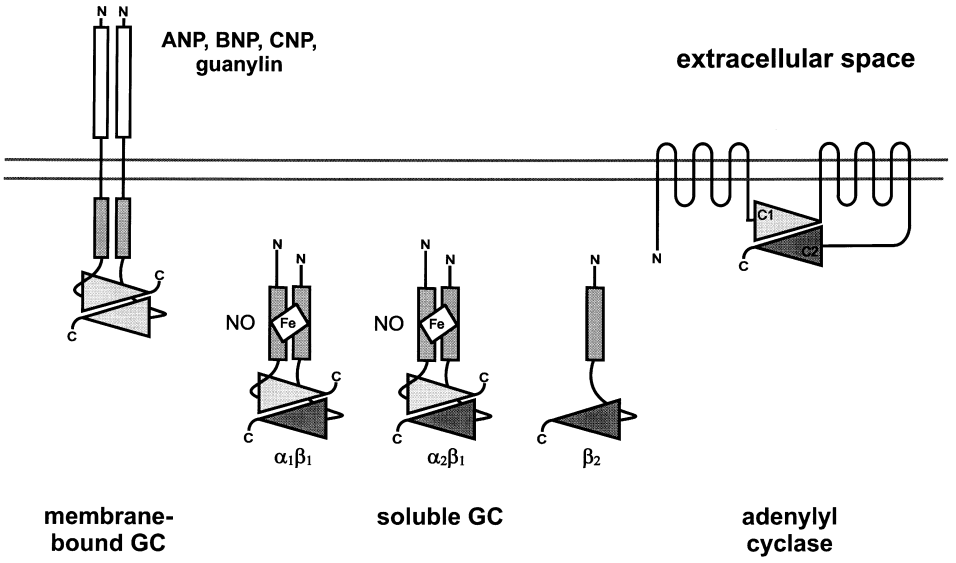


Figure 2 Schematic representation of the structures of guanylyl cyclases and adenylyl cyclases. Shown are the known heterodimers of soluble guanylyl cyclase ($\alpha_1\beta_1$, $\alpha_2\beta_1$), a representative membrane-bound guanylyl cyclase with different ligands at the respective receptor domain, and a representative adenylyl cyclase. The dimerization partner of the β_2 subunit of guanylyl cyclase has not been identified yet. The catalytic domains are symbolized by light gray triangles on the membrane-bound GC, the β subunit of sGC, and, correspondingly, the C2 domain of AC. The dark shaded triangles correspond to the cyclase catalytic domain in the α subunits of sGC and the C1 domain of AC. ANP, natriuretic peptide type A; BNP, natriuretic peptide type B; CNP, natriuretic peptide type C.

Subunits of Soluble Guanylyl Cyclase

Both the α_1 and β_1 subunits of the enzyme purified from lung have been cloned and sequenced. Expression experiments revealed that both subunits are required to form a catalytically active enzyme (Harteneck *et al.,* 1990; Buechler *et al.,* 1991). In lung, brain, and kidney, comparably high mRNA levels for the α_1 and β_1 subunits have been found (Nakane *et al.,* 1990).

The two other sGC subunits, α_2 and β_2, have been found by homology screening (Yuen *et al.,* 1990; Harteneck *et al.,* 1991). The so-called α_3 and β_3 subunits of sGC (Giuili *et al.,* 1992) represent human variants of the α_1 and β_1 subunits; changes in the reading frame account for the differences in amino acid sequences (Zabel *et al.,* 1998). It is noteworthy that the β_2 subunit contains 86 additional amino acids including an isoprenylation consensus sequence (-CVVL) at the C terminus. Thus, the β_2 subunit may be attached to the membrane, and, indeed, the expressed β_2 subunit can be detected in membrane fractions. However, although there is one report on the formation of an active $\alpha_1\beta_2$ heterodimer (Gupta *et al.,* 1997), the role of the β_2 subunit is unclear. Most laboratories have been unable to form a β_2-containing cGMP-synthesizing dimer with any of the known α of β subunits. Furthermore, the physiological occurrence of the β_2 subunit at the protein level has not been demonstrated. In contrast to β_2, the α_2 subunit is able to substitute for the α_1 subunit, as shown by the formation of a catalytically active, NO-sensitive heterodimer on coexpression with the β_1 subunit (Harteneck *et al.,* 1991). In human placenta, the natural occurrence of the α_2 subunit has been demonstrated, and here the β_1 subunit has been identified as its physiological dimerization partner (Russwurm *et al.,* 1998). Comparison of both heterodimers ($\alpha_1\beta_1$, $\alpha_2\beta_1$) did not reveal any differences between the isoforms with respect to the heme content, NO sensitivity, kinetic properties, and the responsiveness toward modulators (see also section on sGC isoforms). Taking ino consideration that the N-terminal thirds of the α subunits share only 27% identical amino acids, this lack of differences between the isoforms is surprising. Right now, the physiological relevance of the two similarly regulated isoforms cannot be explained.

Functional Domains

As previously outlined, four subunits of sGC (α_1, α_2, β_1, β_2) have been identified so far (see Fig. 2). Comparison of the primary structures of all known subunits shows that they can be divided into three distinct domains: an N-terminal region, a central part, and a C-terminal cyclase catalytic domain (Fig. 3).

In comparison to the central and catalytic part of the subunits, the N-terminal regions differ considerably. For example, only 27% identical amino acids are shared by the otherwise highly conserved α_1 and α_2 subunits. Despite these differences in primary structure, either α subunit coexpressed with the β_1 subunit yields enzymes with similar properties (see earlier), indicating that most of the regulatory features of sGC are determined by the β_1 subunit. Within the

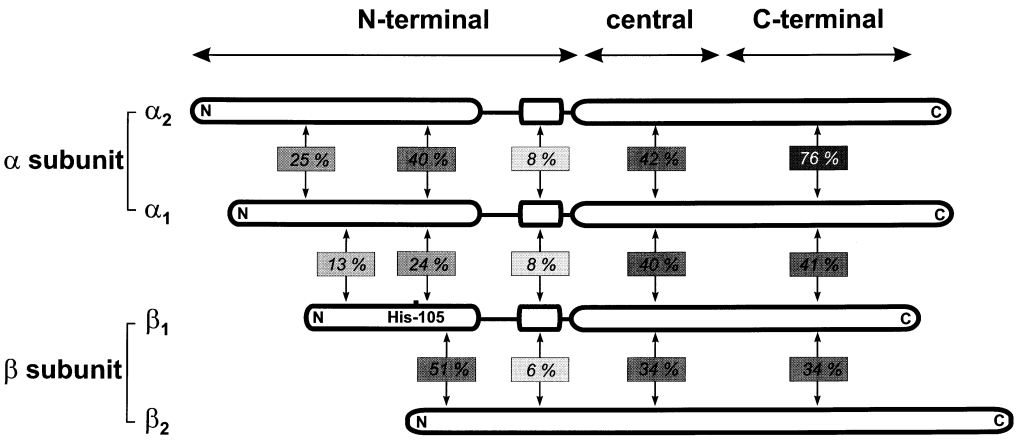


Figure 3 Comparison of the primary structure of the subunits of sGC. The polypeptide chains are shown as white bars, and gaps are indicated by a black line. The numbers between the subunits indicate the amount of identical amino acids shared between the respective subunits in a certain region. The C termini of the subunits of sGC are unrelated. The position of the His-105 as the heme-coordinating residue of the β_1 subunit is given. The regions chosen for comparison are as follows: amino acids 1–161 of α_2, 1–126 of α_1, and 1–56 of β_1 subunits; 162–268 of α_2, 127–234 of α_1, 57–164 of α_2, and 1–101 of β_2 subunits; 269–321 of α_2, 235–282 of α_1, 165–212 of β_1, and 102–208 of β_2 subunits; 322–483 of α_2, 283–441 of α_1, 213–383 of β_1, and 205–368 of β_2 subunits; 484–698 of α_2, 442–660 of α_1, 384–605 of β_1, and 369–581 of β_2 subunits.

N-terminal regions, there is a stretch of about 100 amino acids that shows a higher degree of conservation among the α subunits or the β subunits than between α and β. Conceivably, these regions define the properties of an α or a β subunit. In accordance with that, histidine-105 of the β_1 subunit (see later), which acts as the proximal ligand of the heme group, is located within this region.

The central regions of the subunits of sGC show a high degree of homology. Most likely, these regions are involved in the dimerization of the subunits, as has been shown for the respective parts of the membrane-bound enzymes (Wilson and Chinkers, 1995). Obviously, dimerization itself does not require two different sGC subunits as there is a report of catalytically inactive $\alpha_1\alpha_1$ or $\beta_1\beta_1$ homodimers (Zabel *et al.,* 1999).

The catalytic C-terminal domains of sGC reveal the highest degree of homology. These domains are also very similar to the respective regions in the membrane-bound guanylyl cyclases and in the adenylyl cyclases (see also section on the catalytic domain). With the help of deletion mutants of either of the three cyclic nucleotide-forming enzymes, these domains have been clearly shown to be sufficient to carry out the catalytic reaction. In all cases, association of two catalytic domains was required for the formation of the cyclic nucleotide.

In general, the results obtained with the N-terminally or C-terminally truncated subunits show that the regulatory and catalytic properties of sGC can indeed be attributed to the N-terminal and C-terminal region of the subunits, respectively. The regulatory and catalytic modules are able to work independently of each other, and at least the catalytic domain can be controlled by other regulatory modules, as shown by the receptor-linked guanylyl cyclases or the G-protein-stimulated AC.

THE REGULATORY HEME-BINDING DOMAIN

The N-terminal parts of the subunits of sGC are responsible for heme binding. The heme content of the enzyme has been a matter of debate. Whereas most groups found up to 1 mole of heme per mole of heterodimer (Gerzer *et al.,* 1981a; Humbert *et al.,* 1990; Tomita *et al.,* 1997b), the group of Marletta reported a heme content of 1.5 mole of heme per mole of heterodimer (Stone and Marletta, 1995). However, the latter results were obviously due to an incorrect protein determination, and more recent results obtained by the same group are consistent with a heme to heterodimer ratio of 1:1 (Denninger and Marletta, 1999).

Experiments with truncated subunits showed that the N termini of the α_1 and β_1 subunits are required for proper heme binding, although the two subunits appear to contribute unequally (Foerster *et al.,* 1996). Obviously, the β_1 subunit plays a more important role in heme binding, as it is required for NO stimulation, whereas the N terminus of the α_1 subunit is essential for a normal heme-binding capacity (Foerster *et al.,* 1996). For heme binding, the N terminus of α_1 can also be substituted with the N terminus of the β_1 subunit, as shown by Zhao and Marletta (1997). They demonstrated that the N-terminal half of the β_1 subunit (amino acids 1–385), expressed in *E. coli,* is able to form dimers. These homodimers were shown to be capable of binding heme, resulting in a heme–protein complex with characteristics similar to those of the wild-type enzyme. The exclusive role of the N-terminal region of the β_1 subunit in heme binding is underlined by point mutations of conserved cysteines

(Friebe *et al.,* 1997). These mutations cause a loss of the heme group when performed in the β_1 subunit; mutations of the corresponding cysteines in the α_1 subunit do not alter NO responsiveness.

The heme group of sGC exhibits an absorption maximum at 431 nm, indicative of a histidine as the axial ligand. Using site-directed mutagenesis, histidine-105 of the β_1 subunit has been identified as the axial ligand of the heme group (Wedel *et al.,* 1995; Zhao *et al.,* 1998). Since the mutant lacking histidine-105 remained insensitive to NO even after heme reconstitution, histidine-105 may very likely be required to transduce the NO-induced, heme-mediated activation within the protein.

Although clearly representing a functional domain, the heme binding region appears to be in close association with the catalytic domain, as GTP was shown to induce an increase in NO dissociation from the heme group and to lead to a change of the Raman spectra of NO–sGC (Kharitonov *et al.,* 1997; Tomita *et al.,* 1997a).

CATALYTIC DOMAIN

The C-terminal catalytic domains of the subunits of sGC are also conserved in the membrane-bound GCs and the adenylyl cyclases (ACs). ACs are integral membrane proteins with two hydrophobic domains and two cytosolic domains (see Fig. 2). Each of the hydrophobic domains contains six supposed transmembrane helices. The two cytosolic domains, termed C1 and C2, are homologous to each other and are homologous to the catalytic domains of both soluble and membrane-bound GCs (Sunahara *et al.,* 1996). Like sGC, ACs contain two different but conserved catalytic domains. In the adenylyl cyclases, both domains are located on one molecule, whereas in sGC, each of the two subunits contributes one catalytic domain. Comparison of the primary structures of the catalytic domains of adenylyl cyclases and sGC suggests that the catalytic domains of the α and β subunits correspond to the C1 and C2 domains of the adenylyl cyclases, respectively. This assumption has been very elegantly verified experimentally by Sunahara *et al.* (1998), who turned sGC into a cAMP-producing enzyme by exchanging the three amino acids responsible for the nucleotide specificity (see later).

A tremendous advance in the cyclase research has been the determination of the crystal structure of the catalytic core of the ACs. The analysis of the crystal structure showed that the catalytic center of AC consists of the homologous C_1 and C_2 domains arranged in a head-to-tail fashion. The interface between the C_2 and the C_1 domain forms two pseudosymmetric regions; one of these regions comprises the binding site for the substrate MgATP, and the other one, which has probably evolved from a former ATP binding site, binds the activator forskolin (Fig. 4). Based on this structure, the amino acid residues responsible for nucleotide interaction have been identified (Tesmer *et al.,* 1997); indeed, the exchange of the corresponding residues of sGC into the respective amino acids of the ACs resulted in an sGC that produced cAMP in an NO-sensitive manner (Sunahara *et al.,* 1998). These data underline the structural similarity among the ACs and sGCs. It is amazing to learn that the switch from adenylyl cyclase to guanylyl cyclase has also been performed in nature, as shown to be the case for the *Paramecium* guanylyl cyclase, which has the overall structure of an adenylyl cyclase (Linder *et al.,* 1999). The close relationship of ACs and GCs is further demonstrated by a report of a

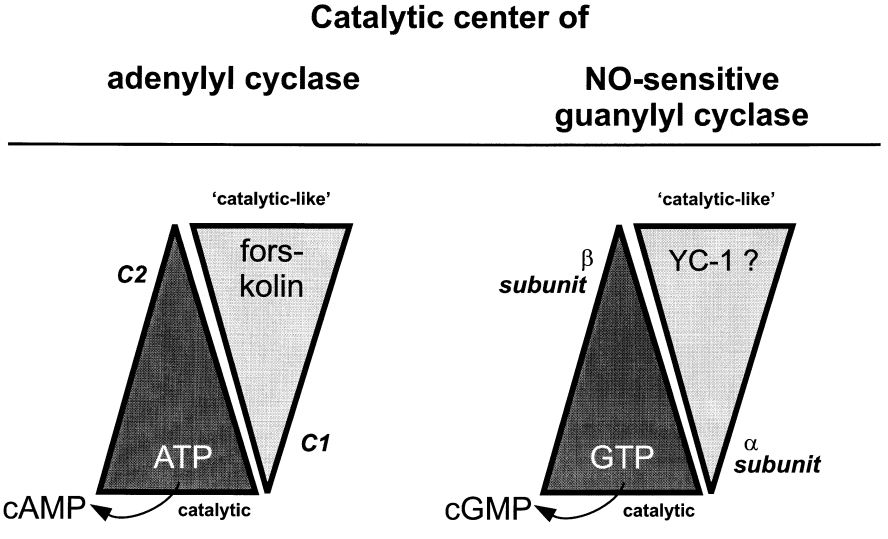


Figure 4 Schematic diagram of the catalytic domains of adenylyl cyclase and soluble guanylyl cyclase. The catalytic regions of heterodimeric AC and sGC are represented as triangles; the C2 domain of AC and the corresponding β subunit of sGC are drawn in dark gray, the C1 and α subunit are light gray. Conversion of ATP to cAMP and of GTP to cGMP are shown for the respective cyclase. The ''catalytic-like'' domain of the AC binds the diterpene activator forskolin. Although the corresponding ligand for sGC has not been identified, it is tempting to speculate on YC-1 as the possible ligand.

chimeric cyclase (Weitmann *et al.,* 1999) in which the C2 domain of an AC forms a cAMP-synthesizing enzyme with the α_1 subunit of sGC.

In addition to one ATP binding site, a pseudosymmetric region that comprises the binding site for the activator forskolin has been identified. As the effects of forskolin are very similar to those of the sGC activator YC-1 (see section on YC-1), and as the catalytic centers of ACs and GCs are very much alike, one may speculate that, analogous to forskolin, YC-1 binds to the corresponding site in sGC (see Fig. 4). Indeed, mutations within this putative YC-1-binding region led to an almost 10-fold increase in activity under nonstimulated conditions (Friebe *et al.,* 1999). Interestingly, the activity of the mutant was further increased by Mn^{2+}, almost reaching the level of the NO-stimulated wild-type enzyme, whereas NO had very little additional effect. In analogy to the YC-1 effect, the mutated enzyme showed a decreased NO dissociation rate (in the presence of Mg^{2+}). The results with the mutant show that the sGC region corresponding to the forskolin binding site has a profound influence on the catalytic rate and the dissociation of NO and suggest that YC-1 may exert its effects by binding to this site or by changing the conformation at this site. In addition, one may speculate about endogenous ligands or posttranslational modifications at this site that would result in alterations of the catalytic rate or the sensitivity of sGC toward NO.

Regulation of Soluble Guanylyl Cyclase

Nitric Oxide

The cyclic GMP-forming activity of the enzyme sGC was first described in 1969 (Hardman and Sutherland, 1969; Schultz *et al.,* 1969; White and Auerbach, 1969; Ishikawa *et al.,* 1969), and hormones which led to an increase of the intracellular calcium concentration were shown to elevate cGMP levels (Schultz *et al.,* 1973). In the late 1970s, NO-containing compounds were identified as potent activators of sGC (Arnold *et al.,* 1977; Böhme *et al.,* 1978). Despite the stimulatory effect of these NO-containing compounds, the physiological significance of NO-induced activation of the enzyme did not become clear until the identification of endothelium-derived relaxing factor (EDRF) as NO (Palmer *et al.,* 1987; Ignarro *et al.,* 1987). After the discovery of NO in the vascular system, NO formation was reported to occur throughout the body and, subsequently, the enzymes responsible for the synthesis of NO were identified and termed NO synthases. More details about further progress in the NO research are given in other chapters of this book.

Carbon Monoxide, a Physiological Activator of Soluble Guanylyl Cyclase?

The role of carbon monoxide (CO) as an activator of sGC has been a matter of debate. CO was shown to induce effects similar to those evoked by NO. There are reports about a role of CO in long-term potentiation, in olfactory signal transduction, in vasorelaxation, as well as in inhibition of platelet aggregation (Brüne and Ullrich, 1987; Brüne *et al.,* 1990; Marks *et al.,* 1991; Maines, 1997). A family of enzymes, the heme oxygenases, appears to be most relevant for the production of CO as signaling molecule. These enzymes catalyze the degradation of heme to biliverdin and CO (Maines, 1988), a reaction which appears to be a rather uneconomical way of producing a signaling molecule. In addition, the currently unknown short-term regulation of the heme oxygenases argues against an involvement in signal transduction (Maines, 1997). The proposed role of CO as a physiological stimulator of sGC stands in contrast to its rather poor sGC-stimulating properties (see section on mechanism of activation of sGC). However, in the presence of the substance YC-1 (see also section on modulators of sGC), CO is able to stimulate sGC to nearly the same extent as NO (Friebe *et al.,* 1996a). Although this capability of CO to stimulate sGC similarly to NO is striking, an endogenously occurring substance with YC-1-like properties would be required for CO to act as a physiological activator of sGC. In addition, even in the presence of YC-1, the CO concentrations required for sGC activation are rather high (Russwurm *et al.,* 1998).

Mechanism of Heme-Mediated Activation of Soluble Guanylyl Cyclase

The prosthetic heme group of sGC acts as the acceptor site for NO (Humbert *et al.,* 1990; Stone and Marletta, 1995), and the presence of the heme group has been shown to be required for the stimulatory effect of NO (Craven and DeRubertis, 1978; Ignarro *et al.,* 1982a; Ohlstein *et al.,* 1982). Removal of the heme group abolishes NO-induced activation, and the stimulatory effect of NO is restored on heme reconstitution of the enzyme (Ignarro *et al.,* 1986; Foerster *et al.,* 1996). The heme group is not covalently bound and exhibits an absorbance maximum at 431 nm (Fig. 5). This peak indicates a five-coordinated ferrous heme with a histidine as the axial ligand at the fifth coordinating position (Stone and Marletta, 1994). Using site-directed mutagenesis, His-105 of the β_1 subunit has been identified as the axial ligand (Wedel *et al.,* 1994; Zhau *et al.,* 1998). NO binds to the sixth coordination position of the heme iron and leads to the breakage of the histidine-to-iron bond, yielding a five-coordinated nitrosyl–heme complex with an absorbance maximum at 398 nm. The opening of the histidine-to-iron bond is thought to initiate a conformational change, resulting in the activation of the enzyme.

The finding that protoporphyrin IX, the iron-free precursor of heme, stimulates sGC independently of NO supports this mechanism of activation (Ignarro *et al.,* 1982b). Protoporphyrin IX, a heme lacking the iron, appears to mimic the structure of the NO–heme complex in which the iron is moved out of the plane of the porphyrin ring. Thus, in the protoporphyrin IX- and in the NO-stimulated enzyme, the axial histidine is unbound. On the other hand, breakage of the histidine-to-iron bond is required but does not appear to

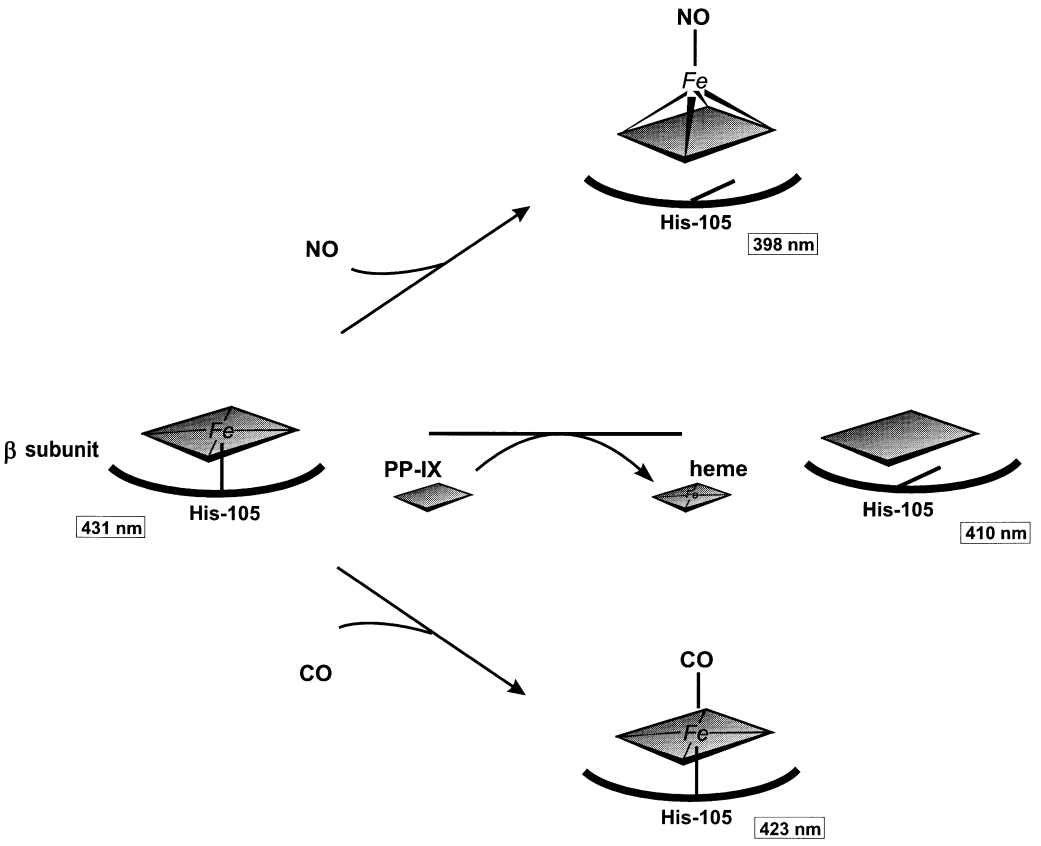


Figure 5 Heme as the NO acceptor site of soluble guanylyl cyclase. In the nonactivated state, the heme of sGC is five-coordinated with the histidine-105 of the β_1 subunit bound to the fifth coordination position of the heme iron (absorption maximum at 431 nm). NO binds to the sixth coordination position of the heme iron and leads to breakage of the histidine-to-iron bond so that the fifth coordination position is now free (absorption maximum at 398 nm). Protoporphyrin IX is able to substitute for the heme group and stimulates the enzyme (absorption maximum at 410 nm). Like NO, CO binds to the sixth coordination position of the heme iron, but, in contrast to NO, CO does not lead to the rupture of the histidine-to-iron bond; therefore, CO binding results in a six-coordinated heme (absorption maximum at 423 nm). For further explanation, see text.

be sufficient for activation, as a mutant without the proximal histidine and, accordingly, without the histidine-to-iron bond did not show an increased catalytic rate. These results indicate that the proximal histidine not only coordinates the heme but also is required to mediate the stimulatory process (see the section on the regulatory heme-binding domain).

Like NO, CO has a very high affinity for heme groups, and thus it also binds to the heme group of sGC. In contrast to NO, which activates sGC up to 200-fold, the stimulation by CO only ranges between four- and six-fold (Stone and Marletta, 1994). CO does not lead to breakage of the heme-to-iron bond, as it yields a six-coordinated heme with an absorption maximum at 423 nm in which the heme-to-iron-bond remains intact. Therefore, the low stimulatory property of CO is in accordance with the assumption that the breakage of the histidine-to-iron bond is a prerequisite for sGC activation.

It is widely accepted that the association of NO to a five-coordinate heme iron is nearly diffusion-controlled, whereas the dissociation of NO is generally rather slow. Studies on the dissociation of NO from sGC revealed that the enzyme has a comparably high NO dissociation rate, yielding a half-life of approximately 5 s at 37°C for the NO–sGC complex (Kharitonov *et al.,* 1997). Apparently, it is a unique property of the heme environment in sGC to allow such fast activation. This half-life is sufficient to allow rapid deactivation of the enzyme in biological systems, although it has to be kept in mind that the presence of an NO scavenger is a prerequisite for such fast deactivation.

Redox Regulation of Soluble Guanylyl Cyclase?

When in the early days of research on sGC the physiological activator of the enzyme NO was unknown, a multitude of different activators and regulators of the enzyme were proposed, most of them exhibiting redox-active properties (for review, see Waldman and Murad, 1987). These substances, which do not act by releasing NO (''non-NO activators''), constitute a rather heterogeneous group; thus, several mechanisms of activation have been postulated and

subsumed as ''redox regulation'' of sGC (Böhme *et al.,* 1984). In contrast to the more than 200-fold stimulation by NO, the stimulatory effect of the substances was only severalfold. Although many of the effects of redox-active compounds have been postulated to be mediated by the cysteine residues of the enzyme (DeRubertis and Craven, 1977; Brandwein *et al.,* 1981; Böhme *et al.,* 1983), point mutation of 15 conserved cysteines impaired neither catalytic activity nor NO stimulation, indicating that the cysteines of sGC are unlikely to be the mediators of this redox regulation (Friebe *et al.,* 1997).

Purified sGC was shown to be sensitive to the small amounts of NO present in the atmosphere (Friebe *et al.,* 1996b). Studies with one of the reported non-NO activators of sGC, superoxide dismutase, revealed that the stimulatory effect is not caused by a direct effect on the enzyme. Rather, superoxide dismutase, which eliminates superoxide (O_2^-), leads to an increase in the concentration of dissolved atmospheric NO in a solution by preventing the inactivation of NO by O_2^- to peroxynitrite (Friebe *et al.,* 1998a). Other non-NO activators of sGC may also exert their action by decreasing the amount of O_2^- and thereby increasing the concentration of NO. Therefore, the redox regulation of sGC may simply be an *in vitro* artifact, arising from the extreme NO sensitivity of sGC and the O_2^--mediated inactivation of the dissolved atmospheric NO.

Modulators of Soluble Guanylyl Cyclase

ODQ, an Inhibitor of the Stimulated Activity of Soluble Guanylyl Cyclase

Methylene blue and LY-83583 have been used as inhibitors of sGC, although they show very little specificity and, for example, interfere with NO formation and NO release from NO synthases (Mayer *et al.,* 1993). A potent and selective inhibitor of sGC, the quinoxalin derivative 1*H*-[1,2,4]oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ), has been identified (Garthwaite *et al.,* 1995). As ODQ was shown not to interfere with NO formation, it appears to represent an ideal tool to discriminate cGMP-dependent and cGMP-independent effects of NO. Originally, ODQ was shown to prevent NO-induced cGMP formation in the brain; now the inhibitory effect of ODQ on sGC has been demonstrated in a variety of other cells and tissues.

Studies with purified sGC revealed that ODQ binds in an NO-competitive manner and leads to an apparently irreversible inhibition of the stimulated enzyme, leaving basal activity almost unchanged. Spectral analysis suggests oxidation of the heme iron as an underlying mechanism of the inhibitory effect of ODQ (Schrammel *et al.,* 1996). The notion that the heme iron is the target of ODQ is confirmed by the finding that ODQ is not able to inhibit the stimulation of sGC induced by the iron-free protoporphyrin IX (Koesling and Friebe, 1999). A derivative of ODQ, NS 2028 (oxadiazolo[3,4-*d*]benz[*b*][1,4]oxazin-1-one), has been reported to have properties similar to those of ODQ (Olesen *et al.,* 1998).

YC-1, a Non-NO Activator of Soluble Guanylyl Cyclase

With YC-1, a non-NO activator of sGC with allosteric effects on the NO-induced activation has been identified. The compound YC-1, a benzylindazol derivative, was first described as an inhibitor of platelet aggregation that causes an increase in the intracellular cGMP concentration (Wu *et al.,* 1995). YC-1 was shown to stimulate purified sGC about 10-fold (Friebe *et al.,* 1996a; Mülsch *et al.,* 1997). Although independent of NO, the presence of the heme group was a prerequisite for YC-1 stimulation. YC-1 exerted dramatic effects on the NO-stimulated enzyme; it increased the maximal NO-induced catalytic rate by about 40% and potentiated the stimulatory effect of submaximally activating NO concentrations. The concentration–response curve of NO was shifted to the left by YC-1, indicating that YC-1 sensitized sGC toward NO. Stimulation of sGC induced by the NO-independent activator protoporphyrin IX was also potentiated by YC-1, clearly demonstrating that YC-1 has an effect on the maximal catalytic rate and an effect on the sensitivity toward ligands at the heme group. The latter is impressively demonstrated by the ability of YC-1 to turn the poor sGC activator CO into an effective stimulator (Friebe *et al.,* 1996a). The YC-1-induced increase in sensitivity toward NO is achieved by a decrease in the dissociation rate (Friebe *et al.,* 1998b). In the case of CO, YC-1 has been reported to increase its association and also to decrease its dissociation from the heme (Sharma *et al.,* 1999).

A direct YC-1–heme interaction is rather unlikely (Friebe and Koesling, 1998), as YC-1 neither changed the Soret absorption of basal nor affected NO-stimulated sGC, and YC-1 caused only a very small shift of the absorption maximum of the CO-stimulated enzyme. In addition, YC-1 was found to bind not only to the nonstimulated but also to the heme-depleted enzyme, suggesting an allosteric site on the enzyme. As mentioned earlier in the section on the catalytic domain, YC-1 may bind to the region corresponding to the forskolin-binding region in the ACs, thereby increasing catalytic rate and decreasing the dissociation of NO.

In addition to the effects on the purified enzyme, YC-1 effects have been demonstrated in a variety of cells and tissues. In vascular smooth muscle cells, YC-1 was reported to induce a concentration-dependent relaxation of endothelial-free rat aortic rings precontracted with phenylephrine and to increase cGMP levels (Mülsch *et al.,* 1997; Wegener and Nawrath, 1997). The synergistic effects of YC-1 and NO have been studied in intact platelets (Friebe *et al.,* 1998b). YC-1 in combination with NO or CO led to complete inhibition of platelet aggregation at concentrations that were ineffective when administered alone. The synergism of YC-1 and NO was emphasized by a drastic increase in intraplatelet cGMP levels. Although the application of either NO or YC-1 at maximally effective concentrations led to only an approximately 13-fold increase in the intracellular cGMP concentrations, both drugs together resulted in an over 1300-fold rise of the cGMP content. When using CO instead of

NO, similar results were obtained. CO alone did not have an effect on platelet aggregation and cGMP formation.

YC-1 was shown to inhibit cGMP-hydrolyzing phosphodiesterases in platelet cytosol (Friebe *et al.,* 1998b; Galle *et al.,* 1999). Thus, YC-1 obviously has dual effects on cGMP levels in platelets, as it increases cGMP formation through stimulation of sGC and prevents cGMP degradation through inhibition of phosphodiesterases, thereby explaining the exceptional increase in the cGMP concentration in platelets. However, even in the presence of the nonspecific inhibitor of phosphodiesterases IBMX, that is, under conditions in which the YC-1 effect on phosphodiesterases should be abolished, YC-1 led to a 10-fold increase in the maximally NO-stimulated sGC activity in platelets. With regard to the increase in the maximal NO-induced catalytic rate, the YC-1 effect in platelets is far more pronounced than that on the purified sGC. Consequently, there may be unknown factors or conditions in an intact cellular surrounding that make sGC more sensitive toward YC-1.

In conclusion, the effects of YC-1 on sGC suggest the existence of a so far unknown allosteric site on the enzyme that modulates the catalytic rate and the responsiveness toward heme ligands. Ligands to this allosteric site may represent a novel class of drugs that may exert vasodilatory, antiaggregatory, and possibly other yet unknown effects by sensitizing sGC toward its physiological activator NO. In contrast to the NO donors commonly used in the treatment of coronary heart disease, YC-1-like substances would exert their action mainly at the sites of endogenous NO production. In addition to the therapeutical implications, it is tempting to speculate about the existence of naturally occurring allosteric modulators of sGC that may alter the sensitivity of the enzyme toward NO. In the case of endogenously generated CO concentrations reaching relevant levels, sGC may be significantly stimulated by CO in the presence of such an endogenous modulator.

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References

Arnold, W., Mittal, C. K., and Murad, F. (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3203–3207.

Böhme, E., Graf, H., and Schultz, G. (1978). Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic cGMP formation in smooth muscle and platelets. *Adv. Cyclic Nucleotide Res.* **9,** 131–143.

Böhme, E., Gerzer, R., Grossmann, G., Herz, J., Mülsch, A., Spies, C., and Schultz, G. (1983). Regulation of soluble guanylyl cyclase activity. *In* "Hormones and Cell Regulation" (J. E. Dumont, J. Nunez, R. M. Denton, eds.), pp. 147–161. Elsevier, Amsterdam.

Böhme, E., Grossmann, G., Herz, J., Mülsch, A., Spies, C., and Schultz, G. (1984). Regulation of cyclic GMP formation by soluble guanylate cyclase: Stimulation by NO-containing compounds. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17,** 259–266.

Brandwein, H. J., Lewicki, J. A., and Murad, F. (1981). Reversible inactivation of guanylate cyclase by mixed disulfide formation. *J. Biol. Chem.* **256,** 2958–2962.

Brüne, B., and Ullrich, V. (1987). Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol.* **32,** 497–504.

Brüne, B., Schmidt, K.-U., and Ullrich, V. (1990). Activation of soluble guanylate cyclase by carbon monoxide and inhibition by superoxide anion. *Eur. J. Biochem.* **192,** 683–686.

Buechler, W. A., Nakane, M., and Murad, F. (1991). Expression of soluble guanylate cyclase activity requires both enzyme subunits. *Biochem. Biophys. Res. Commun.* **174,** 351–357.

Craven, P. A., and DeRubertis, F. R. (1978). Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemeproteins. Evidence for involvement of the paramagnetic nitro–syl–heme complex in enzyme activation. *J. Biol. Chem.* **253,** 8433–8443.

Denninger, J. W., and Marletta, M. A. (1999). Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim. Biophys. Acta* **1411,** 334–350.

DeRubertis, F. R., and Craven, P. A. (1977). Activation of hepatic guanylate cyclase by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. Effects of thiols, *N*-ethylmaleimide, and divalent cations. *J. Biol. Chem.* **252,** 5804–5814.

Foerster, J., Harteneck, C., Malkewitz, J., Schultz, G., and Koesling, D. (1996). A functional heme-binding site of soluble guanylyl cyclase requires intact N-termini of α_1 and β_1 subunits. *Eur. J. Biochem.* **240,** 380–386.

Friebe, A., and Koesling, D. (1998). Mechanism of YC-1-induced activation of soluble guanylyl cyclase. *Mol. Pharmacol.* **53,** 123–127.

Friebe, A., Schultz, G., and Koesling, D. (1996a). Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *EMBO J.* **15,** 6863–6868.

Friebe, A., Malkewitz, J., Schultz, G., and Koesling, D. (1996b). Positive side-effects of pollution? *Nature* **382,** 120.

Friebe, A., Wedel, B., Foerster, J., Harteneck, C., Malkewitz, J., Schultz, G., and Koesling, D., (1997). Function of conserved cysteine residues on soluble guanylyl cyclase. *Biochemistry* **36,** 1194–1198.

Friebe, A., Schultz, G., and Koesling, D. (1998a). Stimulation of soluble guanylyl cyclase by superoxide dismutase is mediated by NO. *Biochem. J.* **335,** 527–531.

Friebe, A., Müllershausen, F., Smolenski, A., Walter, U., Schultz, G., and Koesling, D. (1998b). YC-1 potentiates NO- and CO-induced cGMP effects in human platelets. *Mol. Pharmacol.* **53,** 123–127.

Friebe, A., Russwurm, R., Mergia, E., Koesling, D. (1999). A point-mutated guanylyl cyclase with features of the YC-1-stimulated enzyme: Implications for the YC-1 binding site? *Biochemistry* **38,** 15253–15257.

Galle, J., Zabel, U., Hubner, U., Hatzelmann, A., Wagner, B., Wanner, C., and Schmidt, H. H. H. W. (1999). Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. *Br. J. Pharmacol.* **127,** 195–203.

Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., and Mayer, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ). *Mol. Pharmcol.* **48,** 185–188.

Gerzer, R., Böhme, E., Hofmann, F., and Schultz, G. (1981a). Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett.* **132,** 71–74.

Gerzer, R., Hofmann, F., and Schultz, G. (1981b). Purification of a soluble, sodium-nitroprussid-stimulated guanylate cyclase from bovine lung. *Eur. J. Biochem.* **116,** 479–486.

Giuili, G., Scholl, U., Bulle, F., and Guellaen, G. (1992). Molecular cloning of the cDNAs coding for the two subunits of soluble guanylyl cyclase from human brain. *FEBS Lett.* **304,** 83–88.

Gupta, G., Azam, M., Yang, L., and Danziger, R. S. (1997). The β_2 subunit inhibits stimulation of the α_1/β_1 form of soluble guanylyl cyclase by nitric oxide. Potential relevance to regulation of blood pressure. *J. Clin. Invest.* **100,** 1488–1492.

Hardman, J. G., and Sutherland, E. W. (1969). Guanyl cyclase, an enzyme catalyzing the formation of guanosine 3',5'-monophosphate from guanosine trihosphate. *J. Biol. Chem.* **244,** 6363–6370.

Harteneck, C., Koesling, D., Söling, A., Schultz, G., and Böhme, E. (1990). Expression of soluble guanylate cyclase: Catalytic activity requires two subunits. *FEBS Lett.* **272,** 221–223.

Harteneck, C., Wedel, B., Koesling, D., Malkewitz, J., Böhme, E., and Schultz, G. (1991). Molecular cloning and expression of a new α-subunit of soluble guanylyl cyclase. *FEBS Lett.* **292,** 217–222.

Humbert, P., Niroomand, F., Fischer, G., Mayer, B., Koesling, D., Hinsch, K. D., Gausepohl, H., Frank, R., Schultz, G., and Böhme, E. (1990). Purification of soluble guanylyl cyclase from bovine lung by a new immunoaffinity chromatographic method. *Eur. J. Biochem.* **190,** 273–278.

Ignarro, L. J., Degnan, J. N., Baricos, W. H., Kadowitz, P. J., and Wolin, M. S. (1982a). Activation of purified guanylate cyclase by nitric oxide requires heme. Comparison of heme-deficient, heme-reconstituted and heme-containing forms of soluble enzyme from bovine lung. *Biochim. Biophys. Acta* **718,** 49–59.

Ignarro, L. J., Wood, K. S., and Wolin, M. S. (1982b). Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2870–2873.

Ignarro, L. J., Adams, J. B., Horwitz, P. M., and Wood, K. S. (1986). Activation of soluble guanylate cyclase by NO-hemoproteins involves NO-heme exchange: Comparison of heme-containing and heme-deficient enzyme forms. *J. Biol. Chem.* **261,** 4997–5002.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chandhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Ishikawa, E., Ishikawa, S., Davis, J. W., and Sutherland, E. W. (1969). Determination of guanosine 3',5'-monophosphate in tissues and of guanyl cyclase in rat intestine. *J. Biol. Chem.* **244,** 6371–6376.

Kharitonov, V. G., Russwurm, M., Magde, D., Sharma, V. S., and Koesling, D. (1997). Dissociation of nitric oxide from soluble guanylyl cyclase. *Biochem. Biophys. Res. Commun.* **239,** 284–286.

Koesling, D., and Friebe, A. (1999). Soluble guanylyl cyclase: Structure and function. *Rev. Phys. Biochem. Pharmacol.* **135,** 41–65.

Linder, J. U., Engel, P., Reimer, A., Krüger, T., Schultz, A., and Schultz, J. E. (1999). Guanylyl cyclases with the topology of mammalian adenylyl cyclases and an N-terminal P-type ATPase-like domain in *Paramecium, Tetrahymena* and *Plasmodium. EMBO J.* **18,** 4222–4232.

Maines, M. D. (1988). Heme oxygenase: Function, multiplicity, regulatory mechanism, and clinical applications. *FASEB J.* **2,** 2257–2568.

Maines, M. D. (1997). The heme oxygenase system: A regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* **37,** 517–554.

Marks, G. S., Brien, J. F., Nakatsu, K. B., and McLaughlin, E. (1991). Does carbon monoxide have a physiological function? *Trends Pharmacol. Sci.* **12,** 185–188.

Mayer, B., Brunner, F., and Schmidt, K. (1993). Novel actions of methylene blue. *Eur. Heart J.* **14,** 22–26.

Moncada, S., and Higgs, E. A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* **9,** 1319–1330.

Mülsch, A., Bauersachs, J., Schäfer, A., Stasch, J. P., Kast, R., and Busse, R. (1997). Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. *Br. J. Pharmacol.* **120,** 681–689.

Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W., and Murad, F. (1990). Molecular cloning and expression of cDNAs coding for soluble guanylyl cyclase from rat lung. *J. Biol. Chem.* **265,** 16841–16845.

Ohlstein, E. H., Wood, K. S., and Ignarro, L. J. (1982). Purification and properties of heme-deficient hepatic soluble guanylate cyclase: Effects of heme and other factors on enzyme activation by NO, NO-heme, and protoporphyrin IX. *Arch. Biochem. Biophys.* **218,** 187–198.

Olesen, S. P., Drejer, J., Axelsson, O., Moldt, P., Bang, L., Nielsen-Kudsk, J. E., Busse, R., and Mülsch, A. (1998). Characterization of NS 2028 as a specific inhibitor of soluble guanylyl cyclase. *Br. J. Pharmacol.* **123,** 299–309.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Russwurm, M., Behrends, S., Harteneck, C., and Koesling, D. (1998). Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. *Biochem. J.* **335,** 125–130.

Schmidt, H. H. H. W., and Walter, U. (1994). NO at work. *Cell* **78,** 919–925.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., and Mayer, B. (1996). Characterisation of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) as a heme site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol. Pharmacol.* **50,** 1–5.

Schultz, G., Böhme, E., and Munske, K. (1969). Guanyl cyclase. Determination of enzyme activity. *Life Sci.* **8,** 1323–1332.

Schultz, G., Hardman, J. G., Schultz, K., Baird, C. E., and Sutherland, E. W. (1973). The importance of calcium ions for the regulation of guanosine 3'5'-cyclic monophosphate levels. *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3889–3893.

Sharma, V. S., Magde, D., Kharitonov, V. G., and Koesling, D. (1999). Soluble guanylate cyclase: Effect of YC-1 on ligation kinetics with carbon monoxide. *Biochem. Biophys. Res. Commun.* **254,** 1888–1891.

Stone, J. R., and Marletta, M. A. (1994). Soluble guanylyl cyclase from bovine lung: Activation with nitric oxide and carbon monoxide and spectral characterisation of the ferrous and ferric states. *Biochemistry* **33,** 5636–5640.

Stone, J. R., and Marletta, M. A. (1995). Heme stoichiometry of heterodimeric soluble guanylate cyclase. *Biochemistry* **34,** 14668–14674.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996). Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.* **36,** 461–480.

Sunahara, R. K., Beuve, A., Tesmer, J. J. G., Sprang, S. R., Garbers, D. L., and Gilman, A. G. (1998). Exchange of substrate and inhibitor specificities between adenylyl and guanylyl cyclases. *J. Biol. Chem.* **273,** 16332–16338.

Tesmer, J. J. G., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997). Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G_{s\alpha}$·GTPγS. *Science* **278,** 1907–1916.

Tomita, T., Ogura, T., Tsuyama, S., Imai, Y., and Kitagawa, T. (1997a). Effects of GTP on bound nitric oxide of soluble guanylyl cyclase probed by resonance Raman spectroscopy. *Biochemistry* **36,** 10155–10160.

Tomita, T., Tsuyama, S., Imai, Y., and Kitagawa, T. (1997b). Purification of bovine soluble guanylate cyclase and ADP-ribosylation on its small subunit by bacterial toxins. *J. Biochem.* (*Tokyo*) **122,** 531–536.

Waldman, S. A., and Murad, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.* **39,** 163–196.

Wedel, B. J., and Garbers, D. L. (1997). New insights on the functions of the guanylyl cyclase receptors. *FEBS Lett.* **410,** 29–33.

Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Böhme, E., Schultz, G., and Koesling, D. (1994). Mutation of His-105 of the β_1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2592–2596.

Wedel, B., Harteneck, C., Foerster, J., Friebe, A., Schultz, G., and Koesling, D. (1995). Functional domains of soluble guanylyl cyclase. *J. Biol. Chem.* **270,** 24871–24875.

Wegener, J. W., and Nawrath, H. (1997). Differentiell effects of isoliquiritigenin and YC-1 in rat aortic smooth muscle. *Eur. J. Pharmacol.* **3203,** 89–91.

Weitmann, S., Wursig, N., Navarro, J. M., and Kleuss, C. (1999). A functional chimera of mammalian guanylyl and adenylyl cyclases. *Biochemistry* **16,** 3409–3413.

White, A. A., and Aurbach, G. D. (1969). Detection of guanyl cyclase in mammalian tissues. *Biochim. Biophys. Acta* **191,** 686–697.

Wilson, E. M., and Chinkers, M. (1995). Identification of sequences mediating guanylyl cyclase dimerisation. *Biochemistry* **34,** 4696–4701.

Wu, C. C., Ko, F. N., Kuo, S. C., Lee, F. Y., and Teng, C. M. (1995). YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br. J. Pharmacol.* **116,** 1973–1978.

Yuen, P. S. T., Potter, L. R., and Garbers, D. L. (1990). A new form of guanylyl cyclase is preferentially expressed in rat kidney. *Biochemistry* **29,** 10872–10878.

Zabel, U., Weeger, M., La, M., and Schmidt, H. H. H. W. (1998). Human soluble guanylate cyclase: functional expression and revised isoenzyme family. *Biochem. J.* **335,** 51–57.

Zabel, U., Häusler, C., Weeger, M., and Schmidt, H. H. H. W. (1999). Homodimerisation of soluble guanylyl cyclase subunits. *J. Biol. Chem.* **274,** 18149–18152.

Zhao, Y., and Marletta, M. A. (1997). Localization of the heme binding region in soluble guanylate cyclase. *Biochemistry* **36,** 15959–15964.

Zhao, Y., Schelvis, J. P., Babcock, G. T., and Marletta, M. A. (1998). Identification of histidine 105 in the β_1 subunit of soluble guanylate cyclase as the heme proximal ligand. *Biochemistry* **37,** 4502–4509.

Zhuo, M., and Hawkins, R. D. (1995). Long-term depression: A learning-related type of synaptic plasticity in the mammalian central nervous system. *Rev. Neurosci.* **6,** 259–277.

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CHAPTER 25

Mechanism of Activation of Soluble Guanylyl Cyclase by NO

Allosteric Regulation through Changes in Heme Coordination Geometry

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THE NITRIC OXIDE (NO)–cGMP PATHWAY REGULATES MANY IMPORTANT PHYSIOLOGICAL PROCESSES, INCLUDING BLOOD PRESSURE, HEARING, VISION, AND NEUROTRANSMISSION, AMONG OTHERS. IN MANY CASES THE PHYSIOLOGICAL EFFECTS OF NO ARE MEDIATED THROUGH THE ENZYME RECEPTOR KNOWN AS SOLUBLE GUANYLYL CYCLASE (sGC). THIS CHAPTER PROVIDES AN OVERVIEW OF THE CHEMICAL PROPERTIES OF NO THAT MAKE IT SUCH AN UNUSUAL SENSING MOLECULE, AND IT DESCRIBES HOW NO INTERACTS WITH THE HEME COFACTOR OF NO-SENSING sGC. FIRST, A DESCRIPTION OF THE STRUCTURE AND BONDING OF METAL NITROSYLS IS PRESENTED IN ORDER TO PROVIDE A FOUNDATION FOR UNDERSTANDING THE METALLOBIOCHEMISTRY OF NO. THE REACTIVITY OF NO WITH THE IRON ATOM OF THE HEME PROSTHETIC GROUP IS THEN DESCRIBED IN SOME DETAIL, SINCE NO EXERTS ITS EFFECT BY INTERACTING WITH THE HEME IN sGC. NEXT, THE INTERACTION OF O_2 AND CO WITH OTHER GAS-SENSING HEME PROTEINS, KNOWN TO UNDERGO CONFORMATIONAL CHANGES ON BINDING THEIR MESSENGER MOLECULES, IS PRESENTED AS A PARADIGM FOR NO-SENSING sGC. AN OVERALL PICTURE OF THE MECHANISM OF NO ACTIVATION OF sGC IS DEVELOPED IN LIGHT OF THIS BACKGROUND, USING THE LATEST EXPERIMENTAL DATA ON sGC. FINALLY, INHIBITORS AND ACTIVATORS OF sGC ARE DISCUSSED, BECAUSE A PRACTICAL RESEARCH GOAL IS TO FIND SELECTIVE INHIBITORS OR ACTIVATORS OF sGC THAT MAY BE USED AS THERAPEUTIC AGENTS.

Introduction

The discovery that nitric oxide (NO) is a messenger molecule (Feldman *et al.,* 1993; Ignarro, 1999; Murad, 1999) was a surprise. Most cellular messengers are larger molecules that make specific contacts with their targets via shape selecting, hydrogen bonding, or specific ion pairing interactions. NO, in contrast, has no obvious recognition handles other than its electron distribution and chemical reactivity. NO is able to freely diffuse through aqueous solutions or membranes, reacting rapidly with specific intracellular molecules. One set of targets for NO are the metalloproteins, and

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the best-studied sensor for NO, soluble guanylyl cyclase (sGC), is a heme iron-containing metalloprotein. Metalloproteins are ideal sensors for NO because the metal centers interact both as electron acceptors and as electron donors, forming stable metal nitrosyl complexes.

Since small gaseous molecules, such as NO, exert many of their effects by interacting with the metal cofactors of metalloproteins, it is important to understand how these metal centers interact with gases. Prior to the recognition that they could serve as biological signals, NO and CO were utilized as inhibitors and probes of the active sites of metalloproteins (Antonini and Brunori, 1971). In fact, much of our understanding of how gases interact with the heme cofactor comes from studies intended to probe the function of the O_2 carrier hemoglobin (Hb) (Jameson and Ibers, 1994; Olson and Phillips, 1997). Heme proteins that sense the small molecules O_2 (Fix L) (Gilles-Gonzalez *et al.,* 1995; Tamura *et al.,* 1996; Lukat-Rodgers and Rodgers, 1997), CO (CooA) (Aono *et al.,* 1996; Shelver *et al.,* 1997), and NO (sGC) (Wolin *et al.,* 1982; Stone and Marletta, 1994; Wedel *et al.,* 1994; Yu *et al.,* 1994) have been characterized, and their function is intimately linked to the chemistry of the heme iron–gas molecule interaction. This chapter will focus on the NO-sensing enzyme sGC, the cellular receptor for NO that mediates many of the important biological effects of the NO–cGMP pathway (Ignarro, 1999). This enzyme catalyzes the conversion of guanosine-5'-triphosphate (GTP) to cyclic-3',5'-monophosphate (cGMP) and pyrophosphate (PP_i) (Fig. 1),

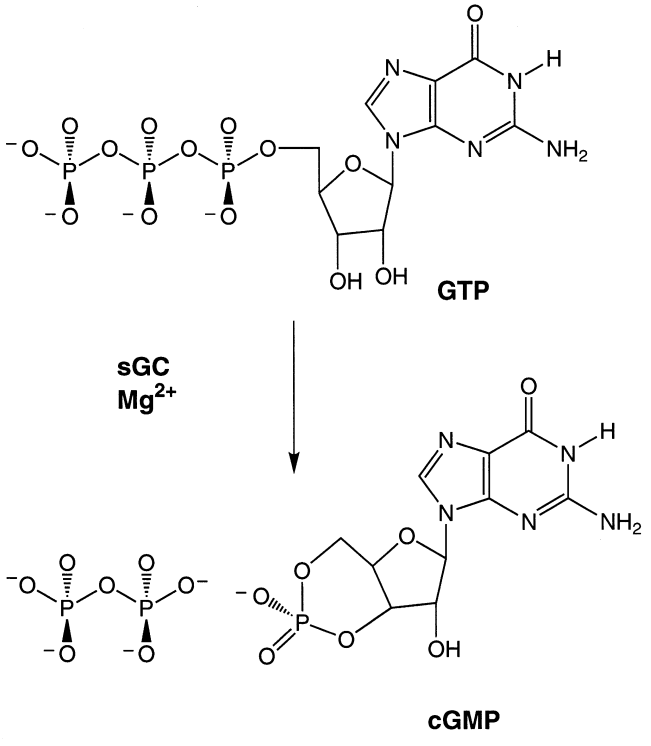


Figure 1 The conversion of GTP to cGMP catalyzed by soluble guanylyl cyclase (sGC). The substrate for the enzyme is $MgGTP^{2-}$, and excess Mg^{2+} is required for maximal catalytic activity.

and, like many other gas-sensing proteins, it contains a heme prosthetic group (Fig. 2), which is the site of NO binding (Wolin *et al.,* 1982; Stone and Marletta, 1994; Yu *et al.,* 1994). NO is believed to trigger a conformational change in sGC on binding to the heme cofactor, and this structural change in turn causes a dramatic increase in the rate of enzyme turnover. Elucidating the NO-sensing mechanism of sGC has proved challenging, owing to the complexity of the enzyme and the lack of purified protein; however, an understanding of how the heme mediates the effect of NO is now emerging (Hobbs, 1997).

This chapter is structured to provide an overview of the chemical properties of NO that make it such an unusual sensing molecule, and in particular, how the properties of NO and its interactions with the heme cofactor can be used to understand the mechanism of NO-sensing by sGC. First, the structure and bonding of metal nitrosyls will be discussed in order to provide a foundation for understanding the metallobiochemistry of NO. The reactivity of NO with the heme prosthetic group will be presented in detail since it is this interaction that mediates many of the biological effects of NO. Next, the interaction of O_2 and CO with other gas-sensing heme proteins known to undergo conformational changes on binding their messenger molecules will be presented as a paradigm for NO-sensing sGC. Finally, an overall picture of the mechanism of NO activation of sGC will be developed in light of this background, using the latest experimental data on sGC. This review is intended to provide the reader with a fundamental understanding of the interactions of NO with heme proteins and, in particular, with an appreciation for how NO triggers the activation of sGC, a crucial step in the physiologically important NO–cGMP signaling pathway in mammals.

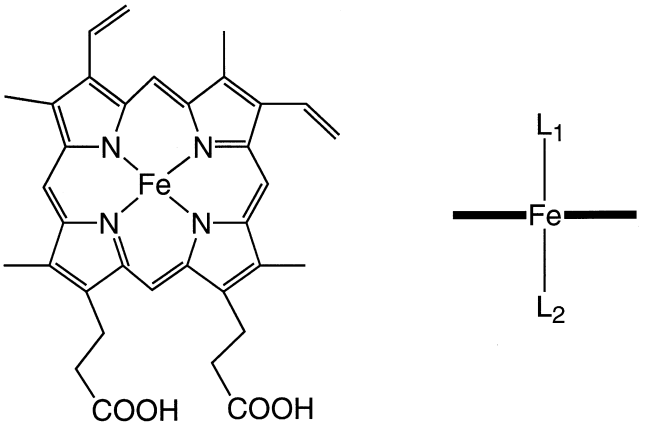


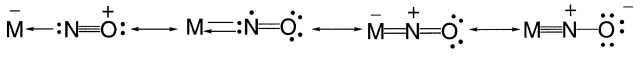
Figure 2 The structure of the heme *b* cofactor [iron(II)protoporphyrin IX] with a side on view of the axial ligands bound to the iron atom. The planar ring structure is common to all porphyrins; other heme cofactors and porphyrins have different peripheral substituents. By analogy to the hemoglobin structure, one ligand (L_2) is termed proximal, and the other (L_1) is termed distal. In subsequent figures the porphyrin is represented by a square of four N atoms, or a heavy bar on either side of the iron atom.

NO and Its Interactions with Metals: The Bonding, Structure, and Properties of Metal Nitrosyl Complexes

The transition metal ions found in metalloproteins react readily with NO to form metal nitrosyl complexes. The biologically relevant transition metals Fe, Mn, Co, Ni, V, Mo, and W all form metal nitrosyl (M–NO) complexes that have been characterized (Richter-Addo and Legzdins, 1992). The bonding in metal nitrosyl complexes has been classically described using valence bond theory by assigning formal oxidation states to the metal and the nitrosyl ligand (Moeller, 1946a,b; Griffith *et al.,* 1958; Lewis *et al.,* 1958; Raynor, 1972). The M–NO unit is either linear with the NO assigned as NO^+ (a net donation of three electrons to the metal) or the NO is bent, with the NO assigned as NO^- (a net donation of one electron to the metal). In valence-bond terms the NO^+ group is considered to be *sp* hybridized, while the NO^- unit is considered to be sp^2 hybridized. The bonding in the M–NO unit is described by a set of resonance structures, as illustrated in Fig. 3. A shortcoming of this valence-bond approach is obvious if one considers the effect of assigning oxidation states to metal nitrosyls using this bonding description. The linear metal nitrosyl complexes $Mn(CO)(NO)_3$ and $Cr(NO)_4$ would exhibit the unreasonably low metal oxidation states of -3 and -4, respectively. Furthermore, experimental and calculational evidence reveals that the M–NO linkage is strongly covalent, not ionic as implied by the valence bond formalism (Feltham and Enemark, 1981; Richter-Addo and Legzdins, 1992).

The covalent bonding in metal nitrosyls is most accurately described using molecular orbitals (Hoffman *et al.,* 1974; Feltham and Enemark, 1981). In this approach the NO ligand donates electron density to the metal through a σ type orbital and accepts electron density from the metal *d* orbitals through backbonding with the π* NO orbitals (Fig. 4). This bonding scheme is analogous to the Dewar–Chatt–Duncanson description of metal carbonyl bonding (Shriver *et al.,* 1994); however, there are distinct differences between CO and NO as ligands for transition metals. NO is considered to be a weaker σ donor but a better π acceptor than CO (Bursten *et al.,* 1981; Bursten and Gatter, 1984). In a M–NO unit the M–N bond is typically strong and the N–O bond is weak; in contrast, in a M–CO unit the M–C bond is weak and the C–O bond is strong (Richter-Addo and Legzdins, 1992). Evidence for these differences can be seen in the differing reactivities of metal nitrosyl and metal carbonyl complexes; metal nitrosyls lose an oxygen atom to form metal nitrides whereas metal carbonyls react to displace or functionalize the CO unit (Richter-Addo and Legzdins, 1992).

(A) Linear NO (NO^+, 3 electron donor)



(B) Bent NO (NO^-, 1 electron donor)

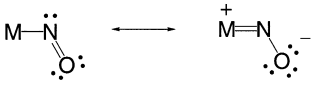
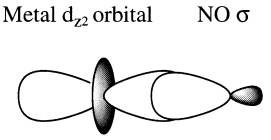


Figure 3 The valence bond description of metal nitrosyls. (A) Resonance structures for a linear metal nitrosyl. (B) Resonance structures for a bent metal nitrosyl.

(A) NO-to-metal σ-bonding



(B) Metal-to-NO π* back-bonding

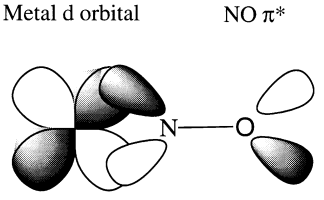


Figure 4 A simple molecular orbital description of metal nitrosyls. The three electrons from NO are distributed among the σ (two electrons) and π* (one electron) symmetry orbitals of NO, and these NO orbitals interact with those of corresponding symmetry on the metal ion. (A) The σ bonding interaction between the NO σ orbital (corresponding to the lone pair on N) and the metal ion d_{z^2} orbital. (B) The π backbonding interaction between a metal orbital of π symmetry (either d_{xz} or d_{yz}) and the NO π* orbital.

A more comprehensive molecular orbital description was developed by Feltham and Enemark (1981) to explain the bonding and properties of a wide variety of metal nitrosyl complexes. The theory explains the existence of both bent and linear M–NO groups without invoking ionic character in the M–NO unit. Feltham and Enemark (1981) proposed the $\{M(NO)_x\}^n$ formalism where the M–NO bonding units are considered first in generating the molecular orbital set; additional ligands can then perturb these M–NO-derived molecular orbitals. In this bonding model, the total number of electrons, *n,* associated with the M–NO unit includes only the *d* electrons from the metal and the π* electrons from NO (one electron for each NO group). For example, $Re(NO)(H)_2(PPh_3)_3$ (where Ph is phenyl) is denoted $\{Re(NO)\}^6$ (five *d* electrons from Re^{2+} and one π* electron from NO). Feltham and Enemark (1981) were able to use this formalism to provide a basis for understanding the structures of a wide variety of metal nitrosyls. Metal nitrosyl

complexes exhibit diverse coordination modes; the NO group can bind to the metal in either a terminal mode (where the NO binds to the metal via the N atom) or a bridging mode (where the NO binds via both the N and O atoms) (Richter-Addo and Legzdins, 1992). Bridging NO ligands are typically found in multimetallic compounds. The $\{M(NO)_x\}^n$ formalism can be effectively applied to understand the bonding in all these structural types; however, we will only discuss monometallic species that contain terminal nitrosyls because it is such a complex that is formed in sGC.

The majority of terminal nitrosyl complexes that have been characterized are six-coordinate, approximately octahedral complexes, although four- and five-coordinate complexes are known (Richter-Addo and Legzdins, 1992). The bonding in these complexes is well described by the Feltham–Enemark (1981) model. In complexes of the type $\{M(NO)\}^n$, where $n = 0$–6 electrons, the valence electrons reside only in bonding and nonbonding molecular orbitals; the antibonding orbitals are empty. In such complexes the NO unit will be linear since maximum overlap can occur between the metal and NO ligand. An example is $Cr(NO)(NO_2)_2(py)_3$ (where py is pyridine) for which $n = 5$ and the M–N–O angle is 180° (Lukehart and Troup, 1977). When $n > 6$, the additional electrons would be expected to occupy a π antibonding orbital shared by M, N, and O. Instead, bending of the M–N–O unit occurs, relieving electronic strain in the complex and lowering the energy of a σ antibonding orbital with metal d_{z^2} and NO σ character (Fig. 5A) (Feltham and Enemark, 1981; Richter-Addo and Legzdins, 1992). The bond-weakening effect of adding additional electrons into an orbital that is antibonding with respect to the metal and the NO is offset by a compensatory bonding interaction between the metal d_{z^2} orbital and the NO π* orbital (Fig. 5B). The metal–NO bond thus remains intact, although the backbonding interactions are weakened by poorer overlap (Fig. 5C and 5D). Examples of complexes with bent M–N–O units include $[Fe(NO)(das)_2(NCS)]BF_4$ [where das is *o*-phenylenebis(dimethylarsine)] for which $n = 7$ and the M–N–O angle is 140° (Enemark *et al.,* 1977), and $[Co(NO)(NH_3)_5]Cl_2$ for which $n = 8$ and the M–N–O angle is 119° (Pratt *et al.,* 1971).

Four- and five-coordinate terminal metal nitrosyl complexes have been characterized. They may be viewed as six-coordinate complexes with vacant sites, since they obey similar rules (Feltham and Enemark, 1981). Five-coordinate nitrosyl complexes can be either square pyramidal or trigonal bipyramidal, with either linear or bent M–NO units. In trigonal bipyramidal complexes, the NO group can be either axial or equatorial. As in the case of six-coordinate complexes, when the electron count in the M–NO unit is less than or equal to six, the M–NO unit is linear, and when the electron count exceeds six, the M–NO unit is bent. The majority of structurally characterized four-coordinate metal nitrosyl complexes are of the $\{M(NO)\}^{10}$ type, and they possess a pseudotetrahedral coordination geometry with linear M–NO groups (Richter-Addo and Legzdins, 1992). An exception is (TP')CuNO [where TP' is tris(3-*tert*-butylpyrazolyl)hydroborate]; in this case, the complex is pseudotetrahedral, with $\{Cu(NO)\}^{11}$ and a Cu–N–O angle of 163° (Ruggiero *et al.,* 1993). Thus, the structure and bonding in metal nitrosyls are easily rationalized by a simplified molecular orbital approach which takes into consideration the covalency of the M–NO unit.

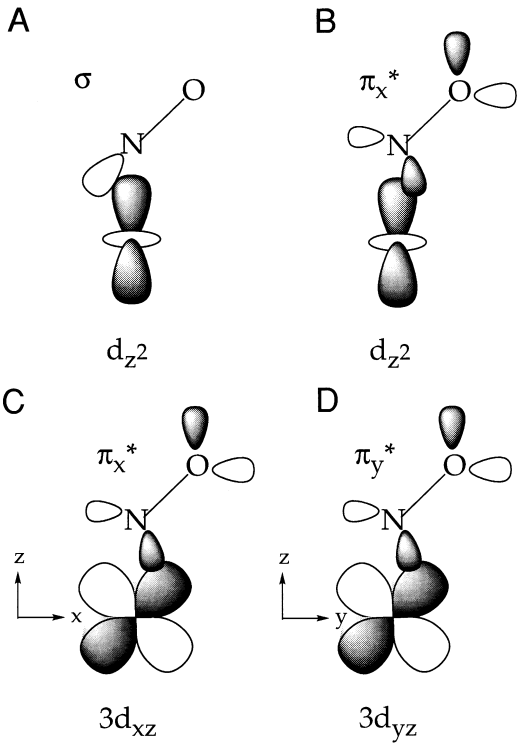


Figure 5 The orbital interactions in a bent metal nitrosyl complex. (A) Bending the NO ligand minimizes the antibonding overlap between the metal d_{z^2} orbital and the NO σ orbital. (B) A compensatory bonding interaction takes place between the NO π* orbital and the metal d_{z^2} orbital. (C and D) Bending reduces the backbonding interactions between the metal and NO π symmetry orbitals by decreasing the orbital overlaps.

Because the nitrosyl formed in sGC is a nitrosyl metalloporphyrin, it is valuable to consider the structure and bonding of nitrosyl metalloporphyrins in more detail. The porphyrin ligand (Fig. 2) bears a −2 charge which is shared equally among the four pyrrole nitrogens. The nitrogens are arranged in a square plane, making the porphyrin an obligate four-coordinate ligand. There are two additional ligand binding sites above and the below the porphyrin plane where small molecules can bind to form five- and six-coordinate complexes. NO binds to metalloporphyrins to form either five-coordinate square pyramidal or six-coordinate pseudo-octahedral complexes (Richter-Addo and Legzdins, 1992). Five-coordinate nitrosyl metalloporphyrins of Groups 6 through 9, (P)M(NO) where P is the dianionic porphyrin core and M a divalent metal ion, have been synthesized (Scheidt, 1977). The M–NO bond geometry is a function of the metal, as predicted by the Feltham–Enemark electron count, and is independent of the identity of the porphyrin (Fig. 6A). The Group 5 metals Cr and Mo form five-coordinate (P)Cr(NO) and (P)Mo(NO) complexes with a

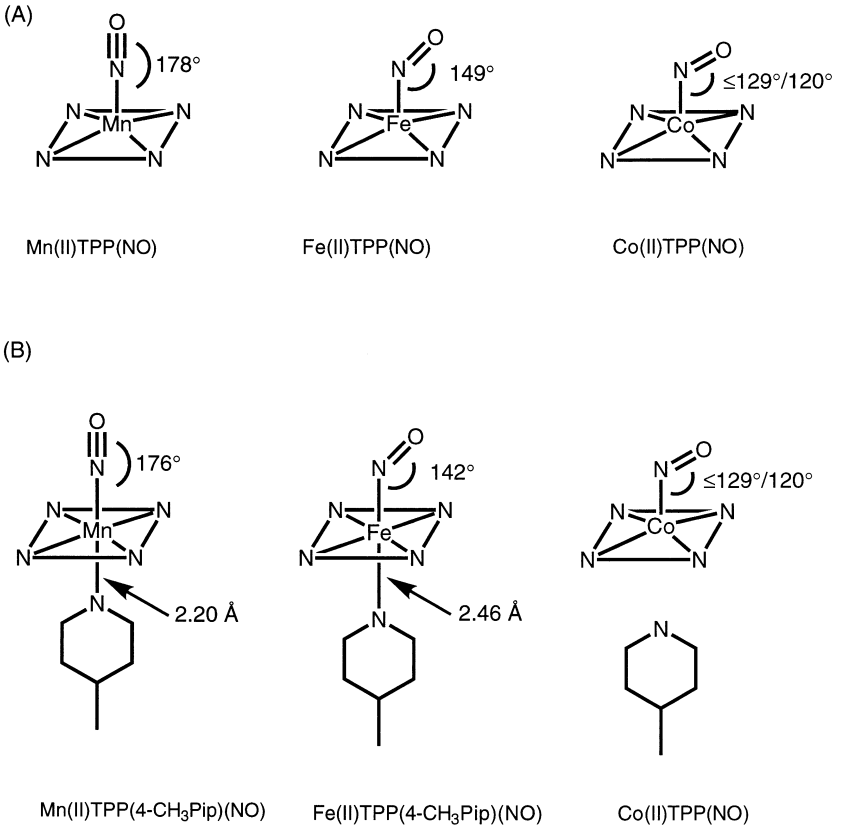


Figure 6 Structures of five- and six-coordinate nitrosyl metalloporphyrins. (A) Five-coordinate nitrosyl metalloporphyrin complexes obey the Feltham–Enemark rules. The M–NO unit in the $\{Mn(NO)\}^6$-type complex is linear, whereas those in $\{Fe(NO)\}^7$- and $\{Co(NO)\}^8$-type complexes are bent. (B) Six-coordinate Fe and Co nitrosyl metalloporphryins exhibit an NO *trans* influence, a lengthening of the bond *trans* to NO. Six-coordinate Mn nitrosyls do not exhibit this bond lengthening. The magnitude of the bond lengthening increases from iron to cobalt, correlating with an increasing population of the antibonding d_{z^2}–σ NO orbital.

$\{M(NO)\}^5$ electron count and linear M–N–O linkages (Diebold *et al.,* 1979; Kelly and Kadish, 1984). The Group 6 Mn ion also forms a five-coordinate (TTP)Mn(NO) complex with $\{M(NO)\}^6$ and a linear M–NO unit (Scheidt *et al.,* 1979). In contrast, the Group 8 atoms Fe and Co form nitrosyl metalloporphyrins that have substantially bent M–N–O groups. (TPP)Fe(NO), $\{Fe(NO)\}^7$, has a M–NO linkage of 149.2° (Scheidt and Frisse, 1975), whereas (TPP)Co(NO), $\{Co(NO)\}^8$, has an M–N–O linkage of 135.2° (Scheidt and Hoard, 1973) or 120° in a more recent crystal structure (Richter-Addo *et al.,* 1996). The Feltham–Enemark description of the structure and bonding in metal nitrosyls thus is valid for five-coordinate nitrosyl metalloporphyrins.

Because five-coordinate nitrosyl complexes, including nitrosyl metalloporphyrins, have a vacant coordination site *trans* to the NO, they are able to bind an additional ligand. Six-coordinate Mn, Fe, and Co nitrosyl complexes have been synthesized, and they follow the structural pattern predicted by the M–NO electron count: the Mn–NO unit is linear whereas the Fe–NO and Co–NO units are bent (Richter-Addo and Legzdins, 1992). Interestingly, six-coordinate Fe nitrosyl complexes display a lengthening of the metal–ligand bond *trans* to NO by approximately 0.2 Å (Nappier *et al.,* 1975; Scheidt and Piciulo, 1976; Enemark *et al.,* 1977; Scheidt *et al.,* 1977). This *trans* influence is clearly a consequence of the additional electron on the NO ligand, as (P)Fe(CO)(L) complexes do not exhibit this effect (Peng and Ibers, 1976; Kim and Ibers, 1991). In contrast, six-coordinate Mn nitrosyl complexes exhibit virtually no *trans* bond lengthening (Tullberg and Vannerberg, 1967; Piciulo *et al.,* 1974). Six-coordinate Co nitrosyl complexes exhibit an even greater *trans* influence than Fe nitrosyl complexes, with a *trans* bond lengthening of approximately 0.3 Å (Snyder and Weaver, 1970; Pratt *et al.,* 1971; Enemark *et al.,* 1975; Johnson *et al.,* 1976). The magnitude of the *trans* influence depends on the Feltham–Enemark electron count and the predicted occupancy of the antibonding metal d_{z^2}–σ NO orbital. As the electron count in the M–NO unit increases above six, the antibonding metal d_{z^2}–σ NO-derived orbital becomes populated, with one electron in the case of Fe–NO and two electrons in the case of Co–NO. This orbital, because of its significant metal d_{z^2} character, is also antibonding with respect to the sixth ligand; therefore, the bond to the ligand *trans* to NO is weakened when the orbital

is populated. The effect of the electron count on the *trans* influence can be clearly seen in Fig. 6B. In Fe(II)TPP(4-MePip)(NO)·$CHCl_3$ the Fe–N bond *trans* to NO is 0.26 Å longer than that in the analogous Mn complex, and 0.33 Å longer than other Fe–N bonds (Piciulo *et al.,* 1974; Scheidt *et al.,* 1977). Co(II)TPP will not bind a sixth ligand at all, existing as five-coordinate Co(II)TPP(NO) even in pure 4-methylpiperidine (Scheidt and Hoard, 1973). As we will discover shortly, the structure and bonding of these nitrosyl metalloporphyrins provides a foundation for understanding the coordination changes that occur when NO binds to the heme of sGC.

The Interaction of NO with the Heme Cofactor of Heme Proteins

Heme-containing proteins are a diverse family found in almost all forms of life. There is a vast literature on heme proteins; some good general reviews are available for those readers who require more information than is provided in this chapter (Durham and Millet, 1994; English, 1994; Goff, 1994; Jameson and Ibers, 1994; Lippard and Berg, 1994; Walker and Simonis, 1994). All heme proteins contain a heme prosthetic group with a single iron atom bound in a square planar array by the four pyrrole nitrogens of the porphyrin (Fig. 2). The oxidation state of the iron is typically either +2 (ferrous) or +3 (ferric), although more highly oxidized states are attainable in certain cases. When the iron is bound in the dianionic porphyrin core, the net charge on a ferrous heme is 0 and on a ferric heme is +1. The protein typically provides one or two additional ligands to the iron atom which bind in the axial positions above and below the porphyrin plane, forming a five- or six-coordinate iron complex. These positions are designated proximal and distal on the basis of their relationship to hemoglobin structure; in hemoglobin the proximal histidine is the heme ligand, and the distal histidine stabilizes the bound oxygen with a hydrogen bond. The heme iron is bound through coordinate covalent bonds to amino acids bearing Lewis basic donor sites. Common heme ligands include histidine, providing an imidazole donor, cysteine, a thiolate donor, tyrosine, a phenolate donor, and methionine, a thioether donor. Less common ligands are the amine on a lysine side chain or the N terminus of the protein. The substituents attached to the porphyrin macrocycle determine the type of heme cofactor. Heme *b,* also known as protoheme or iron protoporphyrin IX, is the cofactor found in hemoglobin and sGC. There are other types of heme cofactors that occur naturally in biology; these include hemes *a* and *d* that contain a polyisoprene tail connected to the porphyrin and hemes *c, c*', and *f* that form covalent thioether bridges to two cysteine thiolate residues of the protein. Hemes *a, b,* and *d* are noncovalently bound to the protein by ionic and hydrophobic interactions between the heme cofactor and the protein matrix.

The electronic state of the heme is modulated by the protein environment and plays an important role in the function of a heme protein (Jameson and Ibers, 1994; Walker and Simonis, 1994). The oxidation state of the heme depends on the conditions and the redox potential of the specific heme protein. The presence of two strong field axial ligands yields a low-spin heme, whereas the presence of a single axial ligand typically yields a high-spin heme. Common strong field ligands include histidine, cysteine (thiolate), tyrosine (phenolate), methionine, OH^-, CN^-, N_3^-, CO, and NO. Weak field ligands include water, F^-, thiols, and alcohols. The possible spin states for the *d* electrons in ferric heme proteins are either high spin ($S = \frac{5}{2}$), intermediate spin ($S = \frac{3}{2}$), or low spin ($S = \frac{1}{2}$), depending on the heme environment. The spin states of ferrous heme are either low spin ($S = 0$) or high spin ($S = 2$); the $S = 1$ intermediate-spin state is not observed in biological ferrous heme proteins (Jameson and Ibers, 1994; Walker and Simonis, 1994). The most important point to emphasize concerning the electronic structures of heme proteins is that the energy differences between spin states are typically small, and the protein environment can exert an enormous influence on the spin state, and thus the function, of the heme cofactor in a protein.

Heme proteins that have only one axial amino acid ligand typically bind to a variety of exogenous ligands, and these ligand interactions have been used to characterize the heme environment. The most widely studied heme proteins are hemoglobin and myoglobin; both contain a high-spin heme bound by a histidine imidazole (Jameson and Ibers, 1994; Lippard and Berg, 1994). Oxygen binds directly to the ferrous heme, forming a six-coordinate complex that retains the histidine ligand. Other neutral ligands such as imidazole and CO can also bind to the heme of ferrous (deoxy) hemoglobin (Antonini and Brunori, 1971; Jameson and Ibers, 1994). Neutral ligands typically bind more tightly to the electron-rich ferrous heme than to ferric heme. In contrast, negatively charged exogenous ligands such as CN^-, N_3^-, or F^- bind preferentially to the more highly charged ferric heme. Addition of a sixth axial ligand to either a ferrous or a ferric heme that is five-coordinate and high spin results in conversion to a six-coordinate, low-spin heme (Antonini and Brunori, 1971; Jameson and Ibers, 1994). The changes in heme coordination environment on binding exogenous ligands can be monitored by a variety of spectroscopic methods, providing a wealth of information on the heme environment, including in many cases the identity of the protein-derived axial ligand.

Nitric oxide reacts readily with the iron of heme proteins, binding even more strongly to ferrous heme than O_2 or CO (Antonini and Brunori, 1971; Traylor and Sharma, 1992; Olson and Phillips, 1997a). The greater affinity of NO for heme, when compared to the affinity of CO for heme, can be attributed to differing dissociation rates; the dissociation rate constant for loss of NO from deoxyhemoglobin is much smaller, 10^{-5} s^{-1}, than that for loss of CO, 20 s^{-1}, whereas the association rates for the two gases are comparable (Doyle and Hoekstra, 1981; Olson *et al.,* 1997). Another important difference between NO and CO is that while CO only binds to Fe(II) hemes, NO binds to both Fe(II) and Fe(III) hemes

(Antonini and Brunori, 1971). On binding to some ferric heme proteins, including hemoglobin, the NO reduces the heme to the ferrous state, and a second NO molecule binds to form the stable Fe(II)–NO adduct (Antonini and Brunori, 1971; Addison and Stephanos, 1986). The effect of NO on the binding of a sixth ligand is readily seen in thermodynamic studies of ligand binding in iron carbonyl and iron nitrosyl heme complexes (Fig. 7) (Traylor and Sharma, 1992). NO thermodynamically favors formation of a five-coordinate NO–heme complex. CO, in contrast, prefers to form a six-coordinate CO-heme, with an axial ligand bound. Also, NO binds to heme in a bent fashion, whereas CO binds to heme in a linear fashion. These observations are easily rationalized in terms of the electron counts of the NO and CO complexes. The Fe(II)–CO unit, with electron count $\{Fe\text{-}CO\}^6$ (the CO donates 0 π^* electrons), forms a linear M–CO unit; a base readily binds to the sixth coordination position. In contrast, the Fe(II)–NO unit, with electron count $\{Fe\text{-}NO\}^7$, forms a bent M–NO unit, and the binding of the sixth ligand is destabilized by the population of the antibonding d_{z^2}–σ NO orbital. The relative instability of the six-coordinate ferrous nitrosyl heme is an important contributor to the mechanism of NO activation of sGC.

The cooperative binding of oxygen to hemoglobin is the paradigm for allosteric regulation of protein function and serves as a foundation for our understanding of sGC. Structural and spectroscopic studies since the 1940s have elucidated the specific changes that occur when O_2 binds to the heme iron (Goff, 1994; Jameson and Ibers, 1994; Lippard and Berg, 1994); it is the interaction of the oxygen molecule with the heme that triggers the allosteric change from the low-affinity T state to the high-affinity R state (Fig. 8). Hemoglobin is an $\alpha_2\beta_2$ tetramer that contains one heme b group per subunit. In the deoxy ferrous state, the hemes are bound by an imidazole nitrogen from the proximal histidine. The high-spin iron atom is displaced somewhat toward the histidine ligand, and when oxygen binds to a ferrous heme, it converts the heme to low spin and pulls the iron atom into the plane of the heme. This motion is triggered by the smaller ionic radius of the low-spin oxy heme. The movement of the heme iron pulls the proximal histidine toward the heme, and the histidine in turn pulls the F helix of the protein. The displacement of the iron-proximal histidine unit by approximately 0.6 Å leads to a 6-Å movement of the hemoglobin subunits due to changes in salt bridges and hydrogen bonding contacts. This conformational change is what enables hemoglobin to cooperatively bind oxygen. The paradigm provided by hemoglobin, which will be invoked to understand sGC, is that it is a change in the heme coordination environment that triggers the allosteric conformational change.

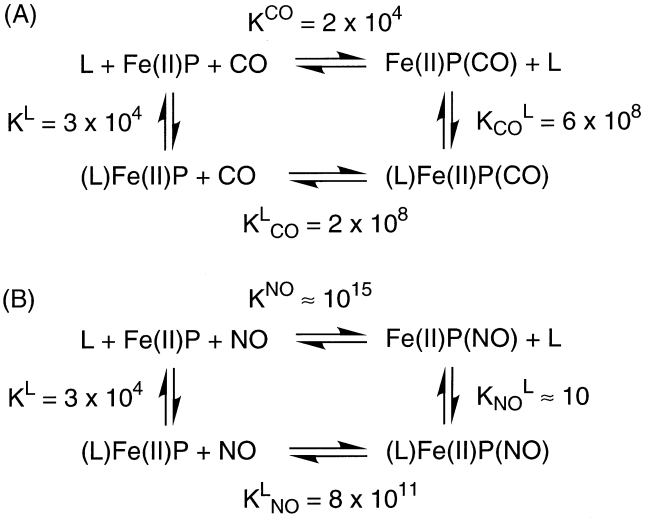


Figure 7 Thermodynamics of CO and NO binding to heme in the presence and absence of an additional axial ligand. (A) The binding affinity of CO for heme is highest in the presence of a sixth ligand. The presence of CO enhances the affinity of the iron porphyrin for the sixth ligand. (B) The binding affinity of NO for heme is highest in the absence of a sixth ligand. The presence of NO decreases the affinity of the iron porphyrin for the sixth ligand. Adapted with permission from Traylor and Sharma (1992). Copyright 1992 American Chemical Society.

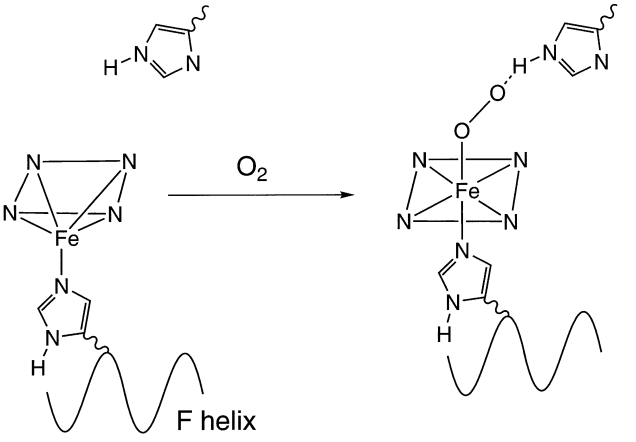


Figure 8 Allosteric control in hemoglobin. The binding of O_2 to a heme triggers a conformational change in the protein that allows hemoglobin to cooperatively bind additional O_2 molecules. Low-spin iron is smaller than high-spin iron and makes shorter bonds to its ligands; in order to maintain ideal bonding distances to the four porphyrin nitrogens, the proximal histidine and the O_2, the iron moves into the porphyrin plane, pulling on the proximal histidine and the F helix.

Other Gas-Sensing Heme Proteins

It has been recognized that there are other heme proteins that serve as gas sensors in prokaryotic organisms, employing allosteric mechanisms similar to that of hemoglobin. The FixL proteins are heme-containing, O_2-sensing protein kinases from plant rhizobial symbionts. FixL phosphorylates transcription factors that control the expression of genes for nitrogen fixation (Gilles-Gonzalez *et al.,* 1995; Tamura *et al.,* 1996; Lukat-Rodgers and Rodgers, 1997). A FixL protein can be divided into three functional domains, a membrane-binding domain, a heme-binding domain, and a kinase domain. Much has been learned about the function of

the FixL protein through the study of the isolated heme domain. The heme cofactor is a high-spin ferrous heme with a single histidine (H194) bound to the iron (Nakamura *et al.,* 1998). Oxygen binding to the heme causes a spin state transition from high-spin to low-spin heme, as occurs in hemoglobin, and this spin state change triggers a conformational change that regulates the activity of the kinase domain (Fig. 9) (Gong *et al.,* 1998). FixL is active as a kinase in the high-spin state and inactive in the low-spin state, regardless of the oxidation state of the heme. Strong field ligands, including O_2, trigger the transition to low-spin heme and inactivate the protein (Gilles-Gonzalez *et al.,* 1994; Gong *et al.,* 1998). FixL thus senses O_2 via a conformational change mediated by the interaction of O_2 with its heme prosthetic group, a mechanism analogous to the cooperative binding of O_2 by hemoglobin.

The first CO-sensing protein, CooA, was purified from the photosynthetic bacterium *Rhodospirillum rubrum* (Aono *et al.,* 1996; Shelver *et al.,* 1997) and provides another important example of a gas-sensing heme protein. CooA is a transcription regulator and a member of the CRP (cAMP receptor protein) and FNR (fumarate nitrate reduction) family of transcription regulators. In the presence of CO, CooA binds to DNA and turns on the expression of a multicomponent CO-oxidation system in *R. rubrum* (Shelver *et al.,* 1995; He *et al.,* 1996). This CO-oxidation system catalyzes the anaerobic conversion of CO to CO_2 and allows growth with CO as the sole energy source (Shelver *et al.,* 1995; He *et al.,* 1996). Unlike FixL and hemoglobin, the heme in CooA is low spin, apparently bearing two protein-derived ligands in both the ferric and ferrous state (Aono *et al.,* 1998; Shelver *et al.,* 1999). In ferric CooA one axial ligand is cysteine (Aono *et al.,* 1998; Reynolds *et al.,* 1998; Shelver *et al.,* 1999); on reduction the cysteine–thiolate is replaced by a histidine (Aono *et al.,* 1998; Shelver *et al.,* 1999; Vogel *et al.,* 1999a). CO induces DNA binding via a conformational change in CooA by displacing one of the ligands from the ferrous heme (Aono *et al.,* 1998; Reynolds *et al.,* 1998; Shelver *et al.,* 1999; Vogel *et al.,* 1999a); available evidence suggests that it is the histidine that is displaced (Fig. 10) (Vogel *et al.,* 1999a). Like hemoglobin and FixL, CooA is proposed to undergo a conformational change that is triggered by the binding of a small molecule, in this case CO, to the heme cofactor.

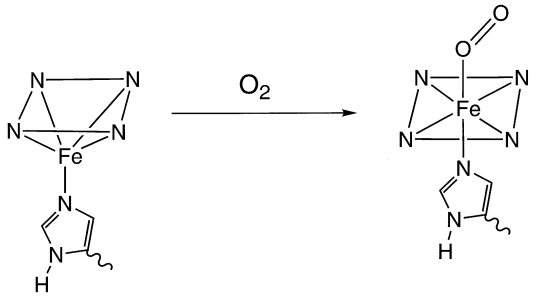


Figure 9 The mechanism of O_2 sensing by FixL. The binding of O_2 to the heme iron of FixL converts the heme iron from high spin to low spin, triggering a conformational change in the protein. O_2 binding to the heme inactivates the kinase activity of FixL.

Soluble Guanylyl Cyclase: A Heme-Dependent NO Sensor

The guanylyl cyclases (GCs) are a diverse family of enzymes that convert guanosine-5'-triphosphate (GTP) to cyclic-3',5'-monophosphate (cGMP) and pyrophosphate (PP_i). There are two genetically distinct subtypes of guanylyl cyclase, the particulate (membrane bound) form (pGC) and the soluble form (sGC) (Garbers *et al.,* 1994; Garbers and Lowe, 1994b). The soluble isoform was discovered in mammalian cells in the late 1960s (Hardman and Sutherland, 1969; Schultz *et al.,* 1969; White and Aurbach, 1969) and has been found in a wide variety of organisms including mammals (Feldman *et al.,* 1993; Hobbs, 1997), the fruitfly *Drosophila melanogaster* (Liu *et al.,* 1995; Shah and Hyde, 1995), the roundworm *Caenorhabditis elegans* (Yu *et al.,* 1997), and the yeast *Saccharomyces cerevisiae* (Kuo *et al.,* 1998). Only the soluble enzyme contains heme and functions as an NO sensor; the activity of sGC is increased several hundredfold on binding NO (Hobbs, 1997).

There is at present a limited amount of information regarding the structure of sGC. The enzyme is an αβ heterodimer composed of two closely related subunits (Koesling *et al.,* 1988, 1990). Enzyme activity is observed only when both subunits are present, implying that only the heterodimer is catalytically active (Wedel *et al.,* 1995). It is not known, however, whether the isolated α and β subunits homodimerize. There appear to be three functional domains in each sGC subunit; an N-terminal heme-binding domain, a C-terminal catalytic domain, and a dimerization domain (Fig. 11). The role of the putative dimerization domain is little studied; the assignment of this region is based simply on homology to pGC. The C termini of the sGC subunits bear strong homology to the C_1 and C_2 catalytic domains of adenylyl cyclase (AC) (Koesling *et al.,* 1988, 1990; Liu *et al.,* 1997). The sGC catalytic domain has been modeled onto the AC catalytic domain structure; the successful fit implies that the sGC active site is formed at the interface of the α and β subunits (Liu *et al.,* 1997). The heme binding domain has been the subject of a number of studies that will be discussed later.

Knowledge of the catalytic mechanism of sGC derives from enzymological studies and structural analogy to the related AC enzymes. The stereochemistry of cyclization for both sGC and AC is known; in both enzymes the reaction proceeds with inversion of configuration at the α phosphate (Gerlt *et al.,* 1980; Eckstein *et al.,* 1981; Senter *et al.,* 1983; Koch *et al.,* 1990). These data are consistent with direct nucleophilic attack of the ribose 3'-hydroxyl on the 5'-α-phosphate of GTP or ATP (Fig. 1). A reasonable mechanism, proposed for AC on the basis of structural analysis, invokes the presence of a catalytic base on the enzyme that would

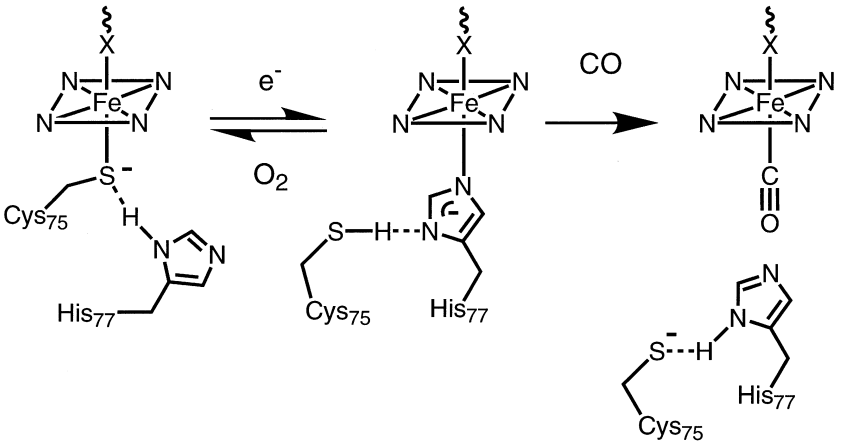


Figure 10 The proposed mechanism for CO sensing by CooA. Oxidized, Fe(III)CooA contains a low-spin heme with two ligands bound to the heme iron. One ligand is a cysteine (Cys^{75}) that is proposed to be hydrogen bonded to a nearby histidine, His^{77}. The identity of the other ligand, labeled X, is unknown. On reduction to Fe(II)CooA, Cys^{75} is displaced by His^{77} to form an imidazolate-bound heme that is presumably stabilized by hydrogen bonding to the nearby Cys^{75}. CO displaces a ligand, most likely the His^{77}–Cys^{75} ligand diad from the heme iron, triggering a conformational change in the protein. When CO is bound, CooA can bind to its target DNA sequence. Adapted with permission from Shelver *et al.* (1999). Copyright 1999 American Chemical Society.

accept the proton from the ribose 3'-hydroxyl, rendering it a more effective nucleophile (Tesmer *et al.,* 1997; Zhang *et al.,* 1997). The leading candidate for this catalytic base is an aspartate residue that is near the ribose 3'-hydroxyl in the crystal structure of AC. An analogous residue appears in all GC isoforms in homology models and has been shown to be essential for catalysis in several AC and GC isoforms (Yuen *et al.,* 1994; Tang *et al.,* 1995; Thompson and Garbers, 1995; Liu *et al.,* 1997).

Essential divalent metal ion cofactors are necessary for cyclase activity and may be involved in binding the negatively charged substrate, stabilizing the transition state, and polarizing the 3'-hydroxyl nucleophile. The AC crystal structure reveals one Mg^{2+} in position to stabilize both the assumed pentavalent transition state and the pyrophosphate leaving group (Tesmer *et al.,* 1997). It is also proposed that a second metal ion, not present in the crystal structures, participates in polarizing the 3'-hydroxyl of ATP (Mitterauer *et al.,* 1998; Zimmerman *et al.,* 1998). Both AC and GC enzymes are known to require Mg^{2+} in excess of the $MgGTP^{2-}$ substrate for maximal activity, implicating additional metal ions in the catalytic mechanism; the absence of a second metal ion in the crystal structures may be a consequence of the fact that the crystals were grown in the absence of substrate or substrate analogs. In addition to metal cations, arginine and asparagine residues in the vicinity of the active site are essential for activity, perhaps by stabilizing the negatively charged transition state and leaving group via hydrogen bonding (Liu *et al.,* 1997; Tesmer *et al.,* 1997). A plausible mechanism for AC is shown in Fig. 12.

Nothing is known about the conformational change that occurs at the active site of cyclase enzymes in response to regulators. All known cyclases, including all AC and GC isoenzymes, are regulated by isoform-specific modulators (Sunahara *et al.,* 1996; Hobbs, 1997; Wedel and Garbers, 1997). Although the specific ligands that activate or inhibit the different enzymes are distinct, there are similarities in the enzyme response. AC and GC enzymes have low activity in the absence of ligands but are typically activated substantially on binding activator ligands (Sunahara *et al.,* 1996; Hobbs, 1997; Wedel and Garbers, 1997). Although the natural divalent cation cofactor is doubtless Mg^{2+}, Mn^{2+} can support catalytic activity (Sunahara *et al.,* 1996; Hobbs, 1997; Wedel and Garbers, 1997). Interestingly, the enzymes are more active in the presence of Mn^{2+} than Mg^{2+}, but are less responsive to activating ligands. It has therefore been suggested that the configuration of the second metal ion binding site is altered in the activated state. Clearly, an important challenge in cyclase enzymology is the elucidation of the structural change that results in heightened catalytic activity.

Early insights into the mechanism by which NO activated sGC were obtained through studies of activation by heme analogs. A number of compounds, including PPIX (protoporphyrin IX, with no metal ion present) and NO-heme [Fe(II)PPIX(NO)] (Ohlstein *et al.,* 1982), activated sGC by a heme-independent mechanism; only enzyme deficient in heme was activated by PPIX and NO-heme. Importantly, PPIX and NO-heme activated heme-deficient sGC in a manner kinetically identical to activation of heme-containing sGC by NO (Wolin *et al.,* 1982). All the activators increased the V_{max} by approximately 40-fold and decreased the K_m for $MgGTP^{2-}$ by approximately one-half (Wolin *et al.,* 1982). These data suggest that there is a single activated conformation formed when the enzyme is activated by different effectors. Other porphyrins and metalloporphyrins

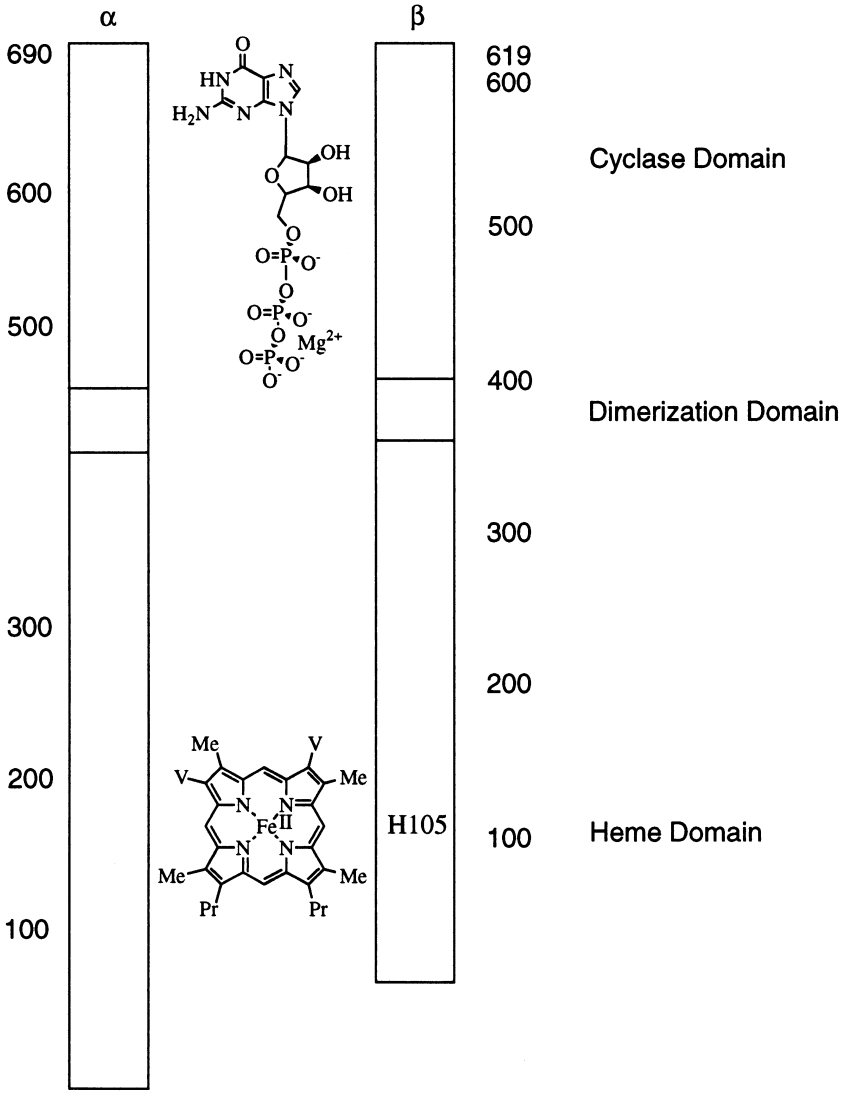


Figure 11 Proposed domain structure for sGC. The enzyme is heterodimeric, composed of three functional domains: a heme domain that binds NO, a cyclase catalytic domain that converts GTP to cGMP, and a dimerization domain that presumably allows the heme and cyclase domains to communicate through a conformational change. The active site within the catalytic domain is formed at the interface of the two subunits.

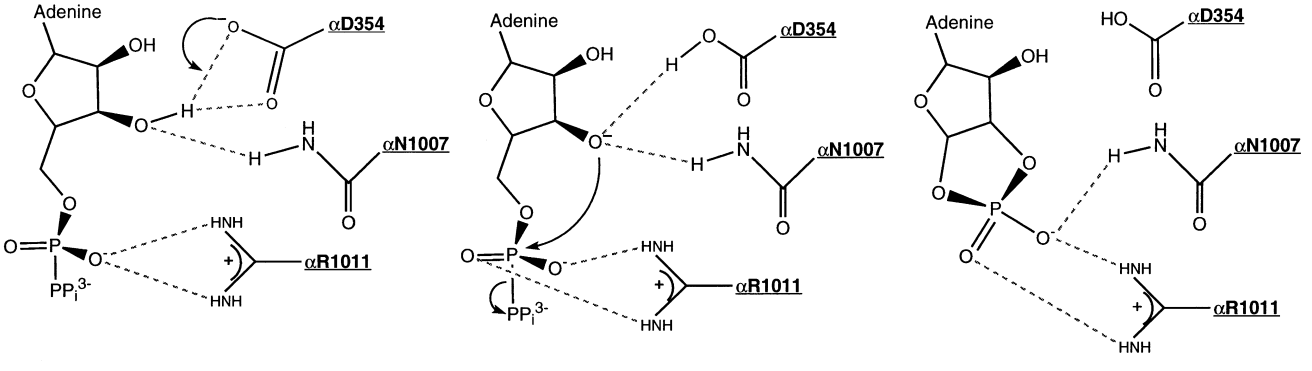


Figure 12 The proposed reaction mechanism for adenylyl cyclase. Aspartate-354 is proposed to serve as the catalytic base, whereas Asn-1007 is thought to play a role in stabilizing the oxyanion nucleophile. The essential Arg-1011 is positioned to stabilize the negative charge of the pentavalent transition state. At least one Mg^{2+} cation also plays a role in catalysis and is believed to stabilize the departing pyrophosphate. Figure adapted with permission from Liu *et al.* (1997). Copyright 1997, National Academy of Sciences, U.S.A.

modulated sGC activity, and the nature of the effect varied with the identity of the metal ion in the porphyrin and with the substitution pattern on the porphyrin periphery (Ignarro *et al.,* 1984a). The metalloporphyrins Fe(II)PPIX, Mn(II) PPIX, and Zn(II)PPIX all inhibited sGC activity (Ignarro *et al.,* 1984a). These metalloporphyrins were competitive with respect to activation by NO-heme (in heme-deficient sGC) and NO (in heme-containing sGC), suggesting that the metalloporphyrins were interacting at the heme binding site (Ignarro *et al.,* 1984b). Similarly, when the vinyl substituents of PPIX were substituted with polar groups or the propionic acids esterified, the porphyrins became competitive inhibitors of activation by PPIX (Ignarro *et al.,* 1984a). Together these results point to the importance of both the metal ion and the porphyrin periphery in accessing the activated conformation of sGC.

Other compounds, including NO, nitroso compounds, and phenylhydrazine, activate sGC in a heme-dependent manner (Craven and DeRubertis, 1978; Ignarro *et al.,* 1984c). Heme-containing enzyme was activated 10-fold by phenylhydrazine in the presence of catalase (Craven and DeRubertis, 1978) and over 100-fold by NO (Ignarro *et al.,* 1984c). NO activation of sGC was potentiated under reducing conditions, in the presence of dithiothreitol, ascorbate, cysteine, or glutathione. In contrast, phenylhydrazine-mediated activation required another heme protein, catalase, and O_2 (Ignarro *et al.,* 1984c). Certain heme proteins, including catalase, are known to form phenyl-heme when exposed to phenylhydrazine in the presence of oxygen; presumably it was the phenyl heme that activated sGC. This observation pointed to a remarkable feature of sGC: the ability of sGC to extract heme from other heme proteins. Indeed, it was subsequently demonstrated that heme-deficient sGC could be fully activated by NO-catalase or NO-myoglobin and that in the activation process, the NO-heme was transferred to sGC (Ignarro *et al.,* 1986).

What conclusions can be drawn from these early studies of sGC activation? The activation of sGC by NO is heme dependent, suggesting that the heme is the site of NO interaction with the protein. NO binds to Fe(II) heme in myoglobin and hemoglobin to form a stable Fe(II)–NO complex under anaerobic conditions (Antonini and Brunori, 1971); therefore, it is logical to conclude that NO is binding to the heme of sGC. The observation that preformed NO-heme activates heme-deficient sGC strongly supports this conclusion. The activation of sGC by the porphyrin PPIX suggests that the central metal is not essential; however, the sensitivity of the enzyme to the porphyrin peripheral substitution suggests that the porphyrin periphery plays an important role. The kinetic similarity between protoporphyrin IX-, NO-, and NO-heme-mediated activation suggests that there is a single conformation for the activated enzyme. These observations are all consistent with a model for NO activation of sGC, shown schematically in Fig. 13, in which NO binds directly to the heme of sGC, forming a five-coordinate NO–heme complex in which the axial ligand is displaced from the heme. The loss of the axial histidine ligand, in the presence of specific protein contacts with the heme periphery, modulates the conformational change to the activated state. This model is strongly supported by data from more recent spectroscopic studies, as will be described in detail later in this chapter.

Because sGC activity is regulated by the interaction of NO with the heme cofactor, there has been great interest in learning about the heme environment in the enzyme. A number of mutagenesis studies have been performed to identify the region of the protein where heme is bound and to identify the liganding amino acids. Wedel *et al.* (1994) mutated seven conserved histidines in the α and β subunits outside the catalytic domains. Interestingly, one variant protein, sGC β-H105F (with the histidine at position 105 of the β subunit changed to phenylalanine), exhibited basal catalytic activity but was unresponsive to NO and did not contain detectable heme. This result provided circumstantial evidence that β-H105 was a ligand to the heme. More recently, Zhao and Marletta (1997) cloned and expressed the N-terminal domain of the β subunit, β1–385, in *Escherichia coli.* This truncated protein contained a stoichiometric amount of heme and exhibited spectral properties similar to those of the intact enzyme (Zhao and Marletta, 1997; Schelvis *et al.,* 1998; Zhao *et al.,* 1998a). When the histidine at position 105 was replaced by glycine, the variant protein did not bind heme;

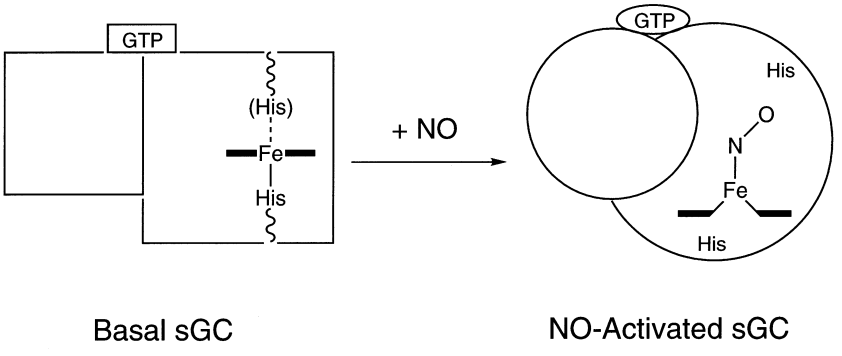


Figure 13 Proposed mechanism of activation of sGC by NO. The binding of NO to the heme of sGC displaces the heme ligand. The change in heme coordination is believed to trigger a structural change in the cyclase catalytic site that dramatically increases the production of cGMP. Figure adapted with permission from Dierks *et al.* (1997). Copyright 1997 American Chemical Society.

however, restoration of heme binding was achieved by the addition of imidazole to the buffer medium (Zhao *et al.,* 1998b). The spectroscopic properties of the restored heme–imidazole complex were identical to those of the wild-type β1–385 fragment. These data provided convincing evidence that β-H105 is indeed the heme ligand. Although the β subunit N terminus appears sufficient for heme binding, it is possible that both the α and β subunit N termini are necessary for configuring the heme binding site in the full-length enzyme. When the N terminus of either the α (131 residues) or the β (64 residues) subunits were removed, alteration of catalytic function and NO sensitivity were observed, supporting the theory that the heme is cooperatively bound by both subunits (Foerster *et al.,* 1996). Because the β1–385 fragment was isolated as a homodimer (Zhao *et al.,* 1998b), the possibility remains that the heme binding site is configured by the homodimeric structure.

Characterization of the heme environment of sGC has revealed inconsistency in behavior. The enzyme has been isolated from bovine lung and spectroscopically characterized in two distinct forms, differing in their heme content and spectroscopic properties. Unlike most other heme proteins that bind heme tightly, the heme in sGC can be lost during purification (Gerzer *et al.,* 1981a). An explanation for this loss has been proposed: oxidation of the heme to the ferric state renders the heme more labile, thus oxidation during purification would result in a heme-deficient protein (Dierks and Burstyn, 1998). The first form of sGC, called sGC_1 in this chapter, contains substoichiometric levels of heme after purification. This form of sGC has been characterized from two sources: direct isolation of the bovine lung enzyme (Yu *et al.,* 1994; Burstyn *et al.,* 1995) and overexpression of the enzyme in insect cells (Fan *et al.,* 1998). The sGC_1 protein can be reconstituted with ferrous heme to yield heme loaded enzyme that is activated by NO (Yu *et al.,* 1994; Burstyn *et al.,* 1995; Fan *et al.,* 1998). The second form, called here sGC_2, has also been isolated from bovine lung and expressed in insect cells; sGC_2 is isolated with stoichiometric amounts of ferrous heme (Stone and Marletta, 1994; Tomita *et al.,* 1997; Makino *et al.,* 1999). Although it was first suggested that sGC_2 contained two hemes per heterodimer (Stone and Marletta, 1995a), more recent analysis suggests that there is only one mole of heme per sGC heterodimer (Brandish *et al.,* 1998). The two forms of the protein exhibit different properties: heme-reconstituted sGC_1 in the ferrous state is readily oxidized in air and subsequently loses heme (Burstyn *et al.,* 1995), whereas sGC_2 in the ferrous state is completely unreactive toward oxygen, and the heme remains stably bound to the protein. The complete lack of reactivity toward oxygen is unusual; most ferrous heme proteins either bind to or are oxidized by oxygen (Antonini and Brunori, 1971). Oxidation of sGC_2 can be achieved with ferricyanide (Stone *et al.,* 1996), and the resulting ferric protein does not lose heme. Both sGC_1 and sGC_2 exhibit similar specific activity and sensitivity to activation by NO (Stone and Marletta, 1995a; Vogel *et al.,* 1999b). It is unclear why sGC isolated from the same source has distinctly different properties; plausible explanations include isolation of different isoforms, or failure of the heme reconstitution process to regenerate the native heme binding conformation. As will be described next, the two forms of the protein have different spectroscopic properties.

Recent spectroscopic studies of sGC have provided the first glimpse into the coordination environment of the heme iron in the presence and absence of NO. Enzyme isolated with less than stoichiometric amounts of heme, sGC_1, was reconstituted under reducing conditions with ferrous heme for spectroscopic study; the reconstituted samples were substantially activated by NO, suggesting that the reconstituted ferrous heme was bound in a functionally correct manner (Burstyn *et al.,* 1995; Dierks *et al.,* 1997). The electronic absorption spectrum of heme-reconstituted sGC_1 exhibited a Soret band at 426 nm and α, β bands at 558 and 528 nm, respectively (Burstyn *et al.,* 1995; Vogel *et al.,* 1999b). This spectrum is typical for ferrous heme bound by two histidines, such as is the case in cytochrome b_5. Additional spectroscopic studies sensitive to the heme environment, including resonance Raman (Yu *et al.,* 1994; Fan *et al.,* 1998; Vogel *et al.,* 1999b) and magnetic circular dichroism studies (Burstyn *et al.,* 1995), revealed a mixture of five- and six-coordinate heme, with the six-coordinate form predominant. The spectral features were consistent with the presence of two histidines bound to the heme iron. One of these ligands was observed to be photolabile, an uncommon feature observed in other heme proteins and attributed to strain (Yu *et al.,* 1994). The second type of sGC isolated from bovine lung, sGC_2, contains one heme per sGC heterodimer, and it is isolated with the heme in the reduced ferrous state. The electronic absorption spectrum of ferrous sGC_2 exhibits a Soret band at 431 nm and a single α/β band at 555 nm (Stone and Marletta, 1994; Tomita *et al.,* 1997; Makino *et al.,* 1999); this spectrum is similar to those of deoxyhemoglobin and myoglobin, proteins known to contain high-spin, histidine-coordinated ferrous heme. Resonance Raman spectra of sGC_2 are also consistent with a high-spin, five-coordinate heme, and direct observation of the Fe–His stretching mode provides conclusive evidence for histidine as the axial ligand (Denium *et al.,* 1996; Tomita *et al.,* 1997). The low frequency of the Fe–His stretch is consistent with a weak or strained Fe–His bond. The ferrous forms of sGC_1 and sGC_2 differ in coordination number at the heme (Fig. 14); sGC_1 contains largely six-coordinate, bishistidine-ligated heme, whereas sGC_2 contains high-spin, five-coordinate heme with a single histidine ligand. At least one of the ligands is weak or under strain in both forms.

The ferric states of sGC_1 and sGC_2 also exhibit different properties, consistent with differing coordination numbers in the two proteins. Heme-reconstituted sGC_1 was observed to rapidly lose heme on oxidation with ferricyanide or O_2, with a half-life of 60 min (Dierks and Burstyn, 1998). The electronic absorption spectrum of ferric sGC_1 exhibited a Soret band at 420 nm and α/β bands at 541 and 572 nm, consistent with the presence of a low-spin, six-coordinate ferric heme (Burstyn *et al.,* 1995). Cyanide (CN^-) did not significantly

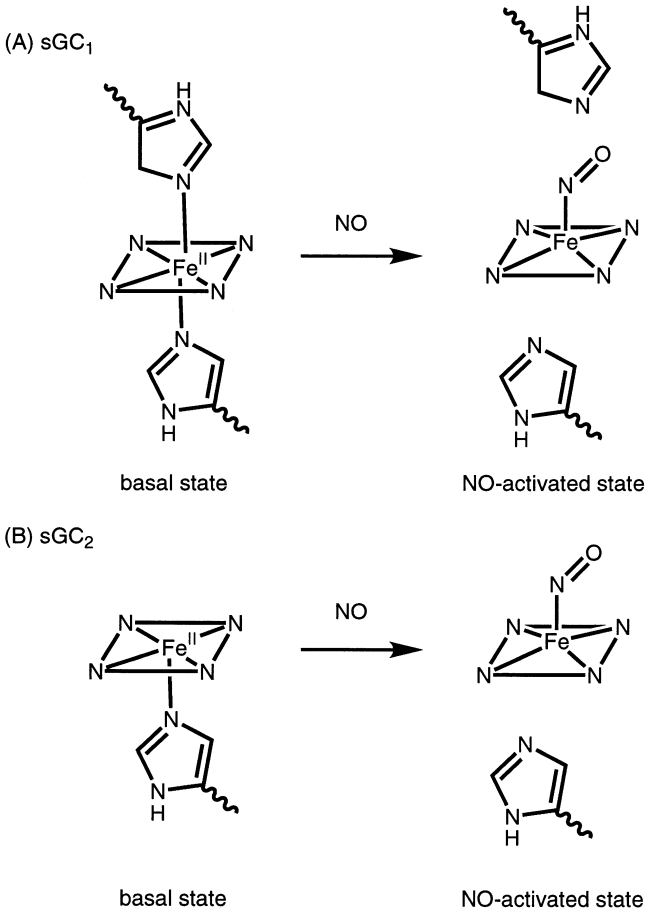


Figure 14 Coordination states of the two types of sGC that have been spectroscopically characterized. (A) sGC_1 is predominantly six-coordinate in the Fe(II) and Fe(III) oxidation states, with two histidine ligands bound to the heme iron. (B) sGC_2 is five-coordinate in both oxidation states with a single histidine bound. Both sGC_1 and sGC_2 react with NO to form a five-coordinate nitrosyl heme, with displacement of the protein derived ligand(s).

alter the electronic or magnetic circular dichroism (MCD) spectra even at 250 m*M,* supporting the conclusion that the ferric state is six-coordinate (Burstyn *et al.,* 1995). In contrast, the ferric state of sGC_2 generated by ferricyanide oxidation did not lose heme and the oxidation/reduction process was reversible (Stone *et al.,* 1996). The electronic spectrum of ferric sGC_2 exhibited a Soret band at 393 nm consistent with a high-spin, five-coordinate heme (Stone and Marletta, 1994). The electron paramagnetic resonance (EPR) spectrum confirmed the presence of a high-spin ferric heme (Stone *et al.,* 1996). Consistent with a five-coordinate geometry, ferric sGC_2 bound typical ferric heme ligands such as cyanide, azide, and fluoride to form six-coordinate adducts (Stone *et al.,* 1996). Thus, the coordination number observed in each enzyme form is consistent between the two redox states, and in most cases the ligand-binding behavior correlates as expected, with only the five-coordinate sGC_2 reacting to form six-coordinate adducts. A surprising exception is the reaction with dioxygen; it is the five-coordinate sGC_2 that is unreactive, whereas six-coordinate sGC_1 is readily autoxidized.

Direct interaction of NO with the heme of sGC has been confirmed through spectroscopic studies. The Fe(II)–NO adduct of sGC, Fe(II)sGC(NO), has been characterized by electronic absorption (Stone and Marletta, 1994; Vogel *et al.,* 1999b), EPR (Stone *et al.,* 1995), and resonance Raman spectroscopy (Yu *et al.,* 1994; Denium *et al.,* 1996; Tomita *et al.,* 1997; Fan *et al.,* 1998; Vogel *et al.,* 1999b). The electronic absorption spectrum of NO bound to both ferrous sGC_1 (Gerzer *et al.,* 1981a; Ignarro *et al.,* 1986; Burstyn *et al.,* 1995; Vogel *et al.,* 1999b) and sGC_2 (Stone and Marletta, 1994; Tomita *et al.,* 1997; Makino *et al.,* 1999) exhibits a shallow, blue-shifted Soret band at ~399 nm characteristic of a five-coordinate nitrosyl heme species (Antonini and Brunori, 1971; Scholler *et al.,* 1979). Resonance Raman spectra of NO bound to either sGC_1 (Fan *et al.,* 1998; Vogel *et al.,* 1999b) or sGC_2 (Denium *et al.,* 1996; Tomita *et al.,* 1997) are similarly indicative of a five-coordinate nitrosyl heme (Choi *et al.,* 1991; Lukat-Rodgers and Rodgers, 1997; Vogel *et al.,* 1999c). The EPR spectrum of Fe(II)sGC(NO) exhibits the three-line rhombic signal characteristic of five-coordinate nitrosyl heme proteins and model compounds (Kon, 1968; Yonetani *et al.,* 1972; Hille *et al.,* 1979; Yoshimura, 1982; Tsai, 1994; Stone *et al.,* 1995; Makino *et al.,* 1999). In both forms of sGC, sGC_1 and sGC_2, the protein-derived ligands(s) are displaced by NO, forming a five-coordinate nitrosyl heme (Fig. 14). The two enzyme forms differ, however, in the apparent polarity of the pocket surrounding the NO ligand (Denium *et al.,* 1996; Vogel *et al.,* 1999b). It is the displacement of the axial histidine ligand or ligands by NO that is believed to trigger the conformational change, resulting in greatly increased enzyme turnover.

The importance of ligand displacement in NO activation of sGC has been demonstrated using nonnative metalloporphyrin substitution. To test the hypothesis that loss of the axial ligand was a key requirement for activation, the ability of NO to displace the axial ligand was systematically altered by varying the electron count at the metal center. Reconstitution of fully heme-depleted sGC_1 with Mn(II)PPIX and Co(II)PPIX produced new proteins, Mn(II)sGC and Co(II)sGC, with novel metal centers (Dierks *et al.,* 1997). These nonnative metalloporphyrins were predicted to form NO complexes with electron counts of $\{Mn\text{-}NO\}^6$ and $\{Co\text{-}NO\}^8$, respectively, and loss of the ligand *trans* to NO was predicted to occur with Co but not with Mn. Characterization of Mn(II)sGC and Co(II)sGC by electronic absorption and resonance Raman spectroscopy revealed that both nonnative metalloporphyrins were five-coordinate with a single histidine ligand (Fig. 15A) (Dierks *et al.,* 1997). A more recent study of Co(II)sGC confirmed the presence of a single nitrogen ligand through the observation of superhyperfine coupling between one ^{14}N and the Co nucleus in the EPR spectrum (Makino *et al.,* 1999). When NO was added, Mn(II)sGC(NO) appeared to retain the histidine ligand, forming a six-coordinate nitrosyl adduct (Fig. 15A), as evidenced by peak positions in the electronic spectrum. In contrast, Co(II)sGC(NO) formed a five-coordinate nitrosyl adduct (Fig. 15B), in which the axial histidine was dis-

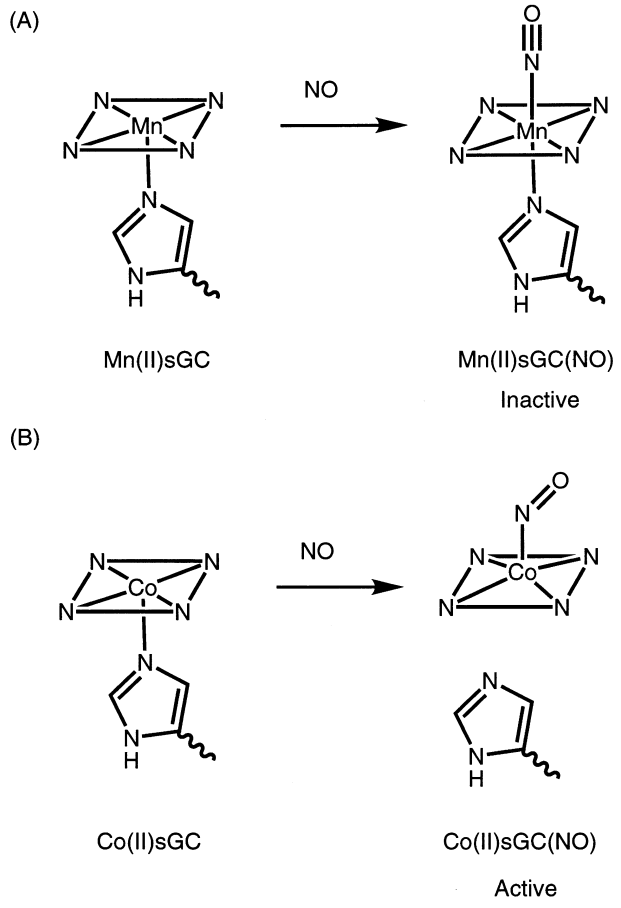


Figure 15 Coordination states of nonnative metalloporphyrins substituted for the heme in sGC. (A) Mn(II)sGC is five-coordinate with a single histidine ligand. Mn(II)sGC reacts with NO to form a six-coordinate nitrosyl, Mn(II)sGC(NO). Mn(II)sGC(NO) exhibits only basal activity. (B) Co(II)sGC is five coordinate with a single histidine ligand. Co(II)sGC reacts with NO to form five-coordinate Co(II)sGC(NO) with displacement of the histidine ligand. Co(II)sGC(NO) is more active than native Fe(II)sGC(NO). Figure adapted with permission from Dierks *et al.* (1997). Copyright 1997, American Chemical Society.

placed as determined from the similarity of the resonance Raman spectrum of Co(II)sGC(NO) to those of model five-coordinate nitrosyl cobalt porphyrin complexes (Dierks *et al.,* 1997; Makino *et al.,* 1999). Thus, the two nonnative metal-substituted enzymes attained the coordination geometry predicted by the Feltham–Enemark electron count when NO was bound. Importantly, the coordination at the metal center correlated with the activity of the enzyme: NO did not activate Mn(II)PPIX-reconstituted sGC, but it did activate Co(II)PPIX-reconstituted sGC (Dierks *et al.,* 1997). The magnitude of activation correlated with the magnitude of metal–ligand bond weakening predicted by the electron count as Co(II)sGC(NO) exhibited consistently greater activity than Fe(II)sGC(NO).

There is direct evidence that an axial histidine ligand is displaced when NO binds to sGC. Experiments on native, heme-containing sGC_2 reveal that a six-coordinate nitrosyl heme is formed initially when NO binds to sGC (Makino *et al.,* 1999). This six-coordinate nitrosyl heme exhibited an EPR spectrum consistent with the presence of an axial histidine ligand *trans* to the NO. Furthermore, this species decayed directly to the five-coordinate nitrosyl heme observed previously. Thus, activation of sGC by NO can be directly linked to displacement of the axial ligand from the heme iron. From all these studies, it can be concluded that nature utilizes the coordination preferences and specificity of NO binding to heme to regulate the conformational change of sGC. NO binding triggers the release of the histidine ligand by weakening the bond between iron and histidine.

Other Regulators of sGC

Molecules other than NO may also regulate sGC activity through interaction with the heme. It has been proposed that CO, another well-known heme ligand, may serve as a physiological regulator of sGC, and biological evidence implicates the enzyme heme oxygenase as a source of CO in a variety of tissues (Maines, 1988). Production of CO leads to elevation of cGMP, and increased cGMP correlates with physiological responses, including vascular relaxation and neurological function (Maines, 1988; Verma *et al.,* 1993). These data imply that sGC activity *in vivo* is responsive to CO. Unfortunately, direct activation of sGC by CO has not been observed *in vitro;* isolated sGC enzyme is activated only minimally (two- to sixfold) by CO (Brune and Ullrich, 1987; Stone and Marletta, 1994; Burstyn *et al.,* 1995; Friebe *et al.,* 1996; Makino *et al.,* 1999; Vogel *et al.,* 1999b). Although CO does not directly activate sGC, it has been discovered that the effect of CO is dramatically enhanced by the xenobiotic 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) (Friebe *et al.,* 1996; Stone and Marletta, 1998). In the presence of YC-1 and CO, sGC activity is comparable to that with NO alone. It is therefore possible that an endogenous compound exists that potentiates activation of sGC by CO.

Consideration of binding affinities suggests that CO is unlikely to compete effectively with NO for the heme of sGC at physiological concentrations of these gases. Available data suggest that NO binds to sGC many orders of magnitude more strongly than does CO. The dissociation rate of CO from the heme of sGC is exceptionally fast (3.5–28 s^{-1}), whereas the association rate is comparable to that of Hb and Mb (3.6×10^4–1.2×10^5 M^{-1} s^{-1}) (Stone and Marletta, 1995b; Kharitonov *et al.,* 1997). The enzyme therefore has a weaker affinity for CO ($K \cong 10^3$ to 10^4 M^{-1}) than Mb and Hb. Comparable data for NO binding to sGC reveal an NO association rate of 7.8×10^8 M^{-1} s^{-1} and a dissociation rate of ~0.01 to 1 s^{-1} (Stone and Marletta, 1996; Kharitonov *et al.,* 1997). Thus, the affinity of NO for sGC is approximately 10^{10}. These data reveal a six order of magnitude preference of the sGC heme for NO over CO, suggesting that under physiological conditions NO will bind to sGC regardless of the CO concentration (Vogel *et al.,* 1999b). It is possible, however, that CO interacts with the heme of sGC in tis-

sues where NO is not present, or under as yet undetermined conditions where the preference of the enzyme is less pronounced.

The CO adduct of sGC has been characterized, and, in contrast to NO, it forms a six-coordinate carbonyl heme in both sGC_1 and sGC_2 (Fig. 16). The electronic spectra of the CO adducts of both sGC_1 (Gerzer *et al.,* 1981b; Burstyn *et al.,* 1995; Vogel *et al.,* 1999b) and sGC_2 (Stone and Marletta, 1994; Tomita *et al.,* 1997) are similar, with a Soret maximum at ~420 nm and well-defined α/β bands, consistent with a low-spin, six-coordinate carbonyl heme in both forms of the enzyme. The electronic spectra are very similar to those of the carbonyl adducts of hemoglobin and myoglobin, where the ligand *trans* to CO is known to be histidine (Antonini and Brunori, 1971). The MCD spectrum of Fe(II)sGC_1(CO) is similar to that of other six-coordinate histidine-bound heme proteins such as the CO adducts of hemoglobin and horseradish peroxidase (Burstyn *et al.,* 1995). The resonance Raman spectra of the CO adducts of sGC_1 (Yu *et al.,* 1994; Fan *et al.,* 1998; Vogel *et al.,* 1999b) and sGC_2 (Kim *et al.,* 1996; Tomita *et al.,* 1997) reveal similarities and some striking differences. Both Fe(II)sGC_1(CO) and Fe(II)sGC_2(CO) exhibit spectra consistent with a six-coordinate histidine-bound heme. The CO ligand appears to be in a hydrophobic environment in the case of sGC_1, but in a negatively polarized environment in the case of sGC_2. The origin of these polarity differences is not known, but the data clearly implicate different heme binding environments in the two forms of sGC. The failure of either enzyme form to become activated in the presence of CO correlates with the presence of an intact iron–histidine bond in the CO adducts, further supporting the idea that loss of the axial ligand is required for activation.

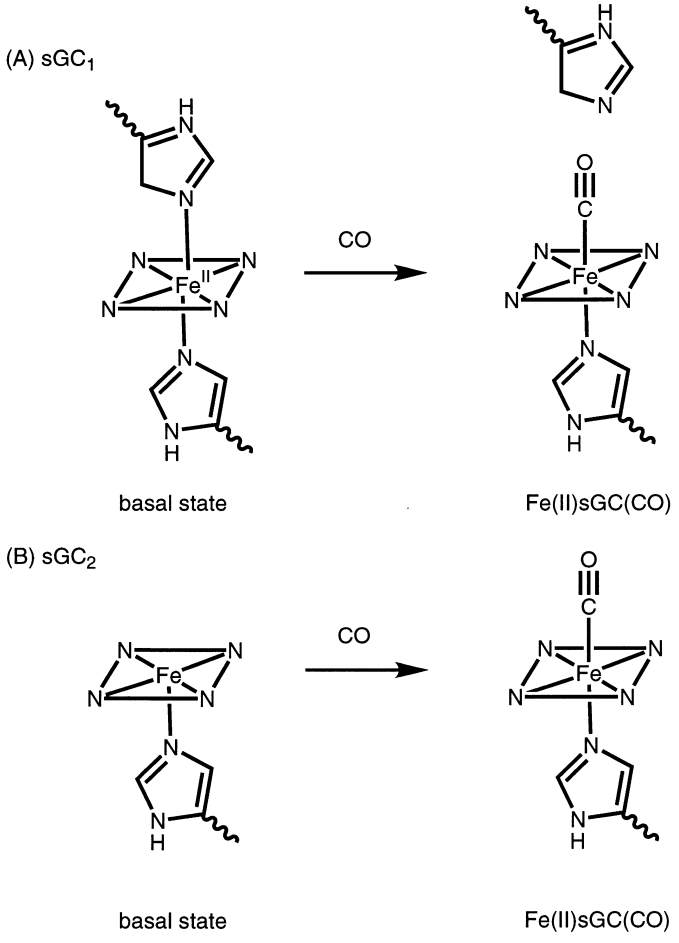


Figure 16 Coordination states of the CO adduct of sGC. CO binds to the hemes of both Fe(II)sGC_1 and Fe(II)sGC_2 to form six-coordinate carbonyl adducts in which the iron–proximal histidine bond remains intact. CO does not significantly activate either sGC_1 or sGC_2.

There are few selective activators or inhibitors of sGC known, making sGC an undeveloped drug target for regulation of the NO–cGMP pathway. Viagra, a drug that has received much recent attention, inhibits the breakdown of cGMP by the cGMP-dependent phosphodiesterase, acting one step downstream from sGC in the NO–cGMP pathway. It is believed that sGC plays an important role in the NO–cGMP pathway and that defects in sGC regulation, resulting in either overactivity or underactivity, may be involved in certain pathologies (Hobbs, 1997; Ignarro, 1999; Murad, 1999). For example, septic shock and migraines result from overactivity of the NO–cGMP pathway, whereas impotence, hypertension, and asthma result from underactivity (Hobbs, 1997). Furthermore, when produced inappropriately, NO can interfere with or destroy critical cellular systems. An important goal in sGC research is to develop sufficient understanding of the protein to enable the development of selectively targeted drugs.

Of the few inhibitors known, a number are redox active compounds that may interact with the heme of sGC. Methylene blue and LY83583 are the most widely used inhibitors of sGC (Fig. 17), but their action is not selective for sGC inhibition (Hobbs, 1997; Dierks and Burstyn, 1998). A plausible explanation for the inhibition of sGC by these redox dyes includes reaction with heme or NO. A new inhibitor, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) (Fig. 17), was shown to be a potent inhibitor of NO-induced smooth muscle relaxation (Brunner *et al.,* 1995; Garthwaite *et al.,* 1995). Studies with purified sGC revealed that ODQ maintains the heme in the oxidized state even in the presence of NO (Schrammel *et al.,* 1996). Although the most specific inhibitor known, ODQ may have limited application *in vivo* given that it interacts with the heme groups of other proteins (Feelisch *et al.,* 1999). The compound YC-1 (Fig. 17) has provided a selective, non-NO-based activator of sGC that is functional *in vivo* (Ko *et al.,* 1994). Although the mechanism of YC-1 activation is not clear, YC-1 may help to stabilize the activated state of sGC by binding to an allosteric site on the protein (Friebe *et al.,* 1996; Stone and Marletta, 1998).

Conclusions

Future work on sGC will doubtless improve our understanding of the structural basis for NO activation of sGC.

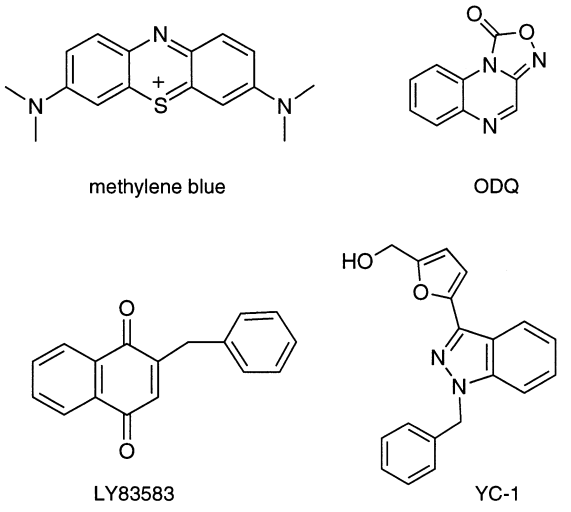


Figure 17 Known inhibitors and activators of sGC. Methylene blue and LY83583 are the most widely used inhibitors of sGC, but their action is not selective. ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) is a more selective inhibitor of sGC, but its usefulness *in vivo* may be limited due to interaction with other heme proteins. The compound YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] is a selective activator of sGC that is functional *in vivo.*

Although the heme of sGC has been conclusively identified as the receptor for NO, and changes in heme coordination geometry on binding NO are clearly important in the activation process, many key questions remain. What structural changes occur in the enzyme upon NO binding to the heme of sGC? What changes in the catalytic site are responsible for the dramatic increase in cyclase activity? How do the heme and the cyclase sites communicate? How is sGC deactivated, since the off rate for NO from hemes is too slow for simple NO dissociation to be relevant? It is hoped that future studies will answer these questions and lead to the development of specific inhibitors and activators of sGC as new therapeutic tools. It is certain that extensive research on sGC in the next few years will vanquish its title as the ''forgotten sibling'' of the cyclases.

References

Addison, A. W., and Stephanos, J. J. (1986). Nitrosyliron (III) hemoglobin: Autoreduction and spectroscopy. *Biochemistry* **25,** 4104–4113.

Antonini, E., and Brunori, M. (1971). ''Hemoglobin and Myoglobin and Their Reaction with Ligands.'' North-Holland Publ., Amsterdam.

Aono, S., Nakajima, H., Saito, K., and Okada, M. (1996). A novel heme protein that acts as a carbon monoxide-dependent transcriptional activator in *Rhodospirillium rubrum. Biochem. Biophys. Res. Commun.* **228,** 752–756.

Aono, S., Ohkubo, K., Matsuo, T., and Nakajima, H. (1998). Redox-controlled ligand exchange of the heme in the CO-sensing transcriptional activator CooA. *J. Biol. Chem.* **273,** 25757–25764.

Brandish, P. E., Buechler, W., and Marletta, M. A. (1998). Regeneration of the ferrous heme of soluble guanylyl cyclase from the nitric oxide complex: Acceleration by thiols and oxyhemoglobin. *Biochemistry* **37,** 16898–16907.

Brune, B., and Ullrich, V. (1987). Inhibition of platelet aggregation by carbon monoxide is meditated by activation of guanylate cyclase. *Mol. Pharmacol.* **32,** 497–504.

Brunner, F., Stessel, H., and Kukovetz, W. R. (1995). Novel guanylyl cyclase inhibitor, ODQ reveals role of nitric oxide but not of cyclic GMP in endothelin-1 secretion. *FEBS Lett.* **376,** 262–266.

Bursten, B. E., and Gatter, M. G. (1984). Molecular orbital studies of organometallic hydride complexes. 1. Acidic vs. hydridic behavior of some (η_5-C5H5)MLηH systems. *J. Am. Chem. Soc.* **106,** 2554–2558.

Bursten, B. E., Jensen, J. R., Gordon, D. J., Treichel, P. M., and Fenske, R. F. (1981). Electronic structure of transition-metal nitrosyls. Xα-SW and configuration interaction calculations of the valence ionization potentials of $Co(CO)_3NO$ and $Mn(CO)_4NO$. *J. Am. Chem. Soc.* **103,** 5226–5231.

Burstyn, J. N., Yu, A. E., Dierks, E. A., Hawkins, B. K., and Dawson, J. H. (1995). Studies of the heme coordination and ligand binding properties of soluble guanylyl cyclase (sGC): Characterization of Fe(II)sGC and Fe(II)sGC(CO) by electronic absorption and magnetic circular dichroism spectroscopies and failure of CO to activate the enzyme. *Biochemistry* **34,** 5896–5903.

Choi, I.-K., Liu, Y., Feng, D., Paeng, K.-J., and Ryan, M. D. (1991). Electrochemical and spectroscopic studies of iron porphyrin nitrosyls and their reduction products. *Inorg. Chem.* **30,** 1832–1839.

Craven, P. A., and DeRubertis, F. R. (1978). Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemoproteins. *J. Biol. Chem.* **253,** 8433–8443.

Denium, G., Stone, J. R., Babcock, G. T., and Marletta, M. A. (1996). Binding of nitric oxide and carbon monoxide to soluble guanylate cyclase as observed with resonance Raman spectroscopy. *Biochemistry* **35,** 1540–1547.

Diebold, T., Schappacher, M., Chevrier, B., and Weiss, R. (1979). Low-valent molybdenum porphyrin derivatives: Synthesis and X-ray crystal structures of dinitrosyl- and methanol(nitrosyl)-*meso*-tetra-*p*-tolylporphyrinatomolybdenum(II) benzene solvates. *J. Chem. Soc., Chem. Commun.,* 693–694.

Dierks, E. A., and Burstyn, J. N. (1998). The deactivation of soluble guanylyl cyclase by redox-active agents. *Arch. Biochem. Biophys.* **351,** 1–7.

Dierks, E. A., Hu, S., Vogel, K. M., Yu, A. E., Spiro, T. G., and Burstyn, J. N. (1997). Demonstration of the role of scission of the proximal histidine-iron bond in the activation of soluble guanylyl cyclase through metalloporphyrin substitution studies. *J. Am. Chem. Soc.* **119,** 7316–7323.

Doyle, M. P., and Hoekstra, J. W. (1981). Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Biochem.* **14,** 351–358.

Durham, B., and Millet, F. S. (1994). Iron: Heme proteins and electron transport. *In* ''Encyclopedia of Inorganic Chemistry'' (R. B. King, ed.), Vol. 4, pp. 1642–1661. Wiley, West Sussex, England.

Eckstein, F., Romaniuk, P. J., Heideman, W., and Storm, D. R. (1981). Stereochemistry of the mammalian adenylate cyclase reaction. *J. Biol. Chem.* **256,** 9118–9120.

Enemark, J. H., Feltham, R. D., Riker-Nappier, J., and Bizot, K. F. (1975). Stereochemical control of valence. IV. Comparison of the structures and chemical reactivities of five- and six-coordinate diarsine complexes of the $\{CoNO\}^8$ group. *Inorg. Chem.* **14,** 624–632.

Enemark, J. H., Feltham, R. D., Huie, B. T., Johnson, P. L., and Bizot Swedo, K. (1977). Stereochemical control of valence. 5. Comparison of the molecular and electronic structures of five- and six-coordinate *o*-phenylenebis(dimethylarsine) complexes of the $\{FeNO\}^7$ Group1. *J. Am. Chem. Soc.* **99,** 3285–3292.

English, A. M. (1994). Iron: Heme proteins, peroxidases and catalases. *In* ''Encyclopedia of Inorganic Chemistry'' (R. B. King, ed.), Vol. 4, pp. 1682–1697. Wiley, West Sussex, England.

Fan, B., Gupta, G., Danziger, R. S., Friedman, J. M., and Rousseau, D. L. (1998). Resonance Raman characterization of soluble guanylate cyclase expressed from baculovirus. *Biochemistry* **37,** 1178–1184.

Feelisch, M., Kotsonis, P., Siebe, J., Clement, B., and Schmidt, H. H. H. W. (1999). The soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one is a nonselective heme protein inhibitor of nitric oxide synthase and other cytochrome P450 enzymes involved in nitric oxide donor bioactivation. *Mol. Pharmacol.* **56,** 243–253.

Feldman, P. L., Griffith, O. W., and Stuehr, D. J. (1993). The surprising life of nitric oxide. *Chem. Eng. News* **71,** 26–38.

Feltham, R. D., and Enemark, J. H. (1981). Structures of metal nitrosyls. *Top. Stereochem.* **12,** 155–215.

Foerster, J., Harteneck, C., Malkewitz, J., Schultz, G., and Koesling, D. (1996). A functional heme-binding site of soluble guanylyl cyclase requires intact N-termini of α_1 and β_1 subunits. *Eur. J. Biochem.* **240,** 380–386.

Friebe, A., Schultz, G., and Koesling, D. (1996). Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *EMBO J.* **15,** 6863–6868.

Garbers, D. L., and Lowe, D. G. (1994). Guanylyl cyclase receptors. *J. Biol. Chem.* **269,** 30741–30744.

Garbers, D. L., Koesling, D., and Schultz, G. (1994). Guanylyl cyclase receptors. *Mol. Biol. Cell* **5,** 1–5.

Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., and Mayer, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cycalse by 1*H*-[1,2,4]oxadizolo[4,3-*a*]quinoxalin-1-one. *Mol. Pharmacol.* **48,** 184–188.

Gerlt, J. A., Coderre, J. A., and Wolin, M. S. (1980). Mechanism of the adenylate cyclase reaction—Stereochemistry of the reaction catalyzed by the enzyme from *Brevibacterium liquefaciens. J. Biol. Chem.* **255,** 331–334.

Gerzer, R., Böhme, E., Hofmann, F., and Schultz, G. (1981a). Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett.* **132,** 71–74.

Gerzer, R., Böhme, E., Hofmann, F., and Schultz, G. (1981b). Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett.* **132,** 71–74.

Gilles-Gonzalez, M. A., Gonzalez, G., Perutz, M. F., Kiger, L., Marden, M. C., and Poyart, C. (1994). Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand binding and autooxidation. *Biochemistry* **33,** 8067–8073.

Gilles-Gonzalez, M. A., Gonzalez, G., and Perutz, M. F. (1995). Kinase activity of oxygen sensor FixL depends on the spin state of its heme iron. *Biochemistry* **34,** 232–236.

Goff, H. M. (1994). Heme proteins and dioxygen transport. *In* ''Encyclopedia of Inorganic Chemistry'' (R. Bruce-King, ed.), Vol. 4, pp. 1635–1642. Wiley, West Sussex, England.

Gong, W., Hao, B., Mansy, S. S., Gonzalez, G., Gilles-Gonzalez, M. A., and Chan, M. K. (1998). Structure of a biological oxygen sensor: A new mechanism for heme-driven signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15177–15182.

Griffith, W. P., Lewis, J., and Wilkinson, G. (1958). Infra-red spectra of transition metal–nitric oxide complexes-II. *J. Inorg. Nucl. Chem.* **7,** 38–44.

Hardman, J. G., and Sutherland, E. W. (1969). Guanylyl cyclase, an enzyme catalyzing the formation of guanosine 3',5'-monophosphate from guanosine triphosphate. *J. Biol. Chem.* **244,** 6363–6370.

He, Y., Shelver, D., Kerby, R. L., and Roberts, G. P. (1996). Characterization of a CO-responsive transcriptional activator from *Rhodospirillum rubrum. J. Biol. Chem.* **271,** 120–123.

Hille, R., Olson, J. S., and Palmer, G. (1979). Spectral transitions of nitrosyl hemes during ligand binding to hemoglobin. *J. Biol. Chem.* **254,** 12110–12120.

Hobbs, A. J. (1997). Soluble guanylate cyclase: The forgotten sibling. *Trends Pharmacol. Sci.* **18,** 484–491.

Hoffman, R., Chen, M. M. L., Elian, M., Rossi, A. R., and Mingos, D. M. P. (1974). Pentacoordinate nitrosyls. *Inorg. Chem.* **13,** 2666–2675.

Ignarro, L. J. (1999). Nitric oxide: A unique endogenous signaling molecule in vascular biology (Nobel lecture). *Angew. Chem. Int. Ed. Engl.* **38,** 1882–1892.

Ignarro, L. J., Ballot, B., and Wood, K. S. (1984a). Regulation of soluble guanylate cyclase activity by porphyrins and metalloporphyrins. *J. Biol. Chem.* **259,** 6201–6207.

Ignarro, L. J., Wood, K. S., and Wolin, M. S. (1984b). Regulation of purified soluble guanylate cyclase by porphyrins and metalloporphyrins: A unifying concept. *Adv. Cyclic Nucleotide Res.* **17,** 267–274.

Ignarro, L. J., Wood, K. S., Ballot, B., and Wolin, M. S. (1984c). Guanylate cyclase from bovine lung: Evidence that enzyme activation by phenylhydrazine is mediated by iron–phenyl hemoprotein complexes. *J. Biol. Chem.* **259,** 5923–5931.

Ignarro, L. J., Adams, J. B., Horwitz, P. M., and Wood, K. S. (1986). Activation of soluble guanylyl cyclase by NO–hemoproteins involves NO–heme exchange. *J. Biol. Chem.* **261,** 4997–5002.

Jameson, G. B., and Ibers, J. A. (1994). Biological and synthetic dioxygen carriers. *In* ''Bioinorganic Chemistry'' (I. Bertini, H. B. Gray, S. J. Lippard, and J. Selverstone Valentine, eds.), pp. 167–252. University Science Books, Mill Valley, California.

Johnson, P. L., Enemark, J. H., Feltham, R. D., and Bizot Swedo, K. (1976). Structural chemistry of the $\{CoNO\}^8$ group. Structure of *trans*-perchloratonitrosylbis(ethylenediamine)cobalt perchlorate, $[Co(NO)(C_2N_2H_8)_2(OClO_3)][ClO_4]$. *Inorg. Chem.* **15,** 2989–2993.

Kelly, S. L., and Kadish, K. M. (1984). Electron-transfer and ligand-addition reactions of $(TPP)CrClO_4$ and (TPP)Cr(NO) in nonaqueous media. *Inorg. Chem.* **23,** 679–687.

Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997). Kinetics of nitric oxide dissociation from five- and six-coordinate nitrosyl hemes and heme proteins, including soluble guanylate cyclase. *Biochemistry* **36,** 6814–6818.

Kim, K., and Ibers, J. A. (1991). Structure of a carbon monoxide adduct of a ''capped'' porphyrin: $Fe(C_2\text{-cap})(CO)$(1-methylimidazole). *J. Am. Chem. Soc.* **113,** 6077–6081.

Kim, S., Deinum, G., Gardner, M. T., Marletta, M. A., and Babcock, G. T. (1996). Distal pocket polarity in the unusual ligand binding site of soluble guanylate cyclase: Implications for the control of NO binding. *J. Am. Chem. Soc.* **118,** 8769–8770.

Ko, F.-N., Wu, C.-C., Kuo, S.-C., Lee, F.-Y., and Teng, C.-M. (1994). YC-1, a novel activator of platelet guanylyl cyclase. *Blood* **84,** 4226–4233.

Koch, K.-W., Eckstein, F., and Stryer, L. (1990). Stereochemical course of the reaction catalyzed by guanylate cyclase from bovine retinal outer segments. *J. Biol. Chem.* **265,** 9659–9663.

Koesling, D., Herz, J., Gausepohl, H., Niroomand, F., Hinsch, K.-D., Mulsch, A., Bohme, E., Schultz, G., and Frank, R. (1988). The primary structure of the 70-kDa subunit of bovine soluble guanylate cyclase. *FEBS Lett.* **239,** 29–34.

Koesling, D., Harteneck, C., Humbert, P., Bosserhoff, A., Frank, R., Schultz, G., and Böhme, E. (1990). The primary structure of the larger subunit of soluble guanylyl cyclase from bovine lung. Homology between the two subunits of the enzyme. *FEBS Lett.* **266,** 128–132.

Kon, H. (1968). Paramagnetic resonance study of nitric oxide hemoglobin. *J. Biol. Chem.* **243,** 4350–4357.

Kuo, W. N., Kanadia, R. N., and Mcnabb, M. (1998). Soluble guanylate cyclase in *Saccharomyces cerevisiae. Biochem. Mol. Biol. Int.* **45,** 125–131.

Lewis, J., Irving, R. J., and Wilkinson, G. (1958). Infra-red spectra of transition metal–nitric oxide complexes-I. *J. Inorg. Nucl. Chem.* **7,** 32–37.

Lippard, S. J., and Berg, J. M. (1994). Atom- and group-transfer chemistry. *In* ''Principles of Bioinorganic Chemistry'' (A. Kelly, ed.), University Science Books, Mill Valley, California.

Liu, W., Yoon, J., Burg, M., Chen, L., and Pak, W. L. (1995). Molecular characterization of two *Drosophila* guanylate cyclases expressed in the nervous system. *J. Biol. Chem.* **270,** 12418–12427.

Liu, Y., Ruoho, A. E., Rao, V. D., and Hurley, J. H. (1997). Catalytic mechanism of the adenylyl and guanylyl cyclases: Modeling and mutational analysis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 13414–13419.

Lukat-Rodgers, G. S., and Rodgers, K. R. (1997). Characterization of ferrous FixL–nitric oxide adducts by resonance Raman spectroscopy. *Biochemistry* **36,** 4178–4187.

Lukehart, C. M., and Troup, J. M. (1977). A 17 electron chromium complex having both nitrosyl and unidentate nitrito ligands. The crystal and molecular structure of dinitronitrosyltripyridinechromium monopyridine solvate, $(ONO)_2(NO)(C_5H_5N)_3Cr\cdot(C_5H_5N)$. *Inorg. Chim. Acta* **22,** 81–86.

Maines, M. D. (1988). Heme oxygenase: Function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* **2,** 2557–2568.

Makino, R., Matsuda, H., Obayashi, E., Shiro, Y., Iizuka, T., and Hori, H. (1999). EPR characterization of axial bond in metal center of native and cobalt-substituted guanylate cyclase. *J. Biol. Chem.* **274,** 7714–7723.

Mitterauer, T., Hohenegger, M., Tang, W.-J., Nanoff, C., and Freissmuth, M. (1998). The C_2 catalytic domain of adenylyl cyclase contains the second metal ion (Mn^{2+}) binding site. *Biochemistry* **37,** 16183–16191.

Moeller, T. (1946a). Inorganic nitrosyl compounds. I. Cationic and anionic compounds. *J. Chem. Educ.* **23,** 441–444.

Moeller, T. (1946b). Inorganic nitrosyl compounds. II. Coordination compounds. *J. Chem. Educ.* **23,** 542–548.

Murad, F. (1999). Discovery of some of the biological effects of nitric oxide and its role in cell signaling (Nobel lecture). *Angew. Chem. Int. Ed. Engl.* **38,** 1856–1868.

Nakamura, H., Saito, K., Ito, E., Tamura, K., Tsuchiya, T., Nishigaki, K., Shiro, Y., and Iizuka, T. (1998). Identification of the hydrophobic amino acid residues required for heme assembly in the rhizobial oxygen sensor protein FixL. *Biochem. Biophys. Res. Commun.* **247,** 427–431.

Nappier, T. E., Feltham, R. D., Enemark, J. H., Kruse, A., and Cooke, M. (1975). Bis[*o*-phenylenebis(dimethylarsine)]iron complexes with NO, CO, CH_3CN, and other small molecules. *Inorg. Chem.* **14,** 806–812.

Ohlstein, E. H., Wood, K. S., and Ignarro, L. J. (1982). Purification and properties of heme-deficient hepatic soluble guanylate cyclase: Effects of heme and other factors on enzyme activation by NO, NO–heme, and protoporphyrin IX. *Arch. Biochem. Biophys.* **218,** 187–198.

Olson, J. S., and Phillips, G. N., Jr. (1997). Myoglobin discriminates between O_2, NO and CO by electrostatic interactions with the bound ligand. *J. Biol. Inorg. Chem.* **2,** 544–552.

Olson, L. J., Knych, E. T., Jr., Herzig, T. C., and Drewett, J. G. (1997). Selective guanylyl cyclase inhibitor reverses nitric oxide-induced vasorelaxation. *Hypertension* **29,** 254–261.

Peng, S.-M., and Ibers, J. A. (1976). Stereochemistry of carbonylmetalloporphyrins. The structure of (pyridine)(carbonyl)(5,10,15,20-tetraphenylporphinato)iron(II). *J. Am. Chem. Soc.* **98,** 8032–8036.

Piciulo, P. L., Rupprecht, G., and Scheidt, W. R. (1974). Stereochemistry of nitrosylmetalloporphyrins. Nitrosyl-α,β,γ,δ-tetraphenylporphinato(1-methylimidazole)iron and nitrosyl-α,β,γ,δ-tetraphenylporphinato(4-methylpiperidine)manganese. *J. Am. Chem. Soc.* **96,** 5293–5295.

Pratt, C. S., Coyle, B. A., and Ibers, J. A. (1971). Redetermination of the structure of nitrosylpenta-amminecobalt(III) dichloride. *J. Chem. Soc. (A),* 2146–2151.

Raynor, J. B. (1972). On the formalism of treating the linear nitric oxide ligand as NO^+. *Inorg. Chim. Acta* **6,** 347–348.

Reynolds, M. F., Shelver, D., Kerby, R. L., Parks, R. B., Roberts, G. P., and Burstyn, J. N. (1998). EPR and electronic absorption spectroscopies of the CO-sensing CooA protein reveal a cysteine-ligated low-spin ferric heme. *J. Am. Chem. Soc.* **120,** 9080–9081.

Richter-Addo, G. B., and Legzdins, P. (1992). ''Metal Nitrosyls.'' Oxford Univ. Press, New York.

Richter-Addo, G. B., Hodge, S. J., Yi, G.-B., Khan, M. A., Ma, T., Caemelbecke, E. V., Guo, N., and Kadish, K. M. (1996). Synthesis, characterization, and spectroelectrochemistry of cobalt porphyrins containing axially bound nitric oxide. *Inorg. Chem.* **35,** 6530–6538.

Ruggiero, C. E., Carrier, S. M., Antholine, W. E., Whittaker, J. W., Cramer, C. J., and Tolman, W. B. (1993). Synthesis and structural and spectroscopic characterization of mononuclear copper nitrosyl complexes: Models for nitric oxide adducts of copper proteins and copper-exchanged zeolites. *J. Am. Chem. Soc.* **115,** 11285–11298.

Scheidt, W. R. (1977). Trends in metalloporphyrin stereochemistry. *Acc. Chem. Res.* **10,** 339–345.

Scheidt, W. R., and Frisse, M. E. (1975). Nitrosylmetalloporphyrins. II. Synthesis and molecular stereochemistry of nitrosyl-α,β,γ,δ-tetraphenylporphinatoiron(II). *J. Am. Chem. Soc.* **97,** 17–21.

Scheidt, W. R., and Hoard, J. L. (1973). Stereochemistry of low-spin cobalt porphyrins. I. Structure and bonding in a nitrosylcobalt porphyrin and their bearing on one rational model for oxygenated protoheme. *J. Am. Chem. Soc.* **95,** 8281–8288.

Scheidt, W. R., and Piciulo, P. L. (1976). Nitrosylmetalloporphyrins. III. Synthesis and molecular stereochemistry of nitrosyl-α,β,γ,δ-tetraphenylporphinato(1-methylimidazole)iron(II). *J. Am. Chem. Soc.* **98,** 1913–1919.

Scheidt, W. R., Brinegar, A. C., Ferro, E. B., and Kirner, J. F. (1977). Nitrosylmetalloporphyrins. 4. Molecular stereochemistry of two crystalline forms of nitrosyl-α,β,γ,δ-tetraphenylporphinato(4-methylpiperidine)iron(II). A structural correlation with ν(NO). *J. Am. Chem. Soc.* **99,** 7315–7322.

Scheidt, W. R., Hatano, K., Rupprecht, G. A., and Piciulo, P. L. (1979). Nitrosylmetalloporphyrins. 5. Molecular stereochemistry of nitrosyl(5,10,15,20-tetratolylporphinato)manganese(II) and nitrosyl(4-methylpiperidine)(5,10,15,20-tetraphenylporphinato)manganese(II). *Inorg. Chem.* **18,** 292–299.

Schelvis, J. P. M., Zhao, Y., Marletta, M. A., and Babcock, G. T. (1998). Resonance Raman characterization of the heme domain of soluble guanylate cyclase. *Biochemistry* **37,** 16289–16297.

Scholler, D. M., Wang, M.-Y. R., and Hoffman, B. M. (1979). Resonance Raman and EPR of nitrosyl human hemoglobin and chains, carp hemoglobin, and model compounds: Implications for the nitrosyl heme coordination state. *J. Biol. Chem.* **254,** 4072–4078.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., and Mayer, B. (1996). Characterization of 1-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol. Pharmacol.* **50,** 1–5.

Schultz, G., Böhme, E., and Munske, K. (1969). Guanylyl cyclase. Determination of enzyme activity. *Life Sci.* **8,** 1323–1332.

Senter, P. D., Eckstein, F., Mülsch, A., and Böhme, E. (1983). The stereochemical course of the reaction catalyzed by soluble bovine lung guanylate cyclase. *J. Biol. Chem.* **258,** 6741–6745.

Shah, S., and Hyde, D. R. (1995). Two *Drosophila* genes that encode the α subunit and the β subunits of the brain soluble guanylate cyclase. *J. Biol. Chem.* **270,** 15368–15376.

Shelver, D., Kerby, R. L., He, Y., and Roberts, G. P. (1995). Carbon monoxide-induced activation of gene expression in *Rhodospirillum rubrum* requires the product of *cooA,* a member of the cyclic AMP receptor protein family of transcriptional regulators. *J. Bacteriol.* **177,** 2157–2163.

Shelver, D., Kerby, R. L., He, Y., and Roberts, G. P. (1997). CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum,* is a CO-binding heme protein. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11216–11220.

Shelver, D., Thorsteinsson, M. V., Kerby, R. L., Chung, S.-Y., Roberts, G. P., Reynolds, M. F., Parks, R. B., and Burstyn, J. N. (1999). Identification of two important heme site residues (Cys^{75} and His^{77}) in CooA, the CO-sensing transcription factor of *Rhodospirillum rubrum. Biochemistry* **38,** 2669–2678.

Shriver, D. F., Atkins, P., and Langford, C. H. (1994). ''Inorganic Chemistry.'' Freeman, New York.

Snyder, D. A., and Weaver, D. L. (1970). The crystal and molecular structure of *trans*-chloronitrosylbis(ethylenediamine)cobalt(III) perchlorate. *Inorg. Chem.* **9,** 2760–2767.

Stone, J. R., and Marletta, M. A. (1994). Soluble guanylyl cyclase from bovine lung: Activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33,** 5636–5640.

Stone, J. R., and Marletta, M. A. (1995a). Heme stoichiometry of heterodimeric soluble guanylate cyclase. *Biochemistry* **34,** 14668–14674.

Stone, J. R., and Marletta, M. A. (1995b). The ferrous heme of soluble guanylate cyclase: Formation of hexacoordinate complexes with carbon monoxide and nitrosomethane. *Biochemistry* **34,** 16397–16403.

Stone, J. R., and Marletta, M. A. (1996). Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* **35,** 1093–1099.

Stone, J. R., and Marletta, M. A. (1998). Synergistic activation of soluble guanylate cyclase by YC-1 and carbon monoxide: Implications for the role of cleavage of the iron–histidine bond during activation by nitric oxide. *Chem. Biol.* **5,** 255–261.

Stone, J. R., Sands, R. H., Dunham, W. R., and Marletta, M. A. (1995). Electron paramagnetic resonance evidence for the formation of a pentacoordinate nitrosyl–heme complex on soluble guanylate cyclase. *Biochem. Biophys. Res. Commun.* **207,** 572–577.

Stone, J. R., Sands, R. H., Dunham, W. R., and Marletta, M. A. (1996). Spectral and ligand-binding properties of an unusual hemoprotein, the ferric form of soluble guanylate cyclase. *Biochemistry* **35,** 3258–3262.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996). Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.* **36,** 461–480.

Tamura, K., Nakamura, H., Tanaka, Y., Oue, S., Tsukamoto, K., Nomura, M., Tsuchiya, T., Adachi, S.-I., Takahasi, S., Iizuka, T., and Shiro, Y. (1996). Nature of endogenous ligand binding to heme iron in oxygen sensor FixL. *J. Am. Chem. Soc.* **118,** 9434–9435.

Tang, W.-J., Stanzel, M., and Gilman, A. G. (1995). Truncation and alanine-scanning mutants of type I adenylyl cyclase. *Biochemistry* **34,** 14563–14572.

Tesmer, J. J. G., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997). Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G_{s\alpha}$·GTPγS. *Science* **278,** 1907–1916.

Thompson, D. K., and Garbers, D. L. (1995). Dominant negative mutations of the guanylyl cyclase-A receptor. Extracellular domain deletion and catalytic domain point mutations. *J. Biol. Chem.* **270,** 425–430.

Tomita, T., Ogura, T., Tsuyama, S., Imai, Y., and Kitagawa, T. (1997). Effects of GTP on bound nitric oxide of soluble guanylate cyclase probed by resonance Raman spectroscopy. *Biochemistry* **36,** 10155–10160.

Traylor, T. G., and Sharma, V. S. (1992). Why NO? *Biochemistry* **31,** 2847–2849.

Tsai, A.-L. (1994). How does NO activate hemeproteins? *FEBS Lett.* **341,** 141–145.

Tullberg, A., and Vannerberg, N.-G. (1967). On the crystal structure of $K_2Mn(CN)_5NO·2H_2O$. *Acta Chem. Scand.* **21,** 1462–1472.

Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V., and Snyder, S. H. (1993). Carbon monoxide: A putative neural messenger. *Science* **259,** 381–384.

Vogel, K. M., Spiro, T. G., Shelver, D., Thorsteinsson, M. V., and Roberts, G. P. (1999a). Resonance Raman evidence for a novel charge relay activation mechanism of the CO-dependent heme protein transcription factor CooA. *Biochemistry* **38,** 2679–2687.

Vogel, K. M., Hu, S., Spiro, T. G., Dierks, E. A., Yu, A. E., and Burstyn, J. N. (1999b). Variable forms of soluble guanylyl cyclase (sGC): Protein ligand interactions and the issue of activation by CO. *J. Biol. Inorg. Chem.* **4,** 804–813.

Vogel, K. M., Rush III, T. S., Kozlowski, P. M., Spiro, T. G., Dou, Y., Ikeda-Saito, M., Olson, J. S., and Zgierski, M. Z. (1999c). FeNO structure in distal pocket mutants of myoglobin from resonance Raman spectroscopy. Manuscript preparation.

Walker, F. A., and Simonis, V. (1994). Iron porphyrin chemistry. *In* ''Encyclopedia of Inorganic Chemistry'' (R. Bruce-King, ed.), Vol. 4, pp. 1785–1847. Wiley, West Sussex, England.

Wedel, B. J., and Garbers, D. L. (1997). New insights on the functions of guanylyl cyclase receptors. *FEBS Lett.* **410,** 29–33.

Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Böhme, E., Schultz, G., and Koesling, D. (1994). Mutation in His-105 in the β_1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2592–2596.

Wedel, B., Harteneck, C., Foerster, J., Friebe, A., Schultz, G., and Koesling, D. (1995). Functional domains of soluble guanylyl cyclase. *J. Biol. Chem.* **270,** 24871–24875.

White, A. A., and Aurbach, G. D. (1969). Detection of guanyl cyclase in mammalian tissues. *Biochim. Biophys. Acta* **191,** 686–697.

Wolin, M. S., Wood, K. S., and Ignarro, L. J. (1982). Guanylate cyclase from bovine lung: A kinetic analysis of the regulation of the purified soluble enzyme by protoporphyrin IX, heme, and nitrosyl–heme. *J. Biol. Chem.* **257,** 13312–13320.

Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S. J., and Reed, G. H. (1972). Electromagnetic properties of hemoproteins. V. Optical and electron paramagnetic resonance characteristics of nitric oxide derivatives of metalloporphyrin–apohemoprotein complexes. *J. Biol. Chem.* **247,** 2447–2455.

Yoshimura, T. (1982). Electron paramagnetic resonance study of nitrosylprotoheme dimethyl ester complexes with aliphatic nitrogenoous bases: Characterization of the axial ligand trans to the nitrosyl group in nitrosylhemoproteins. *Arch. Biochem. Biophys.* **216,** 625–630.

Yu, A. E., Hu, S., Spiro, T. G., and Burstyn, J. N. (1994). Resonance Raman spectroscopy of soluble guanylyl cyclase reveals displacement of distal and proximal heme ligands by NO. *J. Am. Chem. Soc.* **116,** 4117–4118.

Yu, S., Avery, L., Baude, E., and Garbers, D. L. (1997). Guanylyl cyclase expression in specific sensory neurons: A new family of chemosensory receptors. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3384–3387.

Yuen, P. S. T., Doolittle, S. K., and Garbers, D. L. (1994). Dominant negative mutants of nitric oxide-sensitive guanylyl cyclase. *J. Biol. Chem.* **269,** 791–793.

Zhang, G., Liu, Y., Ruoho, A. E., and Hurley, J. H. (1997). Structure of the adenylyl cyclase catalytic core. *Nature* **386,** 247–253.

Zhao, Y., and Marletta, M. A. (1997). Localization of the heme binding region in soluble guanylate cyclase. *Biochemistry* **36,** 15959–15964.

Zhao, Y., Hoganson, C., Babcock, G. T., and Marletta, M. A. (1998a). Structural changes in the heme proximal pocket induced by nitric oxide binding to soluble guanylyl cyclase. *Biochemistry* **37,** 12458–12464.

Zhao, Y., Schelvis, J. P. M., Babcock, G. T., and Marletta, M. A. (1998b). Identification of His 105 in the β_1 subunit of soluble guanylate cyclase as the heme proximal ligand. *Biochemistry* **37,** 4502–4509.

Zimmerman, G., Zhou, D., and Taussig, R. (1998). Mutations uncover a role for two magnesium ions in the catalytic mechanism of adenylyl cyclase. *J. Biol. Chem.* **273,** 19650–19655.

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CHAPTER 26

Cyclic GMP-Mediated Signaling Mechanisms in Smooth Muscle

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IT IS WIDELY APPRECIATED THAT NITRIC OXIDE (NO) IS AN IMPORTANT CELLULAR REGULATOR THAT EXERTS MAJOR EFFECTS ON PROCESSES AS DIVERSE AS THE REGULATION OF VASCULAR TONE AND SYNAPTIC TRANSMISSION AND PLASTICITY. ALTHOUGH NO—COMPOSED OF ONLY TWO ATOMS—IS ONE OF THE SIMPLER CHEMICAL STRUCTURES IN BIOLOGY, ITS EFFECTS ON CELLS ARE UNUSUALLY COMPLEX. THIS CHAPTER EXAMINES THE MOST WIDELY UTILIZED NO SIGNAL TRANSDUCTION PATHWAY, THE CYCLIC GMP/CGMP-DEPENDENT PROTEIN KINASE (PKG) SYSTEM, IN THE REGULATION OF SMOOTH MUSCLE TONE. PARTICULAR EMPHASIS IS PLACED ON THE ROLE OF PKG IN SMOOTH MUSCLE SINCE IT IS NOW APPARENT THAT THIS KINASE IS A CRITICAL CONTROL POINT FOR INTRACELLULAR CALCIUM REGULATION, CONTRACTILE PROTEIN FUNCTION, AND GENE EXPRESSION.

Introduction

The major components of the cGMP signaling pathway in eukaryotic cells were first described in the 1970s. Today, cGMP signaling is one of the more widely studied cellular pathways, owing in part to the importance of nitric oxide (NO) in cellular function. The important contribution of NO to the discipline of cGMP signaling is illustrated in Fig. 1. In Fig. 1A and 1B, the number of publications dealing with cGMP was relatively modest but actually outpaced those dealing with NO until 1992. Publications dealing with either cGMP or cGMP-dependent protein kinase (PKG, Fig. 1C), in fact, had remained somewhat constant or only slightly increased between 1988 and 1992. Since 1992, however, papers dealing with NO have increased logarithmically. As a result of such broad interest in NO, publications dealing with cGMP and particularly PKG have likewise dramatically increased. Thus, prior to the widespread interest in the role of NO in biological systems, a smaller group of investigators held exclusive province in the cGMP/PKG field. The broader interest in cGMP/PKG signaling pathways seen today had to await the emergence of the more translationally applicable NO paradigm.

The ancient history involving cGMP signaling before NO appeared on the scene may seem irrelevant. However, many of the current controversies surrounding the roles of NO, cGMP, and the PKG in cell function might be partially resolved when examined in light of some of the older findings regarding cGMP signaling. This chapter will cover many basic aspects of NO–cGMP signaling, but with emphasis on the role of cGMP and PKG in smooth muscle function. Special consideration will be given to the pitfalls regarding interpretation of the published information from current studies. Limitations on space will by necessity mean that some topics concerning this signal transduction system will not be covered. Nor will all the relevant citations for the work described below be included. Nevertheless, it is hoped that the topics covered will provide a potent stimulus for research on this signaling pathway.

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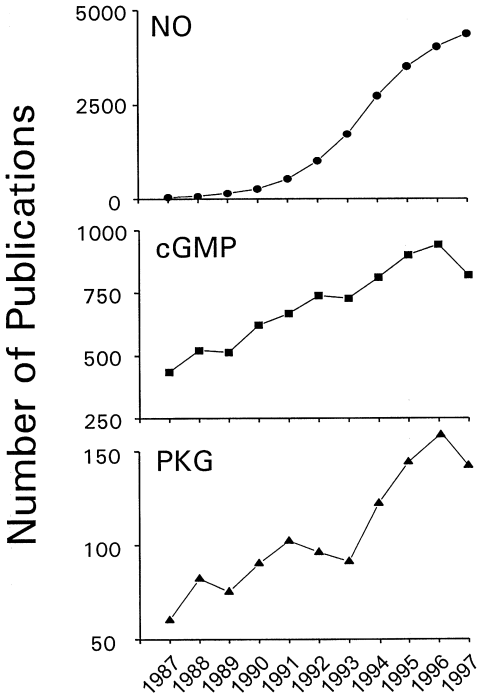


Figure 1 Yearly publications on NO, cGMP, and PKG.

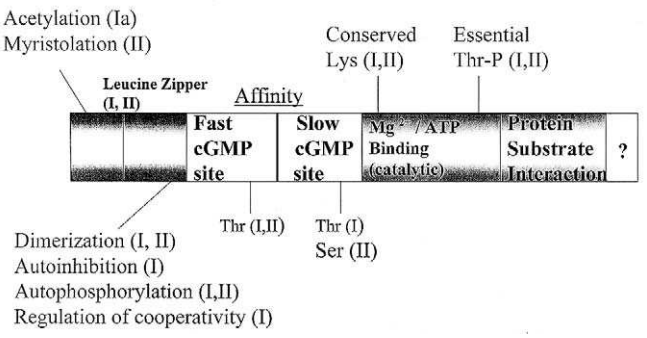


Figure 2 Structure of PKG from vertebrate sources. The domain structure for PKG is illustrated for type I (I) and type II (II) PKG. The positions of the conserved lysine and phosphorylated threonine residues are identified in the catalytic domain. The positions of conserved threonine (serine in type II PKG) in the cGMP binding sites are also shown. The domains for dimerization, autoinhibition, autophosphorylation, and cooperativity are demonstrated also.

NO–Cyclic GMP Signaling via Protein Phosphorylation

Cyclic GMP is a major mediator of NO signaling in the cell by virtue of the high affinity of NO for the soluble form of guanylate cyclase (sCG). Estimates for the affinity of NO for the sGC-bound heme range from 50 p*M* to 1 n*M* (Hobbs, 1997). Binding of NO to sGC increases the V_{max} of the enzyme by more than 200-fold, depending on assay conditions (Hobbs, 1997). Such robust amplification is the hallmark of second messenger signal transduction as originally proposed for hormones that activate adenylate cyclase. The fate of cGMP in the cell is likewise similar to that of cAMP: first, cGMP may be hydrolyzed by cyclic nucleotide phosphodiesterases, some of which are specific for cGMP (Juilfs *et al.,* 1999). Second, cGMP may bind to one of three classes of intracellular receptor proteins (Lincoln and Cornwell, 1993): (i) cyclic nucleotide-dependent protein kinases, which include cAMP-dependent protein kinase (PKA) as well as PKG; (ii) ion channels, primarily nonspecific cation channels whose activity is increased on cGMP binding; and (iii) cyclic nucleotide phosphodiesterases, primarily at allosteric sites located on the enzymes. For the purposes of this review, we will concentrate on the cyclic nucleotide dependent kinases as mediators of NO-cGMP signal transduction.

Cyclic GMP-Dependent Protein Kinase

PROPERTIES

PKG is a modular gene product whose evolution represents the fusion of unrelated coding regions giving rise ultimately to a highly regulated enzyme (for reviews, see Lincoln and Cornwell, 1993, Hofmann *et al.,* 1992, Francis and Corbin, 1994, and Butt *et al.,* 1993). As illustrated in Fig. 2, the enzyme is a serine/threonine kinase consisting of a C-terminal catalytic domain that is homologous with other kinases including tyrosine kinases. A critical lysine, an ATP binding pocket, and an activating phosphorylation site are all present in its structure. The regulatory domain is fused to the catalytic domain via a short hinge sequence and consists of an N-terminal leucine/isoleucine zipper motif, an autophosphorylation/autoinhibitory region, and two homologous cyclic nucleotide binding domains. As illustrated in Table I, there are two genes that encode PKG in mammals: the type I gene and type II gene. The Type I gene gives rise to alternately spliced mRNAs to produce a type Iα PKG and a type Iβ PKG (Francis *et al.,* 1988–1989; Orstavik *et al.,* 1997). In mammals, isoforms of Type II PKG have yet to be described, and they may not exist.

PKG is activated by either low concentrations of cGMP or higher concentrations of cAMP. The concentrations of cyclic nucleotides needed to activate PKG, however, depend on the isoform and the phosphorylation state of the enzyme. All PKG isoforms exist as homodimers; cyclic nucleotides bind to two nonidentical binding sites on each protomer such that four molecules of cyclic nucleotide are bound per active dimer (Francis and Corbin, 1994; Corbin *et al.,* 1986). Substitution of hydrogen with a bulky group at the 8-position of the purine ring (e.g., 8-bromo, 8-chlorophenylthio) renders a cyclic nucleotide more potent in activating the type I isoform of PKG (Francis and Corbin, 1994; Francis *et al.,* 1988). Hence, 8-chlorophenylthio-cAMP is almost as potent an activator of the Iα isoform of PKG than is native cGMP itself. Experiments in which 8-substituted cyclic nucleotides are added to cells or tissues over a period of minutes to hours have resulted in the activation of both cyclic nucleotide kinases, a phenomenon known as ‘‘cross-activation’’ (Francis *et al.,* 1988). Thus, one pitfall with the interpretation of experiments using 8-substituted cyclic nucleotides, especially

Table I Mammalian Cyclic GMP-Dependent Protein Kinases

Species	Name	Form	Chromosome	Subunit M^a	References
Bovine	PRKG1	Iα	Unknown	76,418	Wernet *et al.* (1989)
		Iβ		77,803	Wernet *et al.* (1989)
Human	PRKG1	Iα	10	76,418	Tamura *et al.* (1996)
	PRKG1	Iβ	10	77,803	Sandberg *et al.* (1989)
	PRKG2	II	4	87,4003	Orstavik *et al.* (1996); Fujii *et al.* (1995)
Rat	PRKG2	II	Unknown	87,400	Jarchau *et al.* (1994)
Mouse	PRKG2	II	Unknown	87,400	Uhler (1993)

[a] PKG Iα, Iβ, and II have all been shown to exist as homodimers.

where the Iα isoform is involved, is activation of both cyclic nucleotide-dependent protein kinases. It is advisable to construct dose–response curves using cyclic nucleotide analogs to determine the efficacy, and hence the specific kinase, involved in the biological effect under consideration.

Activation of PKG has been studied most thoroughly for the Type I isoform, and is a complex process involving the binding of cyclic nucleotides to both sites with subsequent induction of phosphorylation of serine and threonine residues in the autoinhibitory region (Hofmann *et al.,* 1985; Landgraf *et al.,* 1986; Smith *et al.,* 1992). The ''slow'' binding site (referred to as such due to the slow dissociation rate for cGMP) has a high affinity for cGMP ($K_{act} \cong 0.1$ μM depending on the isoform), whereas the ''fast'' site has a lower affinity for cGMP. Because basal cGMP levels are generally less than micromolar in cells, it is likely that only the slow site is occupied during resting cellular conditions. This may contribute to the partial activation of the enzyme which is seen both in the intact cell as well as in purified enzyme preparations (Smith *et al.,* 1986). The fast site is then thought to be a ''trigger'' site that induces maximal activation of the enzyme. Although the binding sites for cyclic nucleotides are identical for PKG Iα and Iβ, the Iα isoform is activated by lower cGMP ($K_{act} = 0.1$ μM) than the Iβ isoform ($K_{act} = 1.0$ μM), suggesting perhaps that the autoinhibitory domain of Iβ is more efficient in maintaining basal activity of the enzyme (Francis and Corbin, 1994). The greater susceptibility of the Iα isoform to cyclic nucleotide-dependent activation may account for some of the differences observed when PKG is transfected into cultured cells in order to observe specific biological effects.

Cyclic AMP binds primarily to the slow site of PKG-I, and affinity is increased more than 30-fold by autophosphorylation of the enzyme (Doskeland *et al.,* 1986). Thus, at physiological levels of cAMP (i.e., 0.5 μM), cAMP would be expected to bind to autophosphorylated PKG-I. It has been known since the mid 1970s that the binding of cAMP to PKG is of relatively high affinity because immobilized cAMP columns are routinely used in the purification of PKG. Because the levels of cAMP are some 10- to 20-fold higher than cGMP in most cell types, it is likely that cAMP can trigger PKG activation in the absence of increases in cGMP. Such cross-activation of PKG by physiological increases in cAMP levels has been demonstrated in coronary arterial smooth muscle strips (Jiang *et al.,* 1992).

The molecular basis for selectivity of cyclic nucleotide binding to PKG has been studied by Shabb *et al.* (1990, 1991). On alignment of the cyclic nucleotide binding sites of the regulatory subunits of PKA with the binding sites in the regulatory domains of PKG, a critical role for the hydroxyl group in threonine, found only in PKG isozymes to hydrogen bond with the C-2 amino group of the guanine ring, was described (Fig. 3). Because alanine is substituted for threonine in the regulatory subunit of PKA, no high-affinity hydrogen bonding is available for cGMP. Mutation of the alanine residues in the regulatory subunit of PKA to threonine residues resulted in high affinity binding of cGMP to PKA, providing strong support for the notion that the selectivity for cyclic nucleotide binding to cyclic nucleotide-dependent protein kinases is explained in part by a one amino acid substitution.

The expression pattern of PKG illustrates that this enzyme is far from a ''housekeeping'' gene product. Type Iα is expressed in all smooth muscles, and it is the principal isoform expressed in platelets, cerebellum, and lung—most probably airway and pulmonary vascular smooth muscle cells (VSMC) (Lincoln and Cornwell, 1993; Butt *et al.,* 1993; Lincoln, 1994; Lincoln and Corbin, 1983). PKG-Iβ has been reported to be expressed at higher levels in nonvascular smooth muscle such as uterine and intestinal smooth muscle cells (Keilbach *et al.,* 1992). Type II PKG expression appears to be restricted to intestinal epithelial cells, chondrocytes, and certain regions of the brain (Jarchau *et al.,* 1994; Markert *et al.,* 1995; Uhler, 1993; Pfeifer *et al.,* 1996). And finally, it has been known for decades that many cell types appear to be practically devoid of any isoform of PKG (skeletal muscle myocytes, erythrocytes, possibly hepatocytes, and many cultured cells). Investigators need to be aware of the variable pattern of PKG expression when designing experiments to test the role of this enzyme in cell function. This is especially important given the fact that specific inhibitors and activators of the PKG pathway do not exist (see ''Protein Kinase Inhibitors'').

The variability of PKG levels in tissues suggests that physiological regulation of PKG expression exists. One example of the regulation of PKG expression is found in

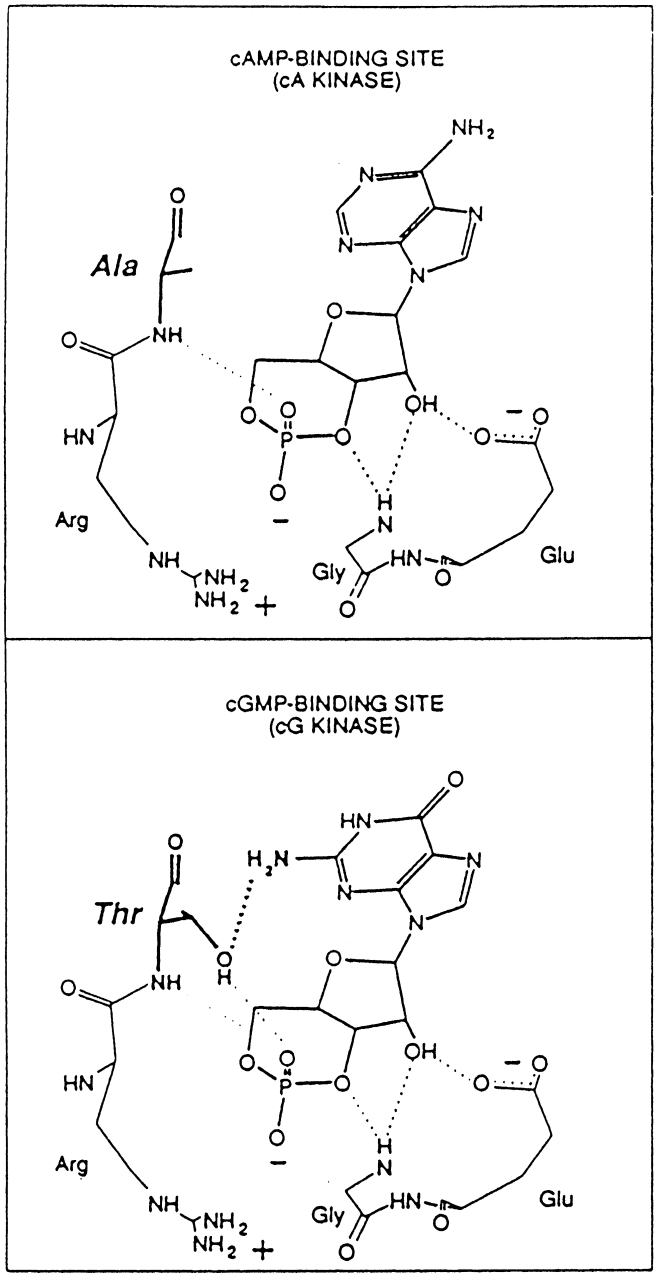


Figure 3 Structure of the cAMP binding site in PKA (top panel) and cGMP binding site in PKG (bottom panel). The model was predicted from the three-dimensional structure of the binding pocket of the cAMP-activator protein (CAP) of *Escherichia coli.* Cyclic GMP-selective binding pockets have threonyl residues located in the positions occupied by alanine in cAMP binding proteins. [From Shabb *et al.* (1990), reprinted with permission from the American Society for Biochemistry and Molecular Biology.]

VSMC. Primary cultures of aortic smooth muscle cells from rodent, for instance, have high levels of PKG-Iα. On passaging, the levels of PKG drop precipitously such that after three or four passages, expression is nearly undetectable (Cornwell and Lincoln, 1989; Cornwell *et al.,* 1994a; Wyatt *et al.,* 1998). On the other hand, high-density culture conditions and cell–cell contact induce PKG expression in VSMC, whereas low-density growth and reduced cell–cell contact causes a loss of expression (Cornwell *et al.,* 1994a). High cell density-dependent expression of PKG may explain why passaging reduces PKG since, by definition, cell–cell contact is disrupted. There may be cell adhesion molecule signaling pathways that regulate PKG expression in VSMC, but these have not been defined.

Another mechanism for the regulation of PKG expression has been uncovered involving NO itself (Soff *et al.,* 1997). As shown in Fig. 4, chronic exposure to high concentrations of NO-donor drugs, *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP), suppresses PKG mRNA in primary cultures of VSMC. The effects of NO-donor drugs on PKG expression were mimicked by exposure of the cells to cyclic nucleotide analogs. It was suggested that chronic NO-induced suppression of PKG may underlie, in part at least, the phenomenon of nitrate tolerance (Soff *et al.,* 1997). Alternatively, chronic NO production such as that which occurs as a result of NO synthase II (iNOS) induction during inflammation may suppress PKG expression in arterial smooth muscle. Reduced PKG expression may be one mechanism leading to the modulation of VSMC from a contractile phenotype to a more synthetic one in response to vascular injury (Boerth *et al.,* 1997) (see ''Cyclic GMP and VSMC Phenotypic Modulation'').

PKG SUBSTRATES

An active area of research concerns substrate proteins for the PKG. In the 1970s, it was noted that PKG and PKA catalyzed the phosphorylation of the same proteins *in vitro* (Lincoln and Corbin, 1977). The substrate specificity for both kinases was defined by a cluster of basic amino acid residues N-terminal to the phosphorylated residue (Lincoln and Corbin, 1977). PKG recognizes both the sequence RKXS as well as RRXS in substrate proteins. Studies by Dostemann and co-workers (Tegge *et al.,* 1995) using combinatorial peptide libraries as substrates for cyclic nucleotide-dependent protein kinases suggest that the arginine–lysine (i.e., RK) combination may be preferred by PKG over the arginine–arginine (RR) combination. PKA, on the other hand, has a strong preference for the arginine–arginine com-

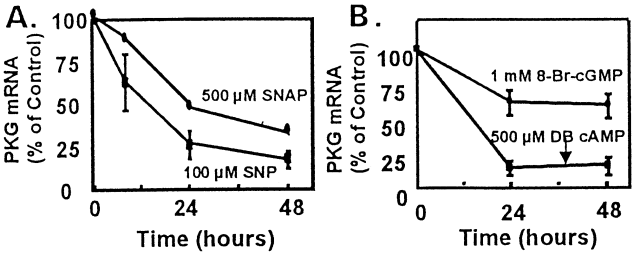


Figure 4 Effects of NO-donor drugs and cyclic nucleotide analogs on PKG expression in cultured bovine aortic smooth muscle cells. (A) Cells were treated with either SNAP or SNP. (B) Cells were treated with either 1 m*M* 8-Br-cGMP or 500 μ*M* dibutyryl-cAMP. PKG mRNA was determined by Northern blot analysis. [From Lincoln *et al.* (1998). *Acta Physiol. Scand.* **164,** 507–515. Reprinted with permission from the Scandinavian Physiological Society.]

bination. Another determinant for PKG, at least in some protein substrates, is a preference for an aromatic residue (i.e., F or Y) four positions C-terminal to the phosphorylatable residue (Colbran *et al.,* 1992a). Despite these findings, no universal paradigm distinguishing PKA and PKG substrate specificity has emerged, suggesting that perhaps primary sequence alone is not the only determinant of substrate specificity for PKG. From the standpoint of experimental protocols, however, it is critical that investigators understand the overlapping nature of substrate specificity between these two enzymes *in vitro,* and to be knowledgeable of the fact that nonphysiological phosphorylation of proteins in the intact cell is likely to occur in response to overexpression of cyclic nucleotide-dependent protein kinases. Such a phenomenon may underlie some of the reported effects of PKG on MAP kinase activity and gene expression (see section on cyclic GMP and cell growth).

Some of the more exciting advances in our understanding of the physiological role of PKG have resulted from the identification of substrate proteins in the intact cell. Table II provides a list of some substrates for PKG likely to be physiologically important. In most these cases, specificity for PKG-dependent phosphorylation has been demonstrated and stoichiometry of phosphorylation has been obtained. Other proteins reported to be phosphorylated by PKG, but not listed in Table II, may be omitted because the studies (i) lacked controls for ruling out preference for PKA-dependent phosphorylation, (ii) demonstrated nonstoichiometric phosphorylation, or (iii) were based mainly on the use of inhibitors. There are molecular processes or events that are known to be regulated by PKG, but in several instances, specific substrate proteins underlying these processes have yet to be identified. Some of these events are provided in Table III and will be discussed in more detail in subsequent sections of this chapter.

Cyclic AMP-Dependent Protein Kinase

As mentioned above, cGMP also activates PKA. Unlike the case for endogenous cAMP activating PKG in the intact cell, it is probably a rare event when cGMP levels reach high enough concentrations to cross-activate PKA. In two pathophysiological instances, however, this has been demonstrated. In human colonic carcinoma T84 cells, bacterial heat-stable enterotoxin (STa) elevated cGMP levels, but not cAMP, resulting in Cl^- and water secretion (Forte *et al.,* 1992). These effects appeared to be mediated by activation of PKA, as the T84 cells were devoid of the type II PKG. Support for this concept was obtained by measuring the endogenous activation of PKA. In a separate study by Cornwell *et al.* (1994b), treatment of cultured VSMC with interleukin 1β (IL-1β) resulted in a large increase in cGMP due to the expression of iNOS. These increases in cGMP were associated with the inhibition of DNA synthesis *in vitro.* Similar to the study described above in T84 cells, the cultured VSMC were devoid of type I PKG. Both IL-1β and

Table II Some Physiologically Important Substrates for PKG

Substrate[a]	Possible functions
VASP[b]	Focal adhesion assembly and integrin signal transduction in cells
Type V PDE[c]	Regulation of PDE activity in smooth muscle and other cells
Type I-IP_3 receptor[d]	Modulate Ca^{2+} release from the SR of smooth muscle cells
Phospholamban[e]	Increase Ca^{2+} uptake into SR of smooth muscle cells
Thromboxane receptor[f]	Inhibit G-protein coupled IP_3 formation in platelets
K_{Ca} channel α subunit[g]	K^+ efflux and hyperpolarize the smooth muscle cell membrane
Myosin binding subunit[h]	Modulate activity of myosin light chain phosphatase
HSP-20[i]	Modulate contractility in smooth muscle cells
CFTR[j]	Activate Cl^- transport in rat intestinal epithelium
DARPP-32[k]	Inhibit protein phosphatases in neural cells

[a] Abbreviations: VASP, vasodilator-stimulated phosphoprotein; PDE, cyclic nucleotide phosphodiesterase; IP_3, inositol 1,4,5-trisphosphate; HSP, heat shock protein; CFTR, cystic fibrosis transmembrane conductance regulator; DARPP, dopamine- and cAMP-regulated phosphoprotein.

[b] Eigenthaler *et al.,* 1998; Horstrup *et al.,* 1994; Eigenthaler and Shattil, 1996.

[c] Geng *et al.,* 1998.

[d] Komalavilas and Lincoln, 1994, 1996; Koga *et al.,* 1994.

[e] Vrolix *et al.,* 1998; Raeymaekers *et al.,* 1988; Sarcevic *et al.,* 1989; Karczewski *et al.,* 1992; Cornwell *et al.,* 1991; Murthy and Makhlouf, 1995; Sulakhe and Vo, 1995; Sabine *et al.,* 1995.

[f] Wong *et al.,* 1998.

[g] Alioua *et al.,* 1998.

[h] Surks *et al.,* 1998.

[i] Beall *et al.,* 1997.

[j] Vaandrager *et al.,* 1997; French *et al.,* 1995.

[k] Tsou *et al.,* 1993.

Table III Some Physiologically Important Signaling Events Regulated by PKG

Cell type	Event
Smooth muscle relaxation	Inhibition of Ca^{2+} levels[a] Activation of K^+ channels[b] Activation of Ca^{2+} sparks[c] Ca^{2+} desensitization[d]
Cytoskeleton signaling	Disassembly of focal adhesions[e] Decreased migration of smooth muscle cells[f] Decreased platelet aggregation and adhesion[e]
VSMC phenotypic modulation	Increased contractile protein expression[f] Decreased extracellular matrix protein expression[f] Decreased proliferation[g]
Gene expression	Increased *c-fos* expression[h] Increased MAP kinase activity[h]
Salt and water secretion	Activation of Cl^- secretion[i]
Bone growth	Inhibition of chondrocyte ossification[i]

[a] See ''Role of PKG in the Regulation of Intracellular Ca^{2+}.''
[b] See ''Activation of K_{Ca} Channels by PKG.''
[c] See ''Regulation of Sarcoplasmic Reticulum Ca^{2+} by PKG.''
[d] See ''Role of PKG in the Regulation of Ca^{2+} Sensitization.''
[e] See ''VASP and HSP-20.''
[f] See ''Cyclic GMP and VSMC Phenotypic Modulation.''
[g] See ''Cyclic GMP and VSMC Phenotypic Modulation'' and ''Cyclic GMP and Cell Growth.''
[h] See ''Cyclic GMP and Cell Growth.''
[i] See ''Studies Based on PKG Null Mice.''

high concentrations of NO-donor drugs activated PKA in these cells even though no increases in cAMP were observed. Hence, in two instances, large increases in cGMP were found to activate PKA in the intact cell. Whether this is a physiological event is not clear; nevertheless, these studies do illustrate the importance of examining the dose–response relationships of cyclic nucleotide ''agonists'' and cyclic nucleotide analogs when examining cellular responses.

Protein Kinase Inhibitors

It is a reasonable assumption that one of the more frustrating problems in defining a role for PKG (or PKA) in cell function is the lack of specific and pharmacologically well-characterized inhibitors (see Smolenski *et al.,* 1998, for extensive review on this subject). For the type I PKG, there are two classes of compounds that purportedly inhibit PKG with reasonable specificity and high affinity: the isoquinolinesulfonamide compounds and the phosphorothioates. The isoquinolinesulfonamides are believed to be competitive inhibitors for the binding of ATP to the catalytic site of the kinase, and are represented by the H compounds (e.g., H-8) and the KT compounds (e.g., KT 5823). The phosphorothioates, on the other hand, are competitive inhibitors of cyclic nucleotide binding, and are represented by the Rp stereoisomers of cGMP phosphorothioates (e.g., Rp-8-pCPT-cGMPS). When incubated with purified (or partially purified) kinases, KT 5823 and Rp-8-pCPT-cGMPS inhibit enzyme activity with a 40-fold and 16-fold selectivity for PKG over PKA, respectively. On the other hand, the specificity of these two classes of compounds for inhibiting PKG in the intact cell is not impressive, and there are even reports that the KT compound does not inhibit PKG. In neutrophils (Wyatt *et al.,* 1991) and platelets (Smolenski *et al.,* 1998), KT 5823 was found to activate rather than inhibit PKG. These studies suggest that perhaps this compound is biotransformed in some way to become an activator of PKG in some cells. Additionally, we reported that KT 5823 accumulates in aortic smooth muscle over a 30-min period to sufficient levels to inhibit PKA activity (Komalavilas and Lincoln, 1996). Clearly, this class of compounds has not been sufficiently characterized pharmacologically to rely on its use in intact cell studies. On the other hand, the Rp stereoisomers of cGMP have a more predictable effect in cells and tissues, but the specificity for inhibiting PKG over PKA is poor (Butt and Walter, 1996). The best outcome that can be expected in interpreting the data using this latter class of compounds is the ability to conclude that a cyclic nucleotide-dependent kinase, as opposed to other kinases, mediates a cell signaling event.

NO-Cyclic GMP Signaling in Vasodilation

Nitrovasodilator drugs such as nitroglycerine have been used clinically for more than a century in the treatment of angina. The mechanism involves peripheral vasodilation,

particularly venous vasodilation, which reduces the preload or end-diastolic volume of the heart. Preload reduction, in turn, decreases myocardial oxygen consumption, which is the basis of the effectiveness of the drug in angina therapy. It would be about 100 years before it was determined that cells make their own endogenous ''nitrate,'' that is, NO, and that a new era of vascular biology would be ushered in.

The role of cGMP in NO-induced vasodilation is clearly established from three major lines of evidence: (i) excellent correlative pharmacological evidence using NO-donor drugs and endogenous NO production to manipulate cGMP levels and relaxation in nearly all vascular tissue; (ii) the consistent findings that cGMP phosphodiesterase inhibitors and cGMP analogs mimic NO-dependent relaxation of various vascular smooth muscle preparations; and (iii) the effects of specific sGC inhibitors to block NO-mediated relaxation. Added to these observations is the study using PKG-I knockout mice, although as discussed later, there are some equivocal concerns with these latter studies.

Role of PKG in the Regulation of Intracellular Ca^{2+}

If the role of NO in vasodilation is clearly established, then the mechanism of action of cGMP and PKG seems less well defined. An early and consistent finding was that NO-donor drugs, cGMP analogs, and PKG (when introduced into smooth muscle cells) all lower intracellular Ca^{2+} levels, especially when elevated with a Ca^{2+}-mobilizing agonist (Cornwell and Lincoln, 1988, 1989; Johnson and Lincoln, 1985; Rashatwar *et al.,* 1987; Felbel *et al.,* 1988; McDaniel *et al.,* 1992; Meisheri *et al.,* 1986). Because Ca^{2+} is the major signal for the activation of myosin light chain (MLC) kinase and cross-bridge cycling in smooth muscle cells, these findings were of interest and significant. There are at least three major mechanisms by which PKG appears to lower intracellular Ca^{2+} in VSMC. First, PKG activates K_{Ca} channels, leading to hyperpolarization and an inhibition of voltage-dependent Ca^{2+} channels; second, it increases uptake of Ca^{2+} into intracellular stores; and third, it inhibits of hormone-dependent induced inositol monophosphate formation.

ACTIVATION OF K_{Ca} CHANNELS BY PKG

The smooth muscle K_{Ca} channel is composed of an α subunit, which is necessary for pore formation and is the actual component of the channel, and the β subunit, which is a regulatory protein for the channel (Tseng-Crank *et al.,* 1994). Activation of K_{Ca} channels by cyclic nucleotide-dependent protein kinases has been proposed to mediate relaxation (Hamaguchi *et al.,* 1991; Thornbury *et al.,* 1991; Kahn *et al.,* 1993; Chen and Rembold, 1996; Robertson *et al.,* 1993; Archer *et al.,* 1994; Sadoshima *et al.,* 1988). The two hypotheses that have been forwarded to account for the effects of PKG are (i) direct phosphorylation of the channel leading to activation (Sadoshima *et al.,* 1988; Kume *et al.,* 1989; Alioua *et al.,* 1995, 1998), and (ii) PKG-mediated dephosphorylation of the channel by activation of a protein phosphatase (White *et al.,* 1993; Zhou *et al.,* 1996). The evidence for the latter mechanism comes from studies using inhibitors of the serine/threonine protein phosphatase, and particularly of protein phosphatase 2A (PP2A), to block the effects of PKG to activate K_{Ca} channels in either cultured pituitary cells (White *et al.,* 1993) or isolated smooth muscle cells (Zhou *et al.,* 1996).

If PP2A activation and subsequent dephosphorylation is the event that activates K_{Ca} channels, then the mechanism of PP2A activation is of paramount importance. The complexity of PP2A is impressive given the large number of genes and spliced variants of subunits expressed in different tissues, but basically the PP2A exists as a heterotrimer: a catalytic subunit, an A subunit generally considered to be a regulatory subunit, and a B subunit which is highly variable in expression and is thought to confer selective activity toward particular protein substrates (Kamibayashi *et al.,* 1994; Csortos *et al.,* 1996). None of the many forms of these subunits have been demonstrated to be phosphorylated by PKG in intact VSMC. It is conceivable that PKG might regulate association or translocation of PP2A to protein substrates such as the K_{Ca} channel in the cell, but this is pure speculation. A study by Zhou *et al.* (1998) seems to confirm the notion that the ''biochemical properties'' of a particular cell dictate whether cyclic nucleotide-dependent protein kinases are capable of regulating K_{Ca} channel activity. Neither PKA nor PKG affected the gating properties of transfected BK channel proteins in CHO cells, whereas both kinases inhibited gating of the channel in transfected myometrial cells. Given the complexity of the number of spliced variants of the K_{Ca} channel, and the importance of the phenotypic properties of cells expressing K_{Ca} channels, the regulation of K^+ currents by cyclic nucleotides remains an area about which there is much to learn. To add further complexity to the situation, several studies now suggest that NO itself can directly activate BK channels in cells, possibly through a mechanism involving *S*-nitrosothiol formation (Krippeit-Drews *et al.,* 1992; Bolotina *et al.,* 1994; Lie *et al.,* 1992). The area of cGMP-independent regulation of cell function is a rapidly growing rea of investigation as well (see section on cGMP and PKG-independent mechanisms of relaxation).

REGULATION OF SARCOPLASMIC RETICULUM CA^{2+} BY PKG

It was demonstrated in the 1980s and 1990s that PKG-catalyzed phosphorylation of sarcoplasmic reticulum (SR) proteins, particularly the protein phospholamban (Vrolix *et al.,* 1988; Raeymaekers *et al.,* 1988; Sarcevic *et al.,* 1989; Karczewski *et al.,* 1992; Cornwell *et al.,* 1991; Murthy and Makhlouf, 1995; Sulakhe and Vo, 1995; Sabine *et al.,* 1995). The importance of this finding was that some forms of the SR Ca^{2+}-transporting ATPases (known as SERCAs) are regulated by phosphorylation of phospholamban. On phosphorylation by either PKA or PKG at serine-16, or by Ca^{2+}-calmodulin-dependent kinase II at serine-17, phospholamban dissociates from the SERCA, thereby activating Ca^{2+} transport into the SR. Evidence that PKG catalyzes phosphorylation of phospholamban in the intact cell comes from studies demonstrating that (i) PKG-catalyzed phospholamban phosphorylation and activation of Ca^{2+}-ATPase occurs concomitantly with reductions in cytosolic Ca^{2+} (Vrolix

et al., 1988; Karczewski *et al.,* 1992; Cornwell *et al.,* 1991), and (ii) blockers of the SR Ca^{2+}-ATPase inhibit NO and cGMP-mediated Ca^{2+} reductions in smooth muscle (Luo *et al.,* 1993; Moritoki *et al.,* 1996; Andriantsitohaina *et al.,* 1995; Cohen *et al.,* 1999).

The importance of phospholamban phosphorylation in the regulation of smooth muscle relaxation *in vivo* has been questioned using studies on phospholamban-deficient mice. These animals demonstrate predictable prolonged cardiac relaxation and enhanced sensitivity of vascular smooth muscle to agonist-evoked contraction (Lalli *et al.,* 1997). On the other hand, the animals do not display impaired cardiac performance nor impaired vascular relaxation in response to NO-donor drugs (Lalli *et al.,* 1999). These results have been interpreted to suggest that phospholamban phosphorylation may play a relatively minor role in the regulation of cyclic nucleotide dependent relaxation.

There is other evidence, however, that phospholamban phosphorylation may be critical for K_{Ca} channel regulation. It has been shown by several laboratories that localized release of Ca^{2+} from the superficial SR located near the plasma membrane in smooth muscle triggers the activation of K_{Ca} channels (Stenho-Bittel and Sturek, 1992; Nelson *et al.,* 1995). The resultant hyperpolarization, termed spontaneous transient outward current or STOC, inhibits Ca^{2+} influx through voltage-dependent channels, which then produces a more global reduction in cytosolic Ca^{2+}. Porter *et al.,* (1998) have demonstrated that cAMP- and cGMP-elevating drugs increase STOCs from vascular smooth muscle cells through a mechanism dependent upon increased uptake and release of Ca^{2+} into the SR. In the phospholamban-deficient mice, there were reduced STOCs in response to NO-donor drugs and forskolin, which resulted in a reduction in smooth muscle relaxation (Wellman *et al.,* 1999).

Based on the studies described in this section, a model illustrating the role of PKA and PKG in smooth muscle relaxation through phosphorylation and STOC generation is shown in Fig. 5. The differences between the *in vivo* studies where cyclic nucleotide-dependent relaxation appeared to be unimpaired (Lalli *et al.,* 1999) and the *in vitro* studies demonstrating impaired STOC generation and relaxation (Porter *et al.,* 1998; Wellman *et al.,* 1999) may be due to the development of embryonic compensatory mechanisms in the phospholamban deficient mice.

Another potential mechanism by which PKG increases STOCs in vascular smooth muscle is phosphorylation of the type I inositol 1,4,5-trisphosphate (IP_3) receptor in the SR. It has been shown that PKG catalyzes phosphorylation of the IP_3 receptor *in vitro* and in intact rat aorta (Komalavilas and Lincoln, 1994, 1996; Koga *et al.,* 1994), whereas other laboratories have demonstrated that PKA- and PKG-dependent phosphorylation of smooth muscle cell IP_3 receptors decreases Ca^{2+} release from the SR (Quinton and Dean, 1992; Murthy *et al.,* 1993; Cavallini *et al.,* 1996). On the other hand, IP_3 receptor phosphorylation by cGMP in hepatocytes appears to increase Ca^{2+} release (Enouf *et al.,* 1987; Bird *et al.,* 1993; Hajnoczky *et al.,* 1993; Rooney *et al.,* 1996). Part of the discrepancy of results may be due to different IP_3 receptor isoforms expressed in smooth muscle cells and hepatocytes. If IP_3 receptor phosphorylation leads to increased Ca^{2+} release from superficial SR, then this could trigger STOCs and decrease global cell Ca^{2+}, similar to the role of

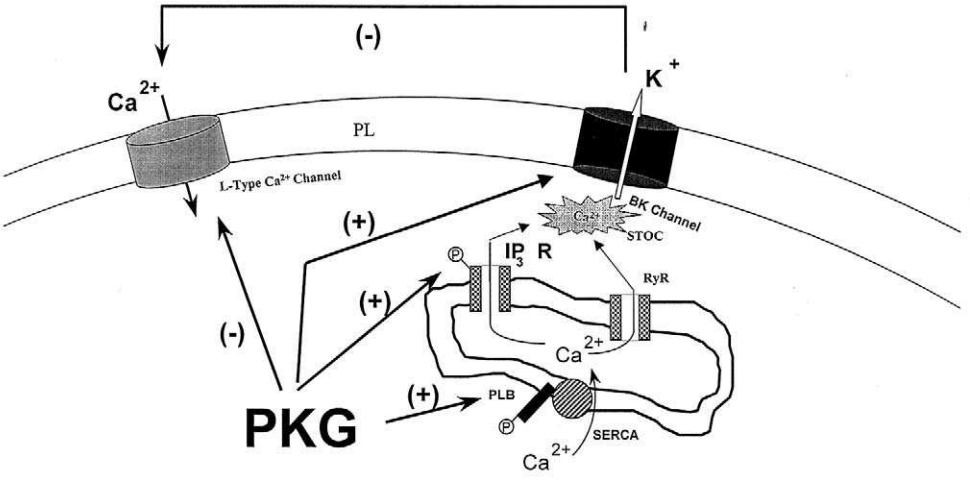


Figure 5 Possible mechanisms of action of PKG on K^+ and Ca^{2+} currents in VSMC. PKG is known to catalyze the phosphorylation of the type I-IP_3 receptor (IP_3 R) and phospholamban (PLB) in the SR of VSMC. Depicted in the figure is the effect of PKG to activate Ca^{2+} uptake into the superficial SR, leading to a more robust release of Ca^{2+} through the IP_3 receptor channel and ryanodine receptor (RyR) channel. Phosphorylation of the IP_3 receptor may also lead to activation of Ca^{2+} release as well. The Ca^{2+} released into the submembrane space leads to activation of K^+-activated Ca^{2+} channels (BK channels) in the plasmalemma (PL) (also known as STOCs) and hyperpolarization. Hyperpolarization decreases Ca^{2+} gating through the L-type Ca^{2+} channel and a decrease in global cell Ca^{2+}. PKG may also have direct effects to activate the BK channel and inhibit the Ca^{2+} channel.

PKG in increasing STOCs through phospholamban phosphorylation. A study by Makhoulf and colleagues also points to a cGMP, but PKG-independent role, in Ca^{2+} release (Murthy and Makhoulf, 1998a). Since cGMP interacts with multiple receptor proteins in the cell (e.g., cyclic nucleotide-regulated ion channels, cyclic nucleotide-regulated phosphodiesterases), it is conceivable that PKG-independent Ca^{2+} release is a component of the NO–cGMP signaling mechanisms in VSMC.

Alternatively, it is possible that IP_3 receptor function has little role in STOC generation in certain cells. Ryanodine-sensitive Ca^{2+} channels are clustered in the superficial SR of smooth muscle cells, raising the possibility that only ryanodine-sensitive Ca^{2+} release is critical for STOCs. Both PKA and PKG are known to catalyze phosphorylation of the ryanodine-sensitive Ca^{2+} channel *in vitro* (Suko *et al.,* 1993; Strand *et al.,* 1993; Mayrleitner *et al.,* 1995). There are no studies at this time, however, demonstrating a PKG-dependent phosphorylation of this channel in the intact smooth muscle cell. Therefore, at the present time, it is unclear whether phosphorylation of Ca^{2+} release channels in smooth muscle SR decreases intracellular Ca^{2+} levels through STOCs or through the inhibition of Ca^{2+} release from the SR. Perhaps both mechanisms operate in smooth muscle cells.

Inhibition of IP_3 Formation

There is a great deal of interest in the role of the NO–cGMP pathway in regulating phospholipase C and the generation of the second messengers IP_3 and diacylglycerol (DAG). In particular, because these second messengers mediate signal transduction in a variety of cells and tissues, demonstration of such regulation would provide a more widespread role for NO–cGMP signaling in regulating cell function. However, investigators have encountered some difficulty actually proving that this occurs. On the one hand, there are several literature citations showing that cGMP and PKG inhibit agonist-evoked increases in IP_3 formation (Rapoport, 1986; Takai *et al.,* 1981; Hirata *et al.,* 1990; Ruth *et al.,* 1993). Inhibition of IP_3 formation would surely provide a mechanism for the inhibition of agonist-evoked contraction of smooth muscle. On the other hand, the precise mechanism by which cGMP and PKG activation inhibit IP_3 or DAG formation is obscure.

Hofmann and co-workers (Ruth *et al.,* 1993; Pfeifer *et al.,* 1995) described the effects of PKG overexpression in Chinese hamster ovary (CHO) cells (normally PKG-deficient cells) on agonist-induced IP_3 formation. PKG activation was particularly effective in blocking thrombin-induced increases in IP_3 and intracellular Ca^{2+} release in CHO cells. These investigators proposed that PKG-mediated phosphorylation of the $G_{i\alpha}$ subunit in CHO cells was responsible for the inhibition of IP_3 formation (Pfeifer *et al.,* 1995). Despite the attractiveness of this hypothesis, PKG-dependent phosphorylation of $G_{i\alpha}$ *in vitro* was only observed using unusually high concentrations of kinase and substrate, and intact cell phosphorylation was reported to be relatively low. It is known that protein kinase C (PKC) catalyzes phosphorylation of $G_{i\alpha}$ quite effectively *in vitro* and in the intact cell (Katada *et al.,* 1985), suggesting that the effects of PKG observed on $G_{i\alpha}$ phosphorylation are in reality PKC-like effects when the PKG is overexpressed.

An alternate explanation for PKG-mediated inhibition of IP_3 formation was proposed by Wong *et al.* (1998). In contrast to $G_{i\alpha}$, it is the thromboxane receptor from human platelets that is stoichiometrically phosphorylated using nanomolar concentrations of PKG *in vitro,* or 8-Br-cGMP in the intact cell. Peptide map analysis indicates that the phosphorylation site is within the cytoplasmic tail where G proteins interact with the serpentine receptor. In human platelets, G_q is the G protein that mediates thromboxane-induced IP_3 formation (Benka *et al.,* 1995). Thus, these data suggest that inhibition of G-protein signaling is indeed responsible for the effects of PKG in platelets, but the mechanism proposed in this case is receptor phosphorylation, not G-protein phosphorylation.

An intriguing study from Murthy and Makhlouf (1998b) suggests that phosphorylation and inhibition of phospholipase A_2 ($cPLA_2$) by PKG inhibits arachidonate- and IP_3-dependent Ca^{2+} mobilization in smooth muscle. From these studies, it is reasonable to conclude, as illustrated in Fig. 6, that multiple mechanisms may be responsible for the inhibition of agonist-induced Ca^{2+} release in smooth muscle cells.

Effects of PKG on Ca^{2+} Channels

There have been numerous studies demonstrating cyclic nucleotide and protein kinase-mediated regulation of the activity of the L-type Ca^{2+} channel in cardiac myocytes (Wahler *et al.,* 1990; Mery *et al.,* 1991; Sumii and Sperelakis, 1995; Rotman *et al.,* 1995). In many cases, these effects may be related to K_{Ca} channel activation and subsequent hyperpolarization as described earlier. However, direct effects on myocyte L-type Ca^{2+} channels have also been reported. Most of these studies were conducted using cGMP analogs in either a whole-cell or an excised patch configuration. In some cases, purified PKG and catalytic subunit of PKA were applied to patches, and I_{Ca} currents were found to be affected (Sumii and Sperelakis, 1995).

If the physiological studies described above suggest an important role for PKG in regulating L-type Ca^{2+} channels, then there are no complementary biochemical studies outlining the mechanism by which PKG-dependent phosphorylation alters channel function. PKA has long been know to catalyze the phosphorylation of specific residues on the α subunit (pore-forming subunit) of the L-type Ca^{2+} channel (see e.g., Rotman *et al.,* 1995). In contrast, there have been no reports of specific PKG-mediated phosphorylation, although PKG catalyzes phosphorylation of sites phosphorylated by PKA *in vitro* (Jahn *et al.,* 1988). This does not necessarily suggest that PKG is incapable of specifically catalyzing the phosphorylation of channel subunits, but rather it alludes to the complexity of the Ca^{2+} channel biology in general. Adding to the complexity of the variability of L-type channel subunit expression in cells is the complexity

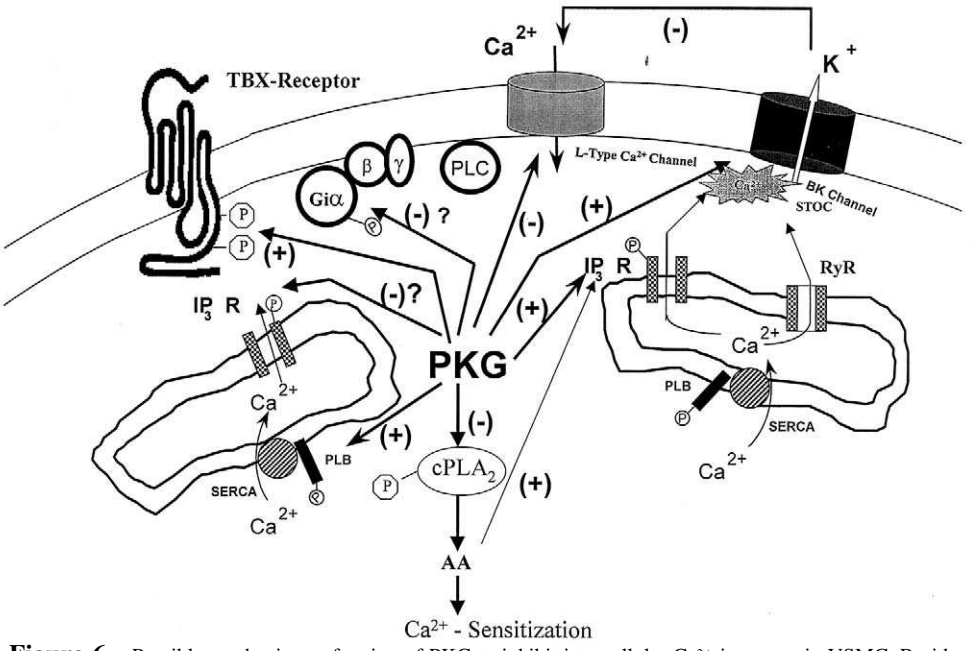


Figure 6 Possible mechanisms of action of PKG to inhibit intracellular Ca^{2+} increases in VSMC. Besides the generation of STOCs, PKG may have other actions to decrease intracellular Ca^{2+}. PKG inhibits the generation of IP_3 by inhibiting receptor coupling to heterotrimeric G proteins. Depicted in the figure are two potential mechanisms: first, the phosphorylation of serpentine-receptor intracellular domains involved in coupling to Gα subunits, and second, the phosphorylation of the Gα subunit itself. In either situation, the activation of phospholipase C (PLC) is inhibited, leading to decreases in the level of intracellular IP_3. Other data suggest that the inhibition of cytosolic phospholipase A_2 ($cPLA_2$) by PKG-dependent phosphorylation may reduce arachidonic acid (AA) levels. Decreased AA may reduce Ca^{2+} release from the SR and decrease contractile protein sensitivity to intracellular Ca^{2+}.

of the cellular background in which the channel functions. In a remarkable study by Zong *et al.* (1995), neither PKA nor the protein kinase inhibitor peptide (PKI) affected L-type channel function when the subunits were expressed alone or in combination in CHO cells or human embryonic kidney (HEK) cells. This was in stark contrast to the effects of PKA in cardiomyocytes. Yet the selective PKA inhibitor H-89 attenuated channel function in CHO and HEK cells. Thus, the L-type Ca^{2+} channel is likely not to be modulated by cyclic nucleotide-dependent kinases in nonexcitable cells as it is in myocytes. The inhibitor studies also point out the likelihood that H-89 has other effects besides blocking PKA activity.

An interesting series of experiments by Keef and coworkers (Ishikawa *et al.,* 1993; Ruiz-Velasco *et al.,* 1998) suggests that cyclic nucleotides may have both opposing and similar actions on Ca^{2+} currents in smooth muscle cells, owing to the phenomenon of cross-activation. Low concentrations of cAMP analogs activate, whereas cGMP analogs inhibit, the I_{Ca} in VSMC. However, higher concentrations of cAMP inhibit Ca^{2+} channels through a mechanism that is blocked by the phosphorothioate PKG inhibitors. Therefore, cross-activation of cyclic nucleotide-dependent protein kinases appears to occur in at least a perforated patch-clamp model of smooth muscle cells.

The phenomenon of cross-activation described previously refers to the capacity of cAMP to activate PKG and cGMP to activate PKA. Cross-activation, first suggested by Corbin and co-workers (Francis *et al.,* 1988; Jiang *et al.,* 1992), was demonstrated to function in isolated rat aortic smooth muscle cells deficient in PKG expression (Lincoln *et al.,* 1990). As suggested from the studies of Keef and coworkers, physiologically relevant concentrations of cAMP appear to be capable of activating PKG (Ruiz-Velasco *et al.,* 1998). Similar conclusions have been obtained by a number of laboratories examining specific physiological events (Murthy *et al.,* 1997; Eckly-Michel *et al.,* 1997; Kawada *et al.,* 1997). In contrast to these studies, cAMP had no effect to either lower Ca^{2+} or relax aortic tissue in the PKG-I null mice, suggesting that cross-activation does not occur under physiological conditions (Pfeifer *et al.,* 1998). However, it should be noted that the usual compensatory mechanisms that occur developmentally in response to gene ablation could occur, thereby masking an important physiological regulatory mechanism.

It is also important to note developmental differences in the regulation of Ca^{2+} channel activity by PKG. Kumar *et al.* (1997) have shown that in neonatal rabbit ventricular myocytes, cGMP activates rather than inhibits I_{Ca}. This effect appears to be mediated by PKG, which is highly expressed in neonatal myocytes compared with adult myocytes. In the adult myocyte, cGMP had little effect on I_{Ca}, neither increasing nor decreasing channel activity. There-

fore, it is important to consider the age of animals in conducting these types of studies, and this may be particularly relevant for cardiac myocyte work that relies heavily on the use of neonatal cells.

Perhaps the most novel mechanism proposed for the regulation of L-type Ca^{2+} channels by cGMP was based on a series of studies in the amphibian heart (Hartzell and Fischmeister, 1986; Mery *et al.,* 1993). In these studies, elevations in the level of cGMP inhibited the β-adrenergic Ca^{2+} current, whereas 8-Br-cGMP had no effect. Paradoxically, inhibitors of cyclic nucleotide phosphodiesterases (PDE) blocked the effects of cGMP rather than enhanced them. These investigators suggested that cGMP, by virtue of activation of a Type II PDE (cGMP-activated PDE), decreased cAMP levels, leading to a decrease in Ca^{2+} current. The importance of these findings is that cyclic nucleotides, and cGMP in particular, are capable of signaling cells through multiple receptor proteins (Lincoln and Cornwell, 1993). In fact, it has been shown that the complex expression of PDEs both activated by cGMP and inhibited by cGMP contribute to cGMP signal transduction in heart (Fischmeister and Hartzell, 1990) and other tissues (Somlyo and Somlyo, 1994). These studies also point out the caution that must be used in interpreting results based solely on the use of protein kinase inhibitors or PDE inhibitors in intact cells and tissues.

Role of PKG in the Regulation of Ca^{2+} Sensitization

An emerging area of research is the phenomenon known as ''Ca^{2+} sensitization'' of contraction. Simply stated, Ca^{2+} sensitization is the capacity of contractile force to be developed in smooth muscle in the presence of diminishing concentrations of intracellular Ca^{2+}, or in the absence of increases in intracellular Ca^{2+} (reviewed in Somlyo and Somlyo, 1994). The technology for quantitating the concentrations of intracellular Ca^{2+} levels has markedly improved over the 1990s, as have the techniques for permeablizing smooth muscle tissue. As a result, investigators have consistently observed that agonist-induced contraction can be achieved in response to smaller increases in intracellular Ca^{2+} in comparison with depolarization-induced contraction. Furthermore, it is now clear that contractile activity, especially the generation of force without cross-bridge cycling, is achieved at near baseline cytosolic Ca^{2+} concentrations. These pivotal findings have led to the notion that low Ca^{2+}-requiring mechanisms exist to increase contractile force. There is now solid evidence that PKG plays an important role in regulating Ca^{2+} sensitization.

Regulation of MLC Phosphatase

With respect to MLC phosphorylation, the incorporation of phosphate into serine-19 in the regulatory light chain of myosin is a result of the balance of activity between MLC kinase and MLC phosphatase. MLC kinase activity is governed principally by its activation by Ca^{2+}–calmodulin; little evidence exists that PKG actually catalyzes the phosphorylation of MLC kinase in the intact cell to inhibit it. MLC phosphatase activity is governed principally by the binding of the catalytic subunit of the enzyme to smooth muscle myosin via a myosin-binding subunit (MBS). MBS is considered an anchoring protein for the phosphatase catalytic subunit, functioning both to bring the substrate (i.e., MLC) closer to the enzyme as well as to activate the phosphatase to dephosphorylate the substrate (see Hartshorne, 1998). Agonists decrease the activity of MLC phosphatase, possibly through a PKC-dependent phosphorylation of MBS (Masuom *et al.,* 1994). Although the detailed mechanism is not clear at this point, evidence suggests that the small GTP-binding protein Rho is involved in MLC phosphatase inhibition as well. It has been proposed that Rho activates a serine/threonine kinase, the *rho* kinase, to catalyze phosphorylation of MBS, resulting in the inhibition of the catalytic subunit (Gong *et al.,* 1996; Uehata *et al.,* 1997).

Other studies have shown that 8-Br-cGMP activates MLC phosphatase in arterial smooth muscle preparations (Lee *et al.,* 1997; Wu *et al.,* 1996). In these cases, PKG causes a Ca^{2+} desensitization of contraction, although the mechanism is unclear. It has also been speculated that cGMP and PKG could interfere with the *rho*-dependent activation of *rho* kinase, perhaps through the inhibition of the translocation of *rho* (or the *rho–rho* kinase complex) to the thick filaments (Somlyo and Somlyo, 1998). Related to these studies is the work of Surks *et al.* (1999) dealing with the potential targeting of PKG-I to MLC phosphatase. Using the yeast two-hybrid screen, these investigators have found that MBS is a major PKG-I anchoring protein in smooth muscle. PKG-dependent phosphorylation of MBS as a consequence of the association of the kinase with the subunit results in an activation of MLC phosphatase *in vitro.*

A different mechanism has been proposed by Somlyo and co-workers (Wu *et al.,* 1999). These investigators have shown that 8-Br-cGMP catalyzes the phosphorylation of the protein telokin in rabbit illeal smooth muscle. Telokin is a particularly abundant protein in this tissue and accelerates MLC dephosphorylation in permeablized strips. Thus, telokin phosphorylation by PKG represents a novel and potentially important mechanism for regulating Ca^{2+} desensitization of contraction. Thus, there may be multiple mechanisms by which PKG regulates Ca^{2+} sensitization of contraction of smooth muscle.

Thin Filament Regulation by PKG

From the discussion above, it is evident that phosphorylation of MLC has been a major focus of Ca^{2+} sensitization mechanisms in smooth muscle. There are also data demonstrating that Ca^{2+}-independent contraction of smooth muscle occurs in the absence of MLC phosphorylation, suggesting a thin filament-dependent regulation of smooth muscle contraction. Proteins that regulate smooth muscle contractility in this fashion include the thin filament-binding proteins caldesmon and calponin. Phosphorylation of both proteins are known to occur in the intact cell, but no role of the NO–cGMP–PKG signaling pathway in thin filament regulation has been established.

VASP AND HSP-20

Of the growing number of proteins whose phosphorylation is increased in response to PKG activation in the intact cell, and perhaps the best characterized, is the actin-binding protein VASP (vasodilator-stimulated phosphoprotein). In addition to actin, VASP binds to cytoskeletal proteins such as profillin and vinculin at the focal adhesion junction of cells. The role of VASP in platelet function, focal adhesion formation, and cell motility has been reviewed by Walter and colleagues (Eigenthaler *et al.,* 1998). Although VASP is expressed in practically every cell type examined, the physiological role of VASP may be best characterized in platelets. Phosphorylation of VASP by PKA and PKG on different sites results in the inhibition of integrin α_{IIB} activation (Horstrup *et al.,* 1994; Eigenthaler and Shattil, 1996). This may be an important mechanism involved in the inhibition of platelet aggregation and adhesion, known to be affected by NO donor drugs. A potentially interesting clinical model of the role of PKG and VASP is human chronic myelocytic leukemia (CML) where PKG expression in platelets is virtually absent (Eigenthaler *et al.,* 1993). Patients with CML demonstrate impaired NO-dependent signal transduction in platelets.

The role of PKG in the regulation of cellular adhesion, migration, and motility is still an area ripe for investigation. There have been several studies published regarding the role of NO and the cGMP signal transduction system in leukocyte migration and degranulation (Pryzwansky *et al.,* 1995a; Wyatt *et al.,* 1993; Elferink and de Koster, 1993; Elferink and Van Uffelen, 1996), but few studies have been directed toward understanding the biochemical mechanisms involving PKG in these physiological processes. In one study on the regulation of focal adhesion formation, activation of PKG was required for matrix protein-dependent focal adhesion disassembly in endothelial and vascular smooth muscle cells (Murphy-Ullrich *et al.,* 1996). Given the potential importance of the cGMP–PKG signaling pathway in the regulation of VSMC growth, migration, and phenotypic expression (see section on cyclic GMP and VSMC phenotypic modulation), it is likely that the elucidation of these processes will increase our understanding of the role of this pathway in vascular diseases.

As mentioned earlier, many of the proteins described as physiological substrates for PKG are uncharacterized. Some of these are relatively small molecular weight entities (i.e., 20–30 kDa) and have been known for some time to be potential substrates for PKG (Rapoport *et al.,* 1982; Li *et al.,* 1996). One such protein has been identified as heat-shock protein 20 or HSP-20 (Beall *et al.,* 1997). Although the precise role of HSP-20 in smooth muscle contraction is unknown, HSP-20 phosphorylation may play a critical role in NO–cGMP-dependent relaxation. HSP-20 is phosphorylated in response to PKA- or PKG-dependent activation in bovine trachealis and carotid artery. Moreover, HSP-20 phosphorylation does not occur in human umbilical artery smooth muscle, a tissue which is insensitive to relaxation by cyclic nucleotides (Bergh *et al.,* 1995; Brophy *et al.,* 1997). These results indicate that HSP-20 may play a role in the regulation of contractile activity in smooth muscle, such as that of regulating myosin phosphorylation or perhaps thin filament function. Clearly more research is needed in this area.

cGMP and PKG-Independent Mechanisms of Relaxation

As is often the case with emerging areas of research, the more scrutiny a topic receives, the more issues arise which at the very least prove to be ''exceptions to the rule.'' In the case of cGMP and PKG-dependent vasodilation in response to NO, there are now reports of NO-dependent and cGMP-independent mechanisms of vasodilation. On the one hand, such reports should hardly be unexpected, as the number of reported cGMP-independent actions of NO seems to be growing faster than those events mediated by cGMP. On the other hand, the importance of cGMP and PKG in vasorelaxation must surely be appreciated based on the published effects of the sGC inhibitor, ODQ (1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one), to block NO-dependent relaxation (Moro *et al.,* 1996) and the effects observed in the PKG-null mice (Pfeifer *et al.,* 1998). The most plausible statement that can be made at this time is that there exist some cGMP-independent actions of NO with regard to cellular function; nevertheless, the exquisite sensitivity of sGC to stimulation by NO indicates that the cGMP–PKG pathway remains a major signaling mechanism for NO in cells.

With regard to cGMP-independent vasorelaxation, Cohen and co-workers (Weisbrod *et al.,* 1998) have identified a cGMP-independent decrease in intracellular Ca^{2+} levels associated with relaxation in the rabbit aorta. This effect may be due to the activation of K_{Ca} channels by NO reported earlier by this laboratory (Bolotina *et al.,* 1994). In a similar vein, Roman and colleagues have demonstrated that NO-dependent vasodilation in renal arteries is not blocked by sGC or PKG inhibitors, but is sensitive to inhibition of P-450 epoxygenase (Sun *et al.,* 1998; Alonso-Galicia *et al.,* 1998). Despite the reservations concerning interpretation of data using inhibitors alone, these results suggest that the inhibition of 20-HETE by NO, in part at least, mediates the vasodilatory effects of NO in these vessels. The attractiveness of this hypothesis lies in the fact that the effects of NO are due to direct binding to the heme moiety of the P-450 epoxygenase. As alluded to earlier, heme-containing enzymes and proteins act as ''receptors'' for NO in a manner analogous to sGC. Because the affinity of sGC for NO is high (nanomolar), and it has yet to be determined whether the affinity of all the other heme-containing proteins for NO is equally as high, it may be premature to speculate that all heme-containing proteins are receptors for NO. Nevertheless, it is an attractive idea that the diversity of NO signaling may be based on the diversity of heme-containing receptor proteins for NO.

Other possible cGMP and PKG-independent actions of NO in vascular and other tissue is based on *S*-nitrosothiol formation. S-Nitrosation has been known since the 1970s from the earlier studies involving NO-donor drug action (Eiserich *et al.,* 1994). There is a great deal of interest in the fact that NO may be both stored as an *S*-nitrosothiol in cells and form *S*-nitrosothiols with proteins to regulate their function (Davisson *et al.,* 1996). However, direct measurement of physiologically important *S*-nitrosothiols in response to NO production is lacking in most studies. Furthermore, because NO activates signaling pathways at nanomolar concentrations, one might not expect to be able to measure generalized S-nitrosation of proteins in cells.

Despite the lack of direct evidence for *S*-nitrosothiol signaling for NO, indirect studies suggest that such specific and sensitive effects of NO occur in response to physiological levels of NO. In cultured VSMC, physiological concentrations of NO induce the expression of the mRNA for glutamylcysteine synthetase (GCS) (Moellering *et al.,* 1998). Importantly, these cells lack PKG, and the effects of NO were neither blocked by ODQ nor mimicked by 8-Br-cGMP. Although the mechanism of the effect of NO is unknown at this point, it is conceivable that nitrosation of some specific cellular component or nitration by NO-derived reactive nitrogen species leads to increased rates of transcription of the GCS gene.

Just how widespread are cGMP/PKG-independent effects of NO in cells? From the pathophysiological point of view, it is clear that cytokine-evoked NO production through iNOS expression could regulate protein function through several mechanisms such as the generation of *S*-nitrosothiols or peroxynitrite. Are physiologically relevant levels of NO capable of regulating cellular function through mechanisms similar to these? To begin to address this question, it may be beneficial to recall one of the basic tenets of intracellular signaling, namely, amplification. The attractive feature of the second messenger hypothesis proposed in the 1950s is that it is amplification of extracellular signals through intracellular enzymatic catalysis that allows minute concentrations of such signals to alter cell function in a specific and efficient manner. sGC activation by NO meets the criterion for amplification; unfortunately, there are only a few other instances in the NO signaling literature which do. In some reports, the levels of NO used in cell signaling experiments are not evaluated for potential physiological significance. Furthermore, the effects of high concentrations of NO on cells may be due to the mere stoichiometric interaction of NO with cellular effector molecules that are not intracellular catalysts. The amplification of signals necessary for specific, reversible, and efficient alterations in cellular function is therefore absent. From a pathophysiological point of view, stoichiometric (as opposed to catalytic) effects of NO may well be significant. From the standpoint of establishing experimental protocols to study the physiological role of NO in cellular function, however, it is important for the experimentalist to approximate the physiologically relevant condition.

Studies Based on PKG Null Mice

Both forms of PKG have been genetically ablated through gene targeted disruption (Pfeifer *et al.,* 1996, 1998). For PKG-II, the phenotypes were more surprising, perhaps because less is known about the biological role of PKG-II. The major physiological disruptions in the PKG-II null mice were reported to be stunted growth and impaired intestinal secretion (Pfeifer *et al.,* 1996). Impaired intestinal secretion in response to *Escherichia coli* enterotoxin and guanylin is a predicted phenotype based on the findings of DeJonge and co-workers that type II PKG catalyzes the phosphorylation and activation of the Cl^- transporter, the cystic fibrosis transmembrane conductance regulator or CFTR, in the basal membranes of intestinal epithelial cells (Vaandrager *et al.,* 1997). However, the dwarfism produced in response to type II PKG deficiency was an unexpected phenotype. Subsequently, these authors demonstrated a high level of expression of PKG-II in chondrocytes in developing bone. Thus, PKG-II appears to play a central role in endochondral ossification at the growth plates.

In contrast to the unpredicted phenotypes of the PKG-II null mice, PKG-I null mice were shown to have ablated NO-dependent vasodilation in aortic VSMC (Pfeifer *et al.,* 1998). Additionally, conscious mice had elevated mean arterial blood pressure (MAP) and reduced vasodilation to NO. And finally, a most dramatic phenotype of the PKG-I null mice was impaired intestinal motility arising from the absence of NO- and cGMP-mediated intestinal smooth muscle relaxation. Overall, these phenotypic differences between PKG +/+ and PKG −/− were predictable based on the known role of the cGMP–PKG pathway in mediating smooth muscle relaxation, and based on the studies reporting deficient vasorelaxation in endothelial NOS (eNOS) (Huang *et al.,* 1995) and atrial natriuretic peptide (ANP) receptor (Lopez *et al.,* 1995) null animals.

There were some unusual findings, however, in the PKG-I null animals that warrant further study. In the first instance, half the animals died of unspecified causes between 35 and 40 days. The effects of PKG ablation on MAP seemed to be confined to animals that were under 32 days of age, because older animals (42 days) were normotensive. Furthermore, even the younger PKG-I null animals when anesthetized did not demonstrate differences in MAP from wild-type animals. It should be noted that even a 42-day-old animal is a juvenile, so that ablation of the PKG-I gene had an apparent lethal effect by a mechanism that was not addressed. And finally, the authors observed that adenosine A2 receptor agonists and cAMP analogs relaxed aortas from the PKG-I null mice in a normal fashion. Although the authors suggested this was evidence against cAMP-dependent cross-activation of PKG, it is more likely that compensatory mechanisms coupled with the lethality of the gene ablation were responsible for the effects of cAMP in the pathologically generated animal. As is always the case with embryonic stem cell mutants, developmental patterns shift to compensate for loss of gene products, and these compensatory mechanisms must be factored into the resultant phenotype.

The Uterus: A Paradox for the NO–cGMP Model for Vasorelaxation

During the early days of discovery in the NO–cGMP field, there were persistent reports that NO-donor drugs and cGMP analogs failed to produce smooth muscle relaxation. The scientific meetings where these findings were presented and heatedly debated made for wonderful entertainment, but the fact is that some smooth muscle tissues are indeed quite refractory to NO-dependent relaxation. The focal point of the controversy was the lack of relaxation of uterine smooth muscle to NO and cGMP.

An early and definitive study on the role of cGMP in mediating NO-dependent relaxation in nonvascular smooth muscle was done by Diamond (1983). In contrast to vascular smooth muscle and gastrointestinal smooth muscle, uterine smooth muscle was refractory to NO-dependent relaxation despite the fact that the drugs utilized elevated cGMP levels. The refractoriness of uterine smooth muscle was a particularly interesting finding, as Word *et al.* (1991) later demonstrated that cGMP decreased intracellular levels of Ca^{2+} and inhibited myosin light chain phosphorylation in human uterine smooth muscle cells.

Other studies have again raised the issue of NO-mediated uterine smooth muscle relaxation. Yallampalli and colleagues (1993, 1994) have published new studies suggesting that relaxation of the uterus by NO and cGMP analogs is particularly sensitive during pregnancy. At first glance, these findings are attractive because (i) they may relate the variable effects of NO and cGMP on relaxation to the hormonal status of the animal, and (ii) they may provide a biochemical mechanism underlying uterine quiescence during pregnancy. However, other laboratories (Bek *et al.,* 1998; Potvin and Varma, 1990; Word and Cornwell, 1998) have found just the opposite effects of pregnancy and hormonal status on uterine sensitivity to NO. In these later studies, uterine smooth muscle from pregnant animals was completely refractory to relaxation by NO, cGMP analogs, or natriuretic peptides when compared to tissue from nonpregnant animals. This was despite the capacity of the NO-donor drugs and the peptides to increase cGMP levels. A mechanism for the greater insensitivity of uterine tissue to relaxation by NO and cGMP from pregnant animals was proposed by Word and Cornwell (1998). These investigators found that progesterone inhibits, whereas estradiol increases, the expression of PKG in uterine smooth muscle cells. These studies were extended to and confirmed in human uterine smooth muscle cells (Cornwell and Word, 1994). Therefore, the role of NO and cGMP in the relaxation of uterine smooth muscle still remains unresolved. Most evidence, however, suggests that the NO–cGMP pathway does not mediate uterine smooth muscle relaxation. This of course begs the question: What is the role of the NO–cGMP pathway in uterine smooth muscle? A new role for cGMP and PKG in regulating smooth muscle cell phenotype is emerging (see section on cyclic GMP and VSMC phenotypic modulation). Because uterine smooth muscle undergoes dramatic phenotypic changes depending on the hormonal status of the animal, it is possible that the NO-signaling pathway plays a key role in regulating gene expression during phenotypic transitions in this tissue.

NO–Cyclic GMP Signaling in Vascular Disease

Vascular disorders including atherosclerosis, peripheral occlusive disease, restenosis following angioplasty and transplantation, and hypertension all fall under the umbrella of vascular disease. The single most significant underlying pathology associated with vascular disorders arising from injury is that the VSMC acquire a fibroproliferative phenotype *in vivo,* which leads to the ''remodeling'' of the vessel wall (see Ross, 1993, for a review). As a result, blood flow is usually reduced, with an accompanying increase in wall stress, platelet activation, and leukocyte extravasation. The physiological mechanisms regulating the ''switch'' that controls the modulation from the muscle phenotype to the fibroproliferative phenotype are unknown, but there is an emerging awareness of the importance of cGMP and PKG in controlling VSMC phenotype.

VSMC Phenotypic Modulation

It has been known since the 1980s that when certain VSMC are placed in culture and grown in medium supplemented with serum-derived growth factors, the cell phenotype changes from one expressing contractile proteins (the contractile phenotype) to one expressing extracellular matrix proteins or ECM (the synthetic phenotype) (Chamley-Campbell *et al.,* 1979, 1981; Hedin *et al.,* 1990; Rovner *et al.,* 1986). The same phenotypic changes occur in the medial layer VSMC in the vessel wall in response to various injury (Campbell and Campbell, 1985; Gown *et al.,* 1986; Majesky *et al.,* 1992; Mosse *et al.,* 1985). This is because the VSMC represent the primary ''wound-healing'' cells in the vessel wall, and they must be able to undergo a change in function from that of regulating the diameter of the lumen of the vessel to repairing the vessel when injured. In culture, as *in vivo* in response to injury, VSMC suppress the expression of the unnecessary contractile proteins and increase the production of ECM proteins such as collagen, fibronectin, osteopontin, and thrombospondin. A variety of growth factors and inflammatory mediators in the vessel wall including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and various cytokines such as IL-1β, tumor necrosis factor α (TNF-α), and γ-interferon (IFN-γ) are believed to promote phenotypic modulation to the synthetic state. It will be recognized that these are typical biological responses, that is, inflammatory responses, to injury.

Cyclic GMP and VSMC Phenotypic Modulation

NO and natriuretic peptides have been shown to inhibit VSMC proliferation both *in vitro* and *in vivo* (Johnson *et al.,*

1988; Abell *et al.,* 1989; Garg and Hassid, 1989a,b; Appel, 1990; Kariya *et al.,* 1989; Nakaki *et al.,* 1990a; McNamara *et al.,* 1993; Cayatte *et al.,* 1994; De Meyer *et al.,* 1995; van der Leyen *et al.,* 1995). With regard to the *in vivo* studies, it has not been clear whether these effects are due to direct actions on VSMC proliferation in the vessel wall or are due to indirect effects such as inhibiting platelet activation and leukocyte infiltration. Indeed, in a broad clinical trial examining the effects of NO-donor drugs on patients following percutaneous transmural coronary angioplasty (PTCA), there were no impressive protective effects of the drugs in preventing restenosis (Lablanche *et al.,* 1997).

There are also problems in interpreting the effects of NO donor drugs on VSMC proliferation *in vitro:* first, the majority of these studies had been performed on multiply passaged cells, usually of adult rodent origin, that were already in the synthetic phenotype and do not express high levels of PKG. Second, and related to this problem, only high micromolar concentrations (usually >100 μM) of NO-donor drugs such as SNP or SNAP were effective in inhibiting adult VSMC proliferation. These questions raise the issues of whether the effects of NO are mediated via the cGMP–PKG pathway and whether they are physiologically relevant. And finally, Hassid *et al.* (1994) have shown that the effects of NO on VSMC proliferation are dependent on the phenotype of the cell; thus, NO inhibits the proliferation of VSMC in the synthetic phenotype at concentrations described above, but lower concentrations of NO stimulate the proliferation of VSMC in the contractile phenotype.

Studies have begun to address the role of NO and cGMP in the regulation of VSMC phenotype and proliferation (Boerth *et al.,* 1997; Cornwell *et al.,* 1994b; Dey *et al.,* 1998; Lincoln *et al.,* 1998). Because rat aortic SMC that have been routinely passaged several times *in vitro* cease to express measurable quantities of PKG, an examination of the effects of PKG restoration to cells deficient in the enzyme were made possible. PKG-Iα, or the catalytically active domain of PKG-I (Boerth and Lincoln, 1994), caused the synthetic cells to resume the contractile morphology (Boerth *et al.,* 1997). As shown in Fig. 7, the PKG-expressing cells become spindle-shaped and grow in the ''hill and valley'' appearance typical of primary cultures of VSMC at confluence. In contrast, the PKG-deficient cells remain fibroblastic in appearance and grow in irregular arrays *in vitro.* Importantly, the expression of biochemical markers for the contractile phenotype (e.g., smooth muscle myosin heavy chain, calponin, desmin, α-actinin) are restored in only the PKG-expressing cells (Boerth *et al.,* 1997), whereas PKG suppresses biochemical markers for the synthetic phenotype (e.g., osteopontin and thrombospondin) (Dey *et al.,* 1998). Thus, it

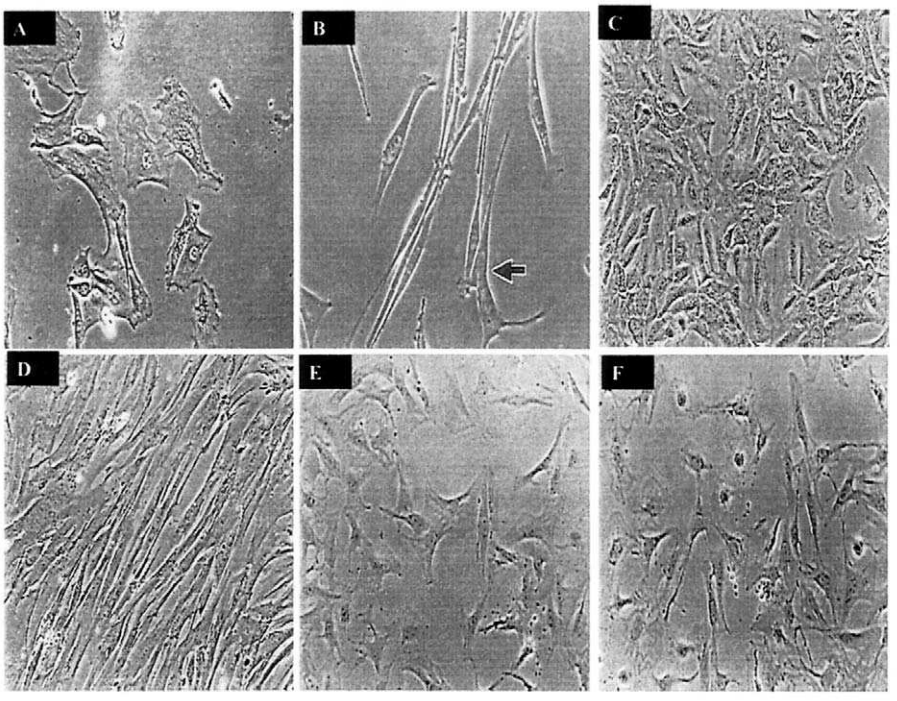


Figure 7 Effect of PKG expression on VSMC morphology. Rat aortic VSMC stably transfected with vector alone (A and C) or with vector containing the PKG catalytic domain (B) or the PKG-Iα holoenzyme (D) were photographed 96 hours after plating. In E and F, cells were treated with or without 1 μM forskolin to activate PKA in PKG-deficient cells. Note the contractile-like morphology displayed by cells expressing PKG or the catalytic domain of PKG compared to the synthetic morphology displayed by cells deficient in the expression of PKG. Note also the lack of effect of cAMP on cellular morphology. [From Lincoln *et al.* (1998). *Acta Physiol. Scand.* **164,** 507–515. Reprinted with permission from the Scandinavian Physiological Society.]

appears that at least one mechanism regulating phenotypic modulation involves the expression of PKG.

The mechanisms regulating PKG-I expression were discussed earlier. Of particular interest for its role in VSMC phenotypic modulation is the potential role of inflammation in suppressing PKG expression. It is well established that mechanical injury, or chronic injury due to oxidative stress and atherosclerosis, leads to an inflammatory response characterized by the activation of leukocytes and other inflammatory cells in the vessel wall (Libby and Galis, 1998, for a review). Hence, immunoreactive IL-1β, TNF-α, and IFN-γ have been shown to be present in vascular lesions. One of the major gene products known to be expressed in response to inflammatory cytokine action is iNOS (Beasley and Eldridge, 1994), and in fact iNOS is robustly expressed in immune cells and VSMC in atherosclerotic and restenotic lesions (Yan and Hansson, 1998). A temporal correlation following iNOS and PKG expression in swine coronary arteries following balloon catheter injury has been performed, demonstrating that iNOS expression increases prior to the suppression of PKG expression in the lesions (Anderson *et al.,* 1996). Additionally, NO-donor drugs and inflammatory cytokines suppress PKG expression in primary cultures of bovine aortic SMC (Soff *et al.,* 1997; Lincoln *et al.,* 1998). Together, these findings support the concept that persistent elevations in NO production suppress PKG expression. This has led to the proposal that one mechanism linking inflammation in response to injury to alteration in cell phenotype, leading to subsequent wound-healing activity and vessel wall remodeling, is the suppression of PKG expression (Soff *et al.,* 1997; Boerth *et al.,* 1997; Cornwell *et al.,* 1994b; Dey *et al.,* 1998; Lincoln *et al.,* 1998). It is predictable that one active area of future research will be which factors control PKG expression—both up and down—in the vessel wall.

Cyclic GMP and Cell Growth

GENE EXPRESSION

From the previous discussion, it is clear that a potentially important role exists for cGMP and PKG in the regulation of gene expression in vascular cells. The first suggestions that cGMP regulates gene expression came from studies in PC12 cells demonstrating that NO and cGMP enhanced Ca^{2+}-dependent regulation of the c-*fos* promoter (Peunova and Enlkolopov, 1993; Haby *et al.,* 1994). Subsequent to these findings, Pilz, Boss, and co-workers have established that transfection of cDNAs encoding PKG-Iβ into baby hamster kidney (BHK) cells lacking the kinase results in a cGMP-mediated activation of the c-*fos* promoter (Pilz *et al.,* 1995; Gudi *et al.,* 1996, 1997; Idriss *et al.,* 1999). The mechanism appears to be mediated by several sequence elements, including the serum response element (SRE), the AP-1 site, and the cAMP response element (CRE) (Gudi *et al.,* 1996; Idriss *et al.,* 1999). The latter element is of considerable interest because several pathways, namely, PKA, Ca^{2+}–calmodulin kinase II, and PKC, lead to activation of the CRE. Phosphorylation of the CREB protein by PKG *in vitro* has in fact been reported, but the effectiveness of PKG compared to PKA is low (Colbran *et al.,* 1992). It is therefore unlikely that CREB is a physiologically relevant target for PKG. It is more likely that PKG catalyzes the phosphorylation of a unique CRE-binding protein that interacts alone or in combination with CREB proteins.

An interesting role for translocation of PKG to the nucleus to regulate gene expression has been suggested. Pryzwansky *et al.* (1995b,c) first had reported the presence of PKG immunofluorescence in the nucleus of neutrophils and monocytes, but the mechanism by which PKG might be localized there was unknown. Studies by Gudi *et al.* (1997) using site-directed mutation of a putative IL-α like nuclear localization sequence (NLS), consisting of amino acids 404–411 in PKG-Iβ (KILKKRHI), prevented PKG from translocating into the nucleus of BHK cells. In the absence of nuclear translocation, PKG was unable to activate c-*fos* promoter activity. These studies suggest a role for PKG in nuclear protein phosphorylation comparable to the role of the catalytic subunit of PKA. In the latter case, however, translocation of the C subunit appears to be controlled by the protein kinase inhibitor protein which acts as a ''chaperon'' to export the C subunit from the nucleus (Harootunian *et al.,* 1993; Wen *et al.,* 1994). For PKG, the mechanisms responsible for PKG exporting the nucleus—if they exist—are unknown. More recently, the role of nuclear translocation of PKG to regulate gene transcription has been questioned (Collins and Uhler, 1999). In the latter study, the holoenzyme of PKG was not translocated to the nucleus on activation by cGMP, and the level of gene transcription using a transfected CRE promoter was exceedingly low compared with PKA. On the other hand, the active catalytic domain of PKG appeared to localize in both the cytoplasm and nucleus of cells due to its smaller size. Overall, these findings support the concept of Harootunian *et al.* (1993) and Wen *et al.* (1994) suggesting the need for a chaperon for PKG if, in fact, its translocation to the nucleus is required for the regulation of gene expression. The mechanisms of gene regulation by PKG will likely be an important issue for further study since import and export from the nucleus is neessary to establish the physiological importance of the translocation event.

GROWTH

Regulation of c-*fos* gene expression is obviously one potential mechanism wherein PKG may regulate growth and differentiation of cells. As discussed earlier, the role of PKG in cell proliferation is highly controversial. Moreover, as c-*fos* gene expression is almost invariably associated with increased cell growth and proliferation, the molecular studies on gene expression do not shed new light on the role of PKG in cell proliferation.

In multiply passaged rat aortic SMC lacking PKG, Cornwell *et al.* (1994b) reported that high concentrations of NO-donors and IL-1β (which induces iNOS in the cells) inhibited PDGF-induced proliferation. The mechanism was apparently related to the effect of cGMP to cross-activate

PKA. Cyclic AMP and PKA activation have long been known to inhibit VSMC proliferation (Fukumoto *et al.,* 1988; Jonzon *et al.,* 1985; Loesberg *et al.,* 1985; Morisaki *et al.,* 1988; Nakaki *et al.,* 1990b; Souness *et al.,* 1992; Southgate and Newby, 1990). In VSMC differentiated to the contractile phenotype by PKG, there were no substantial effects of cGMP on proliferation of the cells (Boerth *et al.,* 1997). These studies support those of Hassid and co-workers (1994), where treatment of primary cultures of adult rat aortic SMC still expressing PKG did not effect proliferation. On the other hand, the proliferation of neonatal aortic SMC that still express PKG but retain a synthetic phenotype was inhibited by low concentrations of NO and cGMP (Hassid *et al.,* 1994). In a study by Chiche *et al.* (1998), it was reported that when passaged pulmonary artery VSMC lacking PKG were transiently transfected with PKG using adenoviral constructs, NO and cGMP dramatically reduced proliferation. These authors concluded that PKG mediates the antiproliferative effects of NO and cGMP in VSMC. One possible explanation for the differences between the effects reported by Boerth *et al.* (1997) and Hassid *et al.* (1994), on the one hand, and Chiche *et al.* (1998), on the other, was the phenotype of the cells. The pulmonary arterial cells infected by adenoviral PKG appeared to be synthetic in phenotype; according to the work of Hassid *et al.* (1994), activation of the NO pathway and PKG would be predicted to inhibit proliferation of cells in this phenotype. The issue of the physiological role of NO, cGMP, and PKG in the regulation of VSMC proliferation is likely to remain controversial, owing to the differential effects of NO and cGMP in cells displaying different phenotypes, the lack of reliable inhibitors to affect PKG, and the ability of the cAMP and cGMP pathways to cross and converge at multiple sites.

MAP KINASES

One of the most widely studied pathways leading to altered growth and proliferation of cells is the mitogen-activated protein kinase (MAP kinase) pathway. There are numerous reviews regarding the components of this pathway, and these will not be recalled here. One thing that is clear from the mountain of evidence generated to date is that the activation of the MAP kinase pathway, and specifically the extracellular regulated kinase or ERK pathway, is necessary for c-*fos* gene expression (Karin, 1994). However, the only reported studies regarding the potential role of cGMP and PKG in regulating the MAP kinase pathway seem to suggest an inhibitory role for cGMP and PKG in regulating ERK activity (Yu *et al.,* 1997; Suhasini *et al.,* 1998). However, there are some possible explanations for these findings when the data are examined in light of the nuances regarding inhibitor studies and the overlapping substrate specificity noted for the cyclic nucleotide-dependent protein kinases.

In one of the first studies conducted to define the role of PKG in regulating the ERK pathway, Yu *et al.* (1997) examined the effects of the KT class of inhibitors on ERK activation in passaged VSMC. On the basis of the actions of these inhibitors, these authors concluded that NO and cGMP, through PKG activation, inhibit ERK activation. The mechanism was proposed to involve the phosphorylation of Raf-1 by PKG since purified PKG catalyzed phosphorylation of Raf-1 in an *in vitro* assay. A similar conclusion was reached by Suhasini *et al.* (1998) using the BHK cell system. These cells are devoid of PKG, so that stable transfection of the kinase into the cells may be used to specifically examine the effects of PKG on cell pathways. In this study, cGMP analogs inhibited ERK activation, and PKG inhibitors reversed these effects. Furthermore, Raf-1 was phosphorylated on serine-43 in the intact BHK cell in response to cGMP in the PKG-expressing cells only. Both papers suggested that a physiologically relevant role of PKG is the phosphorylation of Raf-1 on serine-43, resulting in the inhibition of Ras binding and ERK activation.

There are unanswered questions regarding these studies, however; in the first instance, Raf-1 is a physiologically relevant substrate for PKA, and numerous studies have shown that activation of the PKA pathway results in the phosphorylation of serine-43 on Raf-1 and the inhibition of ERK activation by this mechanism (Graves *et al.,* 1993; Wu *et al.,* 1993; Chuang *et al.,* 1994; Hafner *et al.,* 1994; Erhardt *et al.,* 1995; Kikuchi and Williams, 1996; Mischak *et al.,* 1996; Faure and Bourne, 1995; Yee and Worley, 1997). Furthermore, PKA, but not PKG, is a potent catalyst for the phosphorylation of serine-43 on Raf-1 *in vitro* (T. M. Lincoln and P. Komalavilas, 1998, unpublished). Because it is known that PKG will catalyze phosphorylation of known PKA substrates in cells under appropriate conditions (Lincoln and Cornwell, 1993; Lincoln, 1994; Lincoln and Corbin, 1997, 1983), it is possible that overexpression of PKG in BHK cells has resulted in the activation of this kinase to mimic the more physiologically important effects of PKA on Raf-1 phosphorylation. Because neither study examined the effects of agents that elevate cAMP or cAMP analogs on Raf-1 and ERK activation, it cannot be concluded that the results reported are specific, physiologically relevant effects of PKG activation.

It is also known that phenotypically contractile smooth muscle cells express abundant ERK that is tonically in a relatively activated state (Khalil and Morgan, 1993; Adam *et al.,* 1995; Dessy *et al.,* 1998; Mii *et al.,* 1996; Menice *et al.,* 1997). The role of ERK in contractile smooth muscle function is a subject of much investigation (Adam *et al.,* 1995; Dessy *et al.,* 1998; Gorenne *et al.,* 1998), but it is clearly activated during contraction and not associated with proliferation. In contractile VSMC that express PKG, ERK activity is relatively high compared with synthetic VSMC that are deficient in PKG (Fig. 8). It would appear, therefore, that like the effects of cGMP on proliferation, the effects of NO–cGMP on ERK activity may depend very much on the phenotype of the VSMC. It is likely that in phenotypically contractile VSMC, ERK activity is relatively high as a result of PKG expression. ERK activation by PKG provides a plausible mechanism for c-*fos* promoter activation by PKG. Because c-*fos* expression is one hallmark of increased proliferative capacity of cells, the growth-promoting effects of

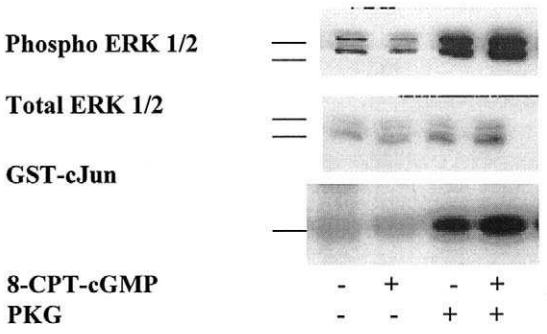


Figure 8 Effects of PKG expression on MAP kinase activity in cultured VSMC. Rat aortic VSMC stably transfected with vector alone (designated by PKG −) or with vector containing PKG-Iα holoenzyme (designated by PKG +) were treated with and without 100 μ*M* 8-chlorophenylthio-cGMP (8-CPT-cGMP) for 15 min. The activation of ERK was assessed by immunoblotting for phospho-ERK (top), whereas the activation of c-Jun N-terminal kinase (JNK) was determined by the phosphorylation of GST–c-Jun protein in the JNK immunoprecipitated complex (bottom). Total ERK was determined using nonselective antibodies for phospho- and dephospho-ERK (middle). Note that ERK ½ and JNK activities are significantly increased in PKG-expressing cells and that 8-CPT-cGMP increases ERK and JNK activation.

PKG activation (Hassid *et al.,* 1994; Sciorati *et al.,* 1997) might well be explained by increased ERK activation and c-*fos* expression in contractile VSMC. Only in the synthetic phenotype expressing low levels of PKG would high concentrations of NO and cGMP inhibit ERK through cross-activation of PKA and phosphorylation of Raf-1 on serine-43. This is clearly a controversial area; but the point to be made is that it is critical to include those important controls that would address the issues of overlapping substrate specificity of kinases and the lack of specificity for activators and inhibitors of the kinases.

Summary and Perspectives

In this chapter we have attempted to achieve two goals: first, to give a synopsis of the role of the NO–cGMP signaling pathway in vascular smooth muscle function, with particular emphasis on the role of PKG, and second, to attempt to highlight some of the pitfalls and controversies surrounding much of the research in this actively growing area. As suggested in the introduction, the cGMP–PKG field is a relatively old one, and there has been much information published concerning the biochemistry and molecular biology of the pathway in general. A knowledge of the key issues surrounding PKG function in cells in order to more precisely interpret the results of the more current literature is important and was a goal of this chapter. With the role of NO assuming greater medical importance, newer studies concerning the role of cGMP–PKG signal transduction pathways are being added to the body of knowledge almost daily. These newer studies need to be interpreted carefully in light of not only the difficulties with using inhibitors and activators of the pathway, but also the cross talk observed with cyclic nucleotide-dependent protein kinase pathways and the ease with which cyclic nucleotide-dependent protein kinases can catalyze phosphorylation of each other's substrates. Our only regret is that we could not cover more of the emerging roles of this fascinating signal transduction system. Such roles as the regulation of renin secretion (Gambaryan *et al.,* 1998), apoptosis (Pollman *et al.,* 1996), and brain function (Arancio *et al.,* 1995) by PKG are likely to be significant areas for basic and clinical research in the coming years.

With these concepts in mind, it is clear that the cGMP–PKG signal transduction pathway will assume major importance for understanding vascular function in physiological and pathophysiological settings. Drug development will almost surely be targeted toward regulating this pathway, as the ground has already been successfully broken with the appearance of sildenafil (Viagra) on the scene (Boolell *et al.,* 1996). Not to diminish in any way the positive impact this drug has had on the quality of human life, perhaps even more important pharmacological agents aimed at regulating the NO pathway, natriuretic peptide pathways, and the intracellular cGMP–PKG pathway in vascular disorders will be seen in the future.

References

Abell, T. J., Richards, A. M., Ikram, H., Espiner, E. A., and Yandle, T. (1989). Atrial natriuretic factor inhibits proliferation of vascular smooth muscle cells stimulated by platelet-derived growth factor. *Biochem. Biophys. Res. Commun.* **160,** 1392–1396.

Adam, L. P., Franklin, M. T., Raff, G. J., and Hathaway, D. R. (1995). Activation of mitogen-activated protein kinase in porcine carotid arteries. *Circ. Res.* **76,** 183–190.

Alioua, A., Huggins, J. P., and Rousseau, E. (1995). PKG-Iα phosphorylates the α-subunit and upregulates reconstituted GK_{Ca} channels from tracheal smooth muscle cells. *Am. J. Physiol.* **268,** L1057–L1063.

Alioua, A., Tanaka, Y., Wallner, M., Hofmann, F., Ruth, P. I., Meera, P., and Toro, L. (1998). The large conductance, voltage-dependent, and calcium sensitive K^+ channel, Hslo, is a target of cGMP-dependent protein kinase phosphorylation in vivo. *J. Biol. Chem.* **273,** 32950–32956.

Alonso-Galicia, M., Sun, C. W., Falck, J. R., Harder, D. R., and Roman, R. J. (1998). Contribution of 20-HETE to the vasodilator actions of nitric oxide in renal arteries. *Am. J. Physiol.* **275,** F370–F378.

Anderson, P. G., Williams, E. L., Cornwell, T. L., and Lincoln, T. M. (1996). *J. Vasc. Res.* **33**(Suppl. 1), 12.

Andriantsitohaina, R., LaGaud, G. J.-L., Andre, A., Muller, B., and Stoclet, J.-C. (1995). Effects of cGMP on calcium handling in ATP-stimulated rat resistance arteries. *Am. J. Physiol.* **268,** H1223–H1231.

Appel, R. G. (1990). Mechanism of atrial natriuretic factor-induced inhibition of rat mesangial cell mitogenesis. *Am. J. Physiol.* **259,** E312–E318.

Arancio, O., Kandel, E. R., and Hawkins, R. D. (1995). Activity-dependent long-term enhancement of transmitter release by presynaptic 3',5'-cyclic GMP in cultured hippocampal neurons. *Nature* **376,** 74–80.

Archer, S. L., Huang, J. M. C., Hampl, V., Nelson, D. P., Shultz, P. J., and Weir, E. K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 7583–7587.

Beall, A. C., Kato, K., Goldenring, J. R., Rasmussen, H., and Brophy, C. M. (1997). Cyclic nucleotide-dependent vasorelaxation is associated with the phosphorylation of a small heat shock-related protein. *J. Biol. Chem.* **272,** 11283–11287.

Beasley, D., and Eldridge, M. (1994). Interleukin 1β and tumor necrosis factor α synergistically induce nitric oxide synthase in rat vascular smooth muscle cells. *Am. J. Physiol.* **266,** R1197–R1203.

Bek, T., Ottesen, B., and Fahrenkrug, J. (1988). The effect of galanin, CGRP and ANP on spontaneous smooth muscle activity of rat uterus. *Peptides* **9,** 497–500.

Benka, M. L., Lee, M., Wang, G.-R., Buckman, S., Burlacu, A., Cole, L., DePina, A., Dias, P., Granger, A., Grant, B., Hayward-Lester, A., Karki, S., Mann, S., Marcu, O., Nussenzweig, A., Piepenhagen, P., Raje, M., Roegiers, F., Rybak, S., Salic, A., Smith-Hall, J., Waters, J., Yamamoto, N., Yanowitz, J., Yeow, K., Busa, W. B., and Mendelsohn, M. E. (1995). The thrombin receptor in human platelets is coupled to a GTP binding protein of the $G\alpha_q$ family. *FEBS Lett.* **363,** 49–52.

Bergh, C. M., Brophy, C. M., Dransfield, D. T., Lincoln, T. M., Goldenring, J. R., and Rasmussen, H. (1995). Impaired cyclic nucleotide-dependent vasorelaxation in human umbilical artery smooth muscle. *Am. J. Physiol.* **268,** H202–H212.

Bird, G. S. J., Burgess, G. M., and Putney, J. W. (1993). Sulfhydryl reagents and cAMP-dependent kinase increase the sensitivity of the inositol 1,4,5-trisphosphate receptor in hepatocytes. *J. Biol. Chem.* **268,** 17917–17923.

Boerth, N. J., and Lincoln, T. M. (1994). Expression of the catalytic domain of cyclic GMP-dependent protein kinase in a baculovirus system. *FEBS Lett.* **342,** 255–260.

Boerth, N. J., Dey, N., Cornwell, T. L., and Lincoln, T. M. (1997). cGMP-dependent protein kinase regulates vascular smooth muscle cell phenotype. *J. Vasc. Res.* **34,** 245–259.

Bolotina V. M., Najibi S., Palacino J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-activated potassium channels in vascular smooth muscle. *Nature* **368,** 850–853.

Boolell, M., Gepi-Attee, S., Gingell, J. C., and Allen, M. J. (1996). Sildenafil, a novel effective oral therapy for male erectile dysfunction. *Br. J. Urol.* **78,** 257–261.

Brophy, C. M., Beall, A., Lamb, S., Dickinson, M., and Ware, D. J. (1997). Small heat shock proteins and vasospasm in human umbillical artery smooth muscle. *Biol. Reprod.* **57,** 1354–1359.

Butt, E., and Walter, U. (1996). Cyclic nucleotides: Measurement and function. *In* "*Platelet Function and Signal Transduction: A Practical Approach*" (K. Authi and S. P. Watson, eds.), pp. 259–278. Oxford Univ. Press, London.

Butt, E., Geiger, J., Jarchau, T., Lohmann, S. M., and Walter, U. (1993). The cGMP-dependent protein kinase—gene, protein and function. *Neurochem. Res.* **18,** 27–42.

Campbell, J. H., and Campbell, G. R. (1985). Smooth muscle cell phenotype changes in arterial wall homeostasis: Implication for the pathogenesis of atherosclerosis. *Exp. Mol. Pathol.* **42,** 136–162.

Cavallini, L., Coassin, M., Borean, A., and Alexandre, A. (1996). Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. *J. Biol. Chem.* **271,** 5545–5551.

Cayatte, A. J., Palacino, J. J., Horten, K., and Cohen, R. A. (1994). Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler. Thromb.* **14,** 753–759.

Chamley-Campbell, J., Campbell, G. R., and Ross, R. (1979). The smooth muscle cell in culture. *Physiol. Rev.* **59,** 1–61.

Chamley-Campbell, J., Campbell, G. R., and Ross, R. (1981). Phenotype-dependent response of cultured aortic smooth muscle cells to serum mitogens. *J. Cell Biol.* **89,** 379–383.

Chen, X.-L., and Rembold, C. M. (1996). Nitroglycerin relaxes rat tail artery primarily by lowering Ca^{2+} sensitivity and partially by repolarization. *Am. J. Physiol.* **271,** H962–H968.

Chiche, J. D., Schlutsmeyer, S. M., Bloch, D. B., de la Monte, S. M., Roberts, J. D., Filippov, G., Janssens, S. P., Rosenzweig, A., and Bloch, K. D. (1998). Adenovirus-mediated gene transfer of cGMP-dependent protein kinase increases the sensitivity of cultured vascular smooth muscle cells to the antiproliferative and pro-apoptotic effects of nitric oxide/cGMP. *J. Biol. Chem.* **273,** 34263–34271.

Chuang, E., Barnard, D., Hettich, L., Zhang, X. F., Avruch, J., and Marshall, M. S. (1994). Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues. *Mol. Cell. Biol.* **14,** 5318–5325.

Cohen, R. A., Weisbrod, R. M., Gericke, M., Yaghoubi, M., Bierl, C., and Bolotina, V. M. (1999). Mechanism of nitric oxide-induced vasodilation. Refilling of intracellular stores by sarcoplasmic reticulum Ca^{2+} ATPase and inhibition of store-operated CA^{2+} influx. *Circ. Res.* **84,** 210–219.

Colbran, J. L., Francis, S. H., Leach, A. B., Thomas, M. K., Jiang, H., McAllister, L. M., and Corbin, J. D. (1992). A phenylalanine in peptide substrates provides for selectivity between cGMP- and cAMP-dependent protein kinases. *J. Biol. Chem.* **267,** 9589–9594.

Colbran, J. L., Roach, P. J., Foil, C. J., Dixon, J. E., Andrisani, O. M., and Corbin, J. D. (1992). *Biochem. Cell Biol.* **70,** 1277–1282.

Collins, S. P., and Uhler, M. D. (1999). Cyclic AMP- and cyclic GMP-dependent protein kinases differ in their regulation of cyclic AMP response element-dependent gene transcription. *J. Biol. Chem.* **274,** 8391–8404.

Corbin, J. D., Ogreid, D., Miller, J. P., Suva, R. H., Jastorff, B., and Doskeland, S. O. (1986). Studies of cGMP analog specificity and function of the two intrasubunit binding sites of cGMP-dependent protein kinase. *J. Biol. Chem.* **261,** 1208–1214.

Cornwell, T. L., and Lincoln, T. M. (1988). Regulation of phosphorylase *a* formation and calcium content in aortic smooth muscle and smooth muscle cells: Effects of atrial natriuretic peptide II. *J. Pharmacol. Exp. Ther.* **247,** 524–530.

Cornwell, T. L., and Lincoln, T. M. (1989). Regulation of intracellular Ca^{2+} levels in cultured vascular smooth muscle cells: Reduction of Ca^{2+} by atriopeptin and 8-bromo-cyclic GMP is mediated by cGMP-dependent protein kinase. *J. Biol. Chem.* **264,** 1146–1155.

Cornwell, T. L., and Word, R. A. (1994). Regulation of cGMP-dependent protein kinase expression by progesterone in myometrial smooth muscle cells. Presented to the Society for Gynecologic Investigation, 41st Annual Meeting, March, 1994.

Cornwell, T. L., Pryzwansky, K. B., Wyatt, T. A., and Lincoln, T. M. (1991). Regulation of sarcoplasmic reticulum phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells. *Mol. Pharmacol.* **40,** 923–931.

Cornwell, T. L., Soff, G. A., Traynor, A. E., and Lincoln, T. M. (1994a). Regulation of the expression of cyclic GMP-dependent protein kinase by cell density in vascular smooth muscle cells. *J. Vasc. Res.* **31,** 330–337.

Cornwell, T. L., Arnold, E., Boerth, N. J., and Lincoln, T. M. (1994b). Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol.* **36,** C1405–C1413.

Csortos, C., Zolnierowicz, S., Bako, E., Durbin, S. D., and DePaoli-Roach, A. A. (1996). High complexity in the expression of the B' subunit of protein phosphatase $2A_o$. *J. Biol. Chem.* **271,** 2578–2588.

Davisson, R. L., Shaffer, R. A., Johnson, A. K., and Lewis, S. J. (1996). Stimulation of lumbar sympathetic nerves may produce hindlimb vasodilation via the release of pre-formed stores of nitrosyl factors. *Neuroscience* **72,** 881–887.

De Meyer, G. R., Bult, H., Ustunes, L., Kockx, M. M., Feelisch, M., and Herman, A. G. (1995). Effect of nitric oxide donors on neointima formation and vascular reactivity in the collared carotid artery of rabbits. *J. Cardiovasc. Pharmacol.* **26,** 272–279.

Dessy, C., Kim, I., Sougnez, C. L., Laporte, R., and Morgan, K. G. (1998). A role for MAP kinase in differentiated smooth muscle contraction evoked by α-adrenoceptor stimulation. *Am. J. Physiol.* **275,** C1081–C1086.

Dey, N. B., Boerth, N. J., Murphy-Ullrich, J. E., Chang, P. L., Prince, C. W., and Lincoln, T. M. (1998). Cyclic GMP-dependent protein kinase inhibits osteopontin and thrombospondin production in rat aortic smooth muscle cells. *Circ. Res.* **82,** 139–146.

Diamond, J. (1983). Lack of correlation between cyclic GMP elevation and relaxation of nonvascular smooth muscle by nitroglycerin, nitroprusside, hydroxylamine and sodium azide. *J. Pharmacol. Exp. Ther.* **225,** 422–426.

Doskeland, S. O., Vintermyr, O. K., Corbin, J. D., and Ogreid, D. (1986). Studies on the interactions between the cyclic nucleotide binding sites of cGMP-dependent protein kinase. *J. Biol. Chem.* **262,** 3534–3540.

Eckly-Michel, A., Martin, V., and Lugnier, C. (1997). Involvement of cyclic nucleotide-dependent protein kinases in cyclic AMP-mediated vasorelaxation. *Br. J. Pharmacol.* **122,** 158–164.

Eigenthaler, M., Ullrich, H., Geiger, J., Horstrup, K., Honig-Liedl, P., Wiebecke, D., and Walter, U. (1993). Defective nitrovasodilator-stimulated protein phosphorylation and calcium regulation in cGMP-dependent protein kinase deficient human platelets of chronic myelocytic leukemia. *J. Biol. Chem.* **268,** 13526–13531.

Eigenthaler, M., and Shattil, S. J. (1996). Integrin signaling and the platelet cytoskeleton. *Curr. Top. Membr.* **43,** 265–291.

Eigenthaler, M., Lohmann, S. M., Walter, U., and Pilz, R. B. (1999). Signal transduction by cGMP-dependent protein kinases and their emerging roles in the regulation of cell adhesion and gene expression. *Rev. Physiol. Biochem. Pharmacol.* **135,** 173–209.

Eiserich, J. P., Patel, R. P., and O'Donnell, V. B. (1994). Pathophysiology of NO and related species: Free radical reactions and modifications of biomolecules. *Mol. Aspects Med.* **19,** 221–357.

Elferink, J. G. R., and de Koster, B. M. (1993). The effect of cyclic GMP and cyclic AMP on migration by electroporated human neutrophils. *Eur. J. Pharmacol.* **246,** 157–161.

Elferink, J. G. R., and Van Uffelen, B. E. (1996). The role of cyclic nucleotides in neutrophil migration. *Gen. Pharmacol.* **27,** 387–393.

Enouf, J., Giraud, F., Bredoux, R., Bourdeau, N., and Levy-Toledano, S. (1987). Possible role of a cAMP-dependent phosphorylation in the calcium release mediated by inositol 1,4,5-trisphosphate in human platelet membrane vesicles. *Biochim. Biophys. Acta* **928,** 76–82.

Erhardt, P., Troppmair, J., Rapp, U. R., and Cooper, G. M. (1995). Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells. *Mol. Cell. Biol.* **15,** 5524–5530.

Faure, M., and Bourne, H. R. (1995). Differential effects of cAMP on the MAP kinase cascade: Evidence for a cAMP-insensitive step that can bypass Raf-1. *Mol. Cell. Biol.* **15,** 5524–5530.

Felbel, J., Trockur, B., Ecker, T., Landgraf, W., and Hofmann, F. (1988). Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. *J. Biol. Chem.* **263,** 16764–16771.

Fischmeister, R., and Hartzell, H. C. (1990). Regulation of calcium current by low K_m cyclic AMP phosphodiesterases in cardiac cells. *Mol. Pharmacol.* **38,** 426–433.

Forte, L. R., Thorne, P. K., Eber, S. L., Krause, W. J., Freeman, R. H., Francis, S. H., and Corbin, J. D. (1992). Stimulation of intestinal Cl^- transport by heat-stable enterotoxin: Activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol.* **263,** C607–C615.

Francis, S. H., and Corbin, J. D. (1994). Structure and function of cyclic nucleotide-dependent protein kinases. *Annu. Rev. Physiol.* **56,** 237–272.

Francis, S. H., Noblett, B. D., Todd, B. W., Well, J. N., and Corbin, J. D. (1988). Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. *Mol. Pharmacol.* **504,** 506–517.

Francis, S. H., Woodford, T. A., Wolfe, L., and Corbin, J. D. (1988–1989). Types Iα and Iβ isozymes of cGMP-dependent protein kinase: Alternative mRNA splicing may produce different inhibitory domains. *Second Messengers Phosphoproteins* **12,** 301–310.

French, P. J., Bijman, J., Edixhoven, M., Vaandrager, A. B., Scholte, B. J., Lohmann, S. M., Nairn, A. C., and de Jonge, H. R. (1995). Isotype-specific activation of cystic fibrosis transmembrane conductance regulator-chloride channels by cGMP-dependent protein kinase II. *J. Biol. Chem.* **270,** 26626–26631.

Fujii, M., Ogata, T., Takahashi, E., Yamada, K., Nakabayashi, K., Oishi, M., and Ayusawa, D. (1995). Expression of the human cGMP-dependent protein kinase II gene is lost upon introduction of SV40 T antigen or immortalization of human cells. *FEBS Lett.* **375,** 263–267.

Fukumoto, Y., Kawahara, Y., Kariya, K., Araki, S., Fukuzaki, H., and Takai, Y. (1988). Independent inhibition of DNA synthesis by protein kinase C, cyclic AMP, and interferon α/β in rabbit aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* **157,** 337–345.

Gambaryan, S., Wagner, C., Smolenski, A., Walter, U., Poller, W., Haase, W., Kurtz, A., and Lohmann, S. M. (1998). Endogenous or overexpressed cGMP-dependent protein kinases inhibit cAMP-dependent renin release from rat isolated perfused kidney, microdissected glomeruli, and isolated juxtaglomerular cells. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9003–9008.

Garg, U. C., and Hassid, A. (1989a). Inhibition of rat mesangial cell mitogenesis by nitric oxide-generating vasodilators. *Am. J. Physiol.* **257,** F60–F66.

Garg, U. C., and Hassid, A. (1989b). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83,** 1774–1777.

Geng, Y., Zhou, L., Thompson, W. J., and Lotz, M. (1998). Cyclic GMP and cGMP-binding phosphodiesterase are required for interleukin-1-induced nitric oxide synthesis in human articular chondrocytes. *J. Biol. Chem.* **273,** 27484–27491.

Gong, M. C., Iizuka, K., Nixon, G., Browne, J. P., Hall, A., Eccleston, J. F., Sugai, M., Kobayashi, S., Somlyo, A. V., and Somlyo, A. P. (1996). The role of G-proteins, ras-family, trimeric or both in Ca^{2+} sensitization of smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1340–1345.

Gorenne, I., Su, X., and Moreland, R. S. (1998). Inhibition of p42 and p44 MAP kinase does not alter smooth muscle contraction in swine carotid artery. *Am. J. Physiol.* **275,** H131–H138.

Gown, A. M., Tsukada, T., and Ross, R. (1986). Human atherosclerosis II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am. J. Pathol.* **125,** 191–207.

Graves, L. M., Bornfeldt, K. E., Raines, E. W., Potts, B. C., MacDonald, S. G., Ross, R., and Krebs, E. G. (1993). Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10300–10304.

Gudi, T., Huvar, I., Meinecke, M., Lohmann, S. M., Boss, G. R., and Pilz, R. B. (1996). Regulation of gene expression by cGMP-dependent protein kinase: Transactivation of the c-*fos* promoter. *J. Biol. Chem.* **271,** 4597–4600.

Gudi, T., Lohmann, S. M., and Pilz, R. B. (1997). Regulation of gene expression by cyclic GMP-dependent protein kinase requires nuclear translocation of the kinase: Identification of a nuclear localization signal. *Mol. Cell. Biol.* **17,** 5244–5254.

Haby, C., Lisovoski, F., Aunis, D., and Zwiller, J. (1994). Stimulation of the cyclic GMP pathway by NO induces expression of the immediate early genes c-*fos* and *junB* in PC12 cells. *J. Neurochem.* **62,** 496–501.

Hafner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994). Mechanism of inhibition of Raf-1 by protein kinase A. *Mol. Cell. Biol.* **14,** 6696–6703.

Hajnoczky, G., Gao, E., Nomura, T., Hoek, J. B., and Thomas, A. P. (1993). Multiple mechanisms by which protein kinase A potentiates inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization in permeablilized hepatocytes. *Biochem. J.* **293,** 413–422.

Hamaguchi, M., Ishibashi, T., and Imai, S. (1991). Involvement of charybdotoxin-sensitive K^+ channel in the relaxation of bovine tracheal smooth muscle by glyceryl trinitrate and sodium nitroprusside. *J. Pharmacol. Exp. Ther.* **262,** 263–270.

Harootunian, A. T., Adams, S. R., Wen, W., Meinkoth, J. L., Taylor, S. S., and Tsien, R. Y. (1993). *Mol. Biol. Cell* **4,** 993–1002.

Hartshorne, D. J. (1998). Myosin phosphatase: Subunits and interactions. *Acta Physiol. Scand.* **164,** 483–493.

Hartzell, H. C., and Fischmeister, R. (1986). Opposite effects of cyclic GMP and cyclic AMP on Ca^{2+} current in single heart cells. *Nature* **323,** 273–275.

Hassid, A., Arabshahi, H., Bourcier, T., Dhaunsi, G. S., and Matthews, C. (1994). Nitric oxide selectively amplifies FGF-2 mitogenesis in primary rat aortic smooth muscle cells. *Am. J. Physiol.* **267,** H1040–H1048.

Hedin, U., Sjolund, M., Hultgardh-Nilsson, A., and Thyberg, J. (1990). Changes in expression and organization of smooth muscle-specific

α-actin during fibronectin-mediated modulation of arterial smooth muscle cell phenotype. *Differentiation* **44,** 222–231.

Hirata, M., Kohse, K. P., Chang, C.-H., Ikebe, T., and Murad, F. (1990). Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. *J. Biol. Chem.* **265,** 1268–1273.

Hobbs, A. J. (1997). Soluble guanylate cyclase: The forgotten sibling. *Trends in Pharmacol. Sci.* **18,** 484–491.

Hofmann, F., Gensheimer, H., and Gobel, C. (1985). cGMP-dependent protein kinase: Autophosphorylation changes the characteristics of binding site 1. *Eur. J. Biochem.* **147,** 361–365.

Hofmann, F., Dostmann, W., Keilbach, A., Landgraf, W., and Ruth, P. (1992). Structure and physiological role of cGMP-dependent protein kinase. *Biochim. Biophys. Acta* **1135,** 51–60.

Horstrup, K., Jablonka, B., Honig-Liedl, P., Just, M., Kochsiek, K., and Walter, U. (1994). Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *Eur. J. Biochem.* **255,** 21–27.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **377,** 239–242.

Idriss, S. D., Gudi, T., Casteel, D. E., Kharitonov, V. G., Pilz, R. B., and Boss, G. R. (1999). Nitric oxide regulation via soluble guanylate cyclase and type I cGMP-dependent protein kinase. *J. Biol. Chem.* **274,** 9489–9493.

Ishikawa, T., Hume, J. R., and Keef, K. D. (1993). Regulation of Ca^{2+} channels by cAMP and cGMP in vascular smooth muscle cells. *Circ. Res.* **73,** 1128–1137.

Jahn, H., Nastainczyk, W., Rohrkasten, A., Schneider, T., and Hofmann, F. (1988). Site specific phosphorylation of the purified receptor for calcium channel blockers by cAMP- and cGMP-dependent protein kinases, protein kinase C, calmodulin-dependent protein kinase II, and casein kinase II. *Eur. J. Biochem.* **178,** 535–542.

Jarchau, T., Hasler, C., Markert, T., Pohler, D., VAndekerckhove, J., De-Jonge, H. R., Lohmann, S. M., and Walter, U. (1994). Cloning, expression and in situ localization of rat intestinal cGMP-dependent protein kinase II. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 9426–9430.

Jiang, H., Colbran, J. L., Francis, S. H., and Corbin, J. D. (1992). Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. *J. Biol. Chem.* **267,** 1015–1019.

Johnson, R. M., and Lincoln, T. M. (1985). Effects of nitroprusside, glyceryl trinitrate and 8-bromo cyclic GMP on phosphorylase *a* formation and myosin light chain phosphorylation in rat aorta. *Mol. Pharmacol.* **27,** 333–342.

Johnson, A., Lermioglu, F., Garg, U. C., Morgan-Boyd, R., and Hassid, A. (1988). A novel biological effect of atrial natriuretic hormone: Inhibition of mesangial cell mitogenesis. *Biochem. Biophys. Res. Commun.* **152,** 893–897.

Jonzon, B., Nilsson, J., and Fredholm, B. B. (1985). Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J. Cell. Physiol.* **124,** 451–456.

Juilfs, D. M., Soderling, S., Burns, F., and Beavo, J. A. (1999). Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases. *Rev. Physiol. Biochem. Pharmacol.* **135,** 67–104.

Kamibayashi, C., Estes, R., Lickteig, R. L., Yang, S. I., Craft, C., and Mumby, M. C. (1994). Comparison of heterotrimeric protein phosphatase 2A containing different B subunits. *J. Biol. Chem.* **269,** 20139–20148.

Karczewski, P., Kelm, M., Hartmann, M., Schrader, J. (1992). Role of phospholamban in NO/EDRF-induced relaxation in rat aorta. *Life Sci.* **51,** 1205–1210.

Karin, M. (1994). Signal transduction from the cell surface to the nucleus through phosphorylation of transcription factors. *Curr. Opin. Biol.* **6,** 415–424.

Kariya, K., Kawahara, Y., Araki, S., Fukuzaki, H., and Takai, Y. (1989). Antiproliferative action of cyclic GMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. *Atherosclerosis* **80,** 143–147.

Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S., and Jakobs, K. H. (1985). Protein kinase C phosphorylates the inhibitory guanine nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur. J. Biochem.* **151,** 431–437.

Kawada, T., Toyosato, A., Islam, M. O., Yoshida, Y., and Imai, S. (1997). cGMP-kinase mediates cGMP- and cAMP-induced Ca^{2+} desensitization of skinned rat artery. *Eur. J. Pharmacol.* **323,** 75–82.

Keilbach, A., Ruth, P., and Hofmann, F. (1992). Detection of cGMP-dependent protein kinase isozymes by specific antibodies. *Eur. J. Biochem.* **208,** 467–473.

Khalil, R. A., and Morgan, K. G. (1993). PKC-mediated redistribution of mitogen-activated protein kinase during smooth muscle cell activation. *Am. J. Physiol.* **265,** C406–C411.

Khan, S. A., Mathews, R., and Meisheri, K. D. (1993). Role of calcium-activated K^+ channels in vasodilation induced by nitroglycerine, acetylcholine and nitric oxide. *J. Pharmacol. Exp. Ther.* **267,** 1237–1335.

Kikuchi, A., and Williams, L. T. (1996). Regulation of interaction of ras p21 with RalGDS and Raf-1 by cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **271,** 588–594.

Koga, T., Yoshida, Y., Cai, J. Q., and Imai, S. (1994). Purification and characterization of a 240-kDa cGMP-dependent protein kinase substrate of vascular smooth muscle: Close resemblance to inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **269,** 11640–11647.

Komalavilas, P., and Lincoln, T. M. (1994). Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic GMP-dependent protein kinase. *J. Biol. Chem.* **269,** 8701–8707.

Komalavilas, P., and Lincoln, T. M. (1996). Phosphorylation of the inositol 1,4,5-trisphosphate receptor: Cyclic GMP-dependent protein kinase mediates cAMP and cGMP dependent phosphorylation in the intact rat aorta. *J. Biol. Chem.* **271,** 21933–21938.

Krippeit-Drews, P., Norel, N., and Godfraind, T. (1992). Effect of nitric oxide on membrane potential and contraction of rat aorta. *J. Cardiovasc. Pharmacol.* **20,** S72–S75.

Kume, H., Takai, A., Tokuno, H., and Tomita, T. (1989). Regulation of Ca^{2+}-dependent K^+ channel activity in tracheal myocytes by phosphorylation. *Nature* **341,** 152–154.

Kumar, R., Namiki, T., and Joyner, R. W. (1997). Effects of cGMP on L-type calcium current of adult and newborn rabbit ventricular cells. *Cardiovasc. Res.* **33,** 573–582.

Lablanche, J. M., Grollier, G., Lusson, J. R., Bassand, J. P., Drobinski, G., Bertrand, B., Battaglia, S., Desveaux, B., Juilliere, Y., Juliard, J. M., Metzger, J. P., Coste, P., Quiret, J. C., Dubois-Rande, J. L., Crochet, P. D., Letac, B., Boschat, J., Virot, P., Finet, G., LeBreton, H., Livarek, B., Leclercq, F., Beard, T., Gikraud, T., McFadden, E. P., and Bertrand, M. E. (1997). Effect of the direct nitric oxide donors linsidomine and molsidomine on angiographic restenosis after coronary balloon angioplasty. The ACCORD study. *Circulation* **95,** 83–89.

Lalli, J., Harrer, J. M., Luo, W., Kranias, E. G., and Paul, R. J. (1997). Targeted ablation of the phospholamban gene is associated with a marked decrease in sensitivity in aortic smooth muscle. *Circ. Res.* **80,** 506–513.

Lalli, M. J., Shimizu, G., Kranias, E. G., and Paul, R. J. (1999). $[Ca^{2+}]_i$ homeostasis and cyclic nucleotide relaxation in aorta of phospholamban-deficient mice. *Am. J. Physiol.* **277,** H693–H670.

Landgraf, W., Hulin, R., Gobel, C., and Hofmann, F. (1986). Phosphorylation of cGMP-dependent protein kinase increases the affinity for cAMP. *Eur. J. Biochem.* **154,** 113–117.

Lee, M. R., Li, L., and Kitazawa, T. (1997). Cyclic GMP causes Ca^{2+} desensitization in vascular smooth muscle by activating the myosin light chain phosphatase. *J. Biol. Chem.* **272,** 5063–5068.

Lei, S. Z., Pan, Z.-H., Aggarwal, S. K., Chen, H. S., Hartman, S., Sucher, N. J., and Lipton, S. A. (1992). Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. *Neuron* **8,** 1087–1089.

Li, H., Liu, J. P., and Robinson, P. J. (1996). Multiple substrates for cGMP-dependent protein kinase from bovine aortic smooth muscle: Purification of p132. *J. Vasc. Res.* **33,** 99–110.

Libby, P., and Galis, Z. S. (1998). Cytokines regulate genes involved in atherogenesis. *Ann. N.Y. Acad. Sci.* 158–170.

Lincoln, T. M. (1994). Cyclic GMP: Biochemistry, Physiology, and Pathophysiology,'' pp. 101–116. R. G. Landes, Austin, Texas.

Lincoln, T. M., and Corbin, J. D. (1983). Characterization and biological role of the cGMP-dependent protein kinase. *Adv. Cyclic Nucleotide Res.* **15,** 139–192.

Lincoln, T. M., and Corbin, J. D. (1977). Adenosine 3':5'-cyclic monophosphate and guanosine 3':5'-cyclic monophosphate-dependent protein kinases: Possible homologous proteins. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3239–3243.

Lincoln, T. M., and Cornwell, T. L. (1993). Intracellular cyclic GMP receptor proteins. *FASEB J.* **7,** 328–338.

Lincoln, T. M., Cornwell, T. L., and Taylor, A. E. (1990). cGMP-dependent protein kinase mediates the reduction of Ca^{2+} by cAMP in vascular smooth muscle cells. *Am. J. Physiol.* **258,** C399–C407.

Lincoln, T. M., Dey, N. B., Boerth, N. J., Cornwell, T. L., and Soff, G. A. (1998). Nitric oxide-cyclic GMP pathway regulates vascular smooth muscle cell phenotypic modulation: Implications in vascular diseases. *Acta Physiol. Scand.* **164,** 507–515.

Loesberg, C., Van Wijk, R., Zandbergen, J., van Aken, W. G., van Mourik, J. A., and de Groot, G. (1985). Cell cycle-dependent inhibition of human vascular smooth muscle cell proliferation by prostaglandin E_1. *Exp. Cell Res.* **160,** 117–125.

Lopez, M. J., Wong, S. K., Kishimoto, I., Dubois, S., Mach, V., Friesen, J., Garbers, D. L., and Beuve, A. (1995). Salt-resistant hypertension in mice lacking the guanyly cyclase-A receptor for atrial natriuretic peptide. *Nature* **378,** 65–68.

Luo, D. L., Nakazawa, M., Ishibashi, T., Kato, K., and Imai, S. (1993). Putative selective inhibitors of the sarcoplasmic reticulum Ca^{2+}-pump ATPase inhibit relaxation by nitroglycerine and atrial natriuretic factor of the rabbit aorta contracted by phenylephrine. *J. Pharmacol. Exp. Ther.* **265,** 1187–1192.

McDaniel, N. L., Chen, X.-L., Singer, H. A., Murphy, R. A., and Rembold, C. M. (1992). Nitrovasodilators relax arterial smooth muscle by decreasing $[Ca^{2+}]_i$ and uncoupling stress from myosin phosphorylation. *Am. J. Physiol.* **263,** C461–C467.

McNamara, D. B., Bedi, B., Aurora, H., Tena, L., Ignarro, L. J., Kadowitz, P. J., and Akers, D. L. (1993). L-Arginine inhibits balloon catheter-induced intimal hyperplasia. *Biochem. Biophys. Res. Commun.* **193,** 291–296.

Majesky, M. W., Giachelli, C. M., Reidy, M. A., and Schwartz, S. M. (1992). Rat carotid neointimal smooth muscle cells re-express a developmentally regulated phenotype during repair of arterial injury. *Circ. Res.* **71,** 759–768.

Markert, T., Vaandrager, A. B., Gambaryan, S., Pohler, D., Hausler, C., Walter, U., DeJonge, H. R., Jarchau, T., and Lohmann, S. (1995). Endogenous expression of Type II cGMP-dependent protein kinase mRNA and protein in rat intestine. *J. Clin. Invest.* **96,** 822–830.

Masuom M., Reardon, S., Ikebe, M., and Kitazawa, T. (1994). A novel mechanism for the Ca^{2+}-sensitizing effect of protein kinase C on vascular smooth muscle: Inhibition of myosin light chain phosphatase. *J. Gen. Physiol.* **104,** 265–286.

Mayrleitner, M., Chandler, R., Schindler, H., and Fleischer, S. (1995). Phosphorylation with protein kinases modulates calcium loading of terminal cisternae of sarcoplasmic reticulum from skeletal muscle. *Cell Calcium* **18,** 1970206.

Meisheri, K. D., Taylor, C. J., and Saneii, H. (1986). Synthetic atrial peptide inhibits intracellular calcium release in smooth muscle. *Am. J. Physiol.* **250,** C171–C174.

Menice, C. B., Hulvershorn, J., Adam, L. P., Wang, C. A., and Morgan, K. G. (1997). Calponin and mitogen-activated protein kinase signaling in differentiated vascular smooth muscle. *J. Biol. Chem.* **272,** 25157–25161.

Mery, P. F., Lohmann, S. M., Walter, U., and Fischmeister, R. (1991). Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 1197–1201.

Mery, P. F., Pavoine, C., Belhassen, L, Pecker, F., and Fischmeister, R. (1993). Nitric oxide regulates cardiac Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J. Biol. Chem.* **268,** 26286–26295.

Mii, S., Khalil, R. A., Morgan, K. G., Ware, J. A., and Kent, K. C. (1996). Mitogen-activated protein kinase and proliferation of human vascular smooth muscle cells. *Am. J. Physiol.* **270,** H142–H150.

Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellerer, M., Philipp, A., and Kolch, W. (1996). Negative regulation of Raf-1 by phosphorylation of serine 621. *Mol. Cell. Biol.* **16,** 5409–5418.

Moellering, D., McAndrew, J., Patel, R. P., Cornwell, T., Lincoln, T., Cao, X., Messina, J. L., Forman, H. J., Jo, H., and Darley-Usmar, V. M. (1998). Nitric oxide-dependent induction of glutathione synthesis through increased expression of γ-glutamylcysteine synthetase. *Arch. Biochem. Biophys.* **358,** 74–82.

Morisaki, N., Kanzaki, T., Motoyama, N., Saito, Y., and Yoshida, S. (1988). Cell cycle-dependent inhibition of DNA synthesis by prostaglandin I_2 in cultured rabbit aortic smooth muscle cells. *Atherosclerosis* **71,** 165–171.

Moritoki, H., Hisayama, T., Takeuchi, S., Kondoh, W., Inoue, S., and Kida, K. (1996). Inhibition by SK&F 96365 of NO-mediated relaxation induced by Ca^{2+}-ATPase inhibitors in rat thoracic aorta. *Br. J. Pharmacol.* **117,** 1544–1548.

Moro, M. A., Russell, R. J., Cellek, S., Lizasoain, I., Su, Y., Darley-Usmar, V. M., Radomski, M. W., and Moncada, S. (1996). cGMP mediates the vascular and platelet actions of nitric oxide: Confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1480–1485.

Mosse, P. R., Campbell, G. R., Wang, Z. L., and Campbell, J. H. (1985). Smooth muscle phenotypic expression in human carotid arteries I. Comparison of cells from diffuse intimal thickenings adjacent to atheromatous plaques with those of the media. *Lab. Invest.* **53,** 556–562.

Murphy-Ullrich, J. E., Pallero, M. A., Boerth, N., Greenwood, J. A., Lincoln, T. M., and Cornwell, T. L. (1996). Cyclic GMP-dependent protein kinase is required for thrombospondin and tenascin mediated focal adhesion disassembly. *J. Cell Sci.* **109,** 2499–2508.

Murthy, K. S., and Makhlouf, G. M. (1995). Interaction of cA-kinase and cG-kinase in mediating relaxation of dispersed smooth muscle cells. *Am. J. Physiol.* **268,** C171–C180.

Murthy, K. S., and Makhlouf, G. M. (1998a). cGMP-mediated Ca^{2+} release from IP_3-insensitive Ca^{2+} stores in smooth muscle. *Am. J. Physiol.* **274,** C1199–C1205.

Murthy, K. S., and Makhlouf, G. M. (1998b). Differential regulation of phsopholipase A_2 (PLA_2)-dependent Ca^{2+} signaling in smooth muscle by cAMP- and cGMP-dependent protein kinases. *J. Biol. Chem.* **273,** 34519–34526.

Murthy, K. S., Severi, C., Grider, J. R., and Makhlouf, G. M. (1993). Inhibition of IP_3 an IP_3-dependent Ca^{2+} mobilization by cyclic nucleotides in isolated gastric muscle cells. *Am. J. Physiol.* **264,** G967–G974.

Murthy, K. S., Jin, J. G., Grider, J. R., and Makhlouf, G. M. (1997). Characterization of PACAP receptors and signaling pathways in rabbit gastric muscle cells. *Am. J. Physiol.* **272,** G1391–G1399.

Nakaki, T., Nakayama, M., and Kato, R. (1990a). Inhibition by nitric oxide and nitric oxide-producing vasodilators of DNA synthesis in vascular smooth muscle cells. *Eur. J. Pharmacol.* **189,** 347–353.

Nakaki, T., Nakayama, M., Yamamoto, S., and Kato, R. (1990b). α-adrenergic stimulation and β-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol. Pharmacol.* **37,** 30–36.

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J., and Lederer, W. J. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* **270,** 633–637.

Orstavik, S., Solberg, R., Tasken, K., Nordahl, M., Altherr, M. R., Hansson, V., Jahnsen, T., and Sandberg, M. (1996). Molecular cloning, cDNA structure, and chromosomal localization of the human type II cGMP-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **220,** 759–765.

Orstavik, S., Natarajan, V., Tasken, K., Jahnsen, T., and Sandberg, M. (1997). Characterization of the human gene encoding the Type Iα and

Type Iβ cGMP-dependent protein kinase (PRKG1). *Genomics* **42,** 311–318.

Peunova, N., and Enlkolopov, G. (1993). Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells. *Nature* **364,** 450–453.

Pfeifer, A., Nurnberg, B., Kamm, S., Uhde, M., Schultz, G., Ruth, P., and Hofmann, F. (1995). Cyclic GMP-dependent protein kinase blocks pertussis toxin-sensitive hormone receptor signaling pathways in Chinese Hamster Ovary Cells. *J. Biol. Chem.* **270,** 9052–9059.

Pfeifer, A., Aszodi, A., Seidler, U., Ruth, P., Hofmann, F., and Fassler, R. (1996). Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II. *Science* **274,** 2082–2086.

Pfeifer, A., Klatt, P., Massberg, S., Ny, L., Sausbier, M., Hirneiss, C., Wang, G. X., Korth, M., Aszodi, A., Andersson, K. E., Krombach, F., Mayerhofer, A., Ruth, P., Fassler, R., and Hofmann, F. (1998). Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J.* **11,** 3045–3051.

Pilz, R. B., Suhasini, M., Idriss, S., Meinkoth, J. L., and Boss, G. R. (1995). Nitric oxide and cGMP analogs activate transcription from AP-1-responsive promoters in mammalian cells. *FASEB J.* **9,** 552–558.

Pollman, M. J., Yamada, T., Horiuchi, M., and Gibbons, G. H. (1996). Vasoactive substances regulate vascular smooth muscle cell apoptosis. Countervailing influences of nitric oxide and angiotensin II. *Circ. Res.* **79,** 748–756.

Porter, V. A., Bonev, A. D., Knot, H. J., Heppner, T. J., Stevenson, A. S., Kleppisch, T., Lederer, W. J., and Nelson, M. T. (1998). Frequency modulation of Ca^{2+} sparks is involved in regulation of arterial diameter by cyclic nucleotides. *Am. J. Physiol.* **274,** C1346–C1355.

Potvin, W., and Varma, D. R. (1990). Refractoriness of the gravid rat uterus to tocolytic and biochemical effects of atrial natriuretic peptide. *Br. J. Pharmacol.* **100,** 341–347.

Pryzwansky, K. B., Wyatt, T. A., and Lincoln, T. M. (1995a). Cyclic guanosine monophosphate dependent protein kinase is targeted to intermediate filaments and phosphorylates vimentin in A23187-stimulated human neutrophils. *Blood* **85,** 222–230.

Pryzwansky, K. B., Wyatt, T. A., and Lincoln, T. M. (1995b). Cyclic guanosine monophosphate dependent protein kinase is targeted to intermediate filaments and phosphorylates vimentin in A23187-stimulated human neutrophils. *Blood* **85,** 222–230.

Pryzwansky, K. B., Kidao, S., Wyatt, T. A., Reed, W., and Lincoln, T. M. (1995c). Localization of cyclic GMP-dependent protein kinase in human mononuclear phagocytes. *J. Leukocyte Biol.* **57,** 670–678.

Quinton, T. M., and Dean, W. L. (1992). Cyclic AMP-dependent phosphorylation of the inositol 1,4,5-trisphosphate receptor inhibits Ca^{2+} releases from platelet membranes. *Biochem. Biophys. Res. Commun.* **184,** 893–899.

Raeymaekers, L., Hofmann, F., and Casteels, R. (1988). Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle. *Biochem. J.* **252,** 269–273.

Rapoport, R. M. (1986). Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in rat aorta. *Circ. Res.* **58,** 407–410.

Rapoport, R. M., Draznin, M. B., and Murad, F. (1982). Sodium nitroprusside-induced protein phosphorylation in intact rat aorta is mimicked by 8-bromo cyclic GMP. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 6470–6474.

Rashatwar, S. S., Cornwell, T. L., and Lincoln, T. M. (1987). Effects of 8-bromo-cGMP on Ca^{2+} levels in vascular smooth muscle cells: Possible regulation of Ca^{2+}-ATPase by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 5685–5689.

Robertson, B. E., Schubert, R., Hescheler, J., and Nelson, M. T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.* **265,** C299–C303.

Rooney, T. A., Joseph, S. K., Queen, C., and Thomas, A. P. (1996). Cyclic GMP induces oscillatory calcium signals in rat hepatocytes. *J. Biol. Chem.* **271,** 19817–19825.

Ross, R. (1993). The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **362,** 801–809.

Rotman, E. I., Murphy, B. J., and Catterall, W. A. (1995). Sites of selective cAMP-dependent phosphorylation of the L-type calcium channel α1 subunit from intact rabbit skeletal muscle myotubes. *J. Biol. Chem.* **270,** 16371–16377.

Rovner, A. S., Murphy, R. A., and Owens, G. K. (1986). Expression of smooth muscle and nonmuscle myosin heavy chains in cultured vascular smooth muscle cells. *J. Biol. Chem.* **261,** 14740–14745.

Ruiz-Velasco, V., Zhong, J., Hume, J. R., and Keef, K. D. (1998). Modulation of Ca^{2+} channels by cyclic nucleotide cross-activation of opposing protein kinases in rabbit portal vein. *Circ. Res.* **82,** 557–565.

Ruth, P., Wang, G.-X., Boekhoff, I., May, B., Pfeifer, A., Penner, R., Korth, M., Breer, H., and Hofmann, F. (1993). Transfected cGMP-dependent protein kinase suppresses calcium transients by inhibition of inositol 1,4,5-trisphosphate production. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2623–2627.

Sabine, B., Willenbroch, R., Haase, H., Karczewski, P. l., Wallukat, G., Dietz, R., and Krause, E. G. (1995). Cyclic GMP-mediated phospholamban phosphorylation in intact cardiomyocytes. *Biochem. Biophys. Res. Commun.* **214,** 75–80.

Sadoshima, J., Akaike, N., Kanaide, H., and Nakamura, M. (1988). Cyclic AMP modulates Ca-activated K channel in cultured smooth muscle cells of rat aortas. *Am. J. Physiol.* **255,** H754–H759.

Sandberg, M., Natarajan, V., Ronander, I., Kalderon, D., Walter, U., Lohmann, S. M., and Jahnsen, T. (1989). Molecular cloning and predicted full-length amino acid sequence of the type Iβ isozyme of cGMP-de pendent protein kinase from human placenta. *FEBS Lett.* **255,** 321–329.

Sarcevic, B., Brookes, V., Martin, T. J., Kemp, B. E., and Robinson, P. J. (1989). Atrial natriuretic peptide-dependent phosphorylation of smooth muscle cell particulate fraction proteins is mediated by cGMP-dependent protein kinase. *J. Biol. Chem.* **264,** 20648–20654.

Sciorati, C., Nistico, G., Meldolesi, J., and Clementi, E. (1997). Nitric oxide effects on cell growth: cGMP-dependent stimulation of the AP-1 transcription complex and cGMP-independent slowing of cell cycling. *Br. J. Pharmacol.* **122,** 687–697.

Shabb, J. B., Ng, L., and Corbin, J. D. (1990). One amino acid change produces a high affinity cGMP-binding site in cAMP-dependent protein kinase. *J. Biol. Chem.* **265,** 16031–16034.

Shabb, J. B., Buzzeo, B. D., Ng, L., and Corbin, J. D. (1991). Mutating protein kinase cAMP-binding sites into cGMP-binding sites. *J. Biol. Chem.* **266,** 24320–24326.

Smith, J. A., Francis, S. H., and Corbin, J. D. (1992). Activation of type Iβ of cGMP-dependent protein kinase by preincubation with MgATP and cAMP. *FASEB J.* **6,** A315.

Smith, J. A., Francis, S. H., Walsh, K. A., Kumar, S., and Corbin, J. D. (1996). Autophosphorylation of type Iβ cGMP-dependent protein kinase increases basal catalytic activity and enhances allosteric activation by cGMP or cAMP. *J. Biol. Chem.* **271,** 20756–20762.

Smolenski, A., Burkhardt, M., Eigenthaler, M., Butt, E., Gambaryan, S., Lohmann, S. M., and Walter, U. (1998). Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **358,** 134–139.

Soff, G., Cornwell, T. L., Cundiff, D. L., Wade, E. L., and Lincoln, T. M. (1997). Smooth muscle cell expression of type I cyclic GMP-dependent protein kinase is suppressed by continuous exposure to nitrovasodilators, theophylline, cyclic GMP, and cyclic AMP. *J. Clin. Invest.* **100,** 2580–2587.

Somlyo, A. P., and Somlyo, A. V. (1994). Signal transduction and regulation in smooth muscle. *Nature* **372,** 231–236.

Somlyo, A. P., and Somlyo, A. V. (1998). From pharmacomechanical coupling to G-proteins and myosin phosphatase. *Acta Physiol. Scand.* **164,** 437–448.

Souness, J. E., Hassall, G. A., and Parrott, D. P. (1992). Inhibition of pig aortic smooth muscle cell DNA synthesis by selective type III and type IV cyclic AMP phosphodiesterase inhibitors. *Biochem. Pharmacol.* **44,** 857–866.

Southgate, K., and Newby, A. C. (1990). Serum-induced proliferation of rabbit aortic smooth muscle cells from the contractile state is inhibited by 8-Br-cAMP but not 8-Br-cGMP. *Atherosclerosis* **82,** 113–123.

Stenho-Bittel, L., and Sturek, M. (1992). Spontaneous sarcoplasmic reticulum calcium release and extrusion from bovine, not porcine, coronary artery smooth muscle. *J. Physiol.* **451,** 49–78.

Strand, M. A., Louis, C. F., and Mickelson, J. R. (1993). Phosphorylation of the porcine skeletal muscle and cardiac muscle sarcoplasmic reticulum ryanodine receptor. *Biochim. Biophys. Acta* **1175,** 319–326.

Suhasini, M., Li, H., Lohmann, S. M., Boss, G. R., and Pilz, R. B. (1998). Cyclic-GMP-dependent protein kinase inhibits the Ras/Mitogen-Activated Protein Kinase Pathway. *Mol. Cell. Biol.* **18,** 6983–6994.

Suko, J, Maurer-Fogy, I. L., Plank, B., Bertel, O., Wyskovsky, W., Hohenegger, M., and Hellmann, G. (1993). Phosphorylation of serine 2843 in ryanodine receptor-calcium release channel of skeletal muscle by cAMP-, cGMP-, and CaM-dependent protein kinase. *Biochim. Biophys. Acta* **1175,** 193–206.

Sulakhe, P. V., and Vo, X. T. (1995). Regulation of phospholamban and troponin-I phosphorylation in the intact rat cardiomyocytes by adrenergic and cholinergic stimuli: Roles of cyclic nucleotides, calcium, protein kinases and phosphatases, and depolarization. *Mol. Cell. Biochem.* **149–150,** 103–126.

Sumii, K., and Sperelakis, N. (1995). cGMP-dependent protein kinase regulation of the L-type calcium current in rat ventricular myocytes. *Circ. Res.* **77,** 803–812.

Sun, C. W., Alonso-Galicia, M., Taheri, M. R., Falck, J. R., Harder, D. R., and Roman, R. J. (1998). Nitric oxide-20-hydroxyeicosatetraenoic acid interaction in the regulation of K^+ channel activity and vascular tone in renal arterioles. *Circ. Res.* **83,** 1069–1079.

Surks, H. K., Mochizucki, N., Kasai, Y., Georgescu, S. P., Tang, M., Ito, M., Lincoln, T. M., and Mendelsohn, M. E. (1999). Regulation of myosin phosphatase by a specific interaction with cGMP-dependent protein kinase 1α. *Science* **286,** 1583–1587.

Takai, Y., Kaibuchi, K., Matsubara, T., and Nishizuka, Y. (1981). Inhibitory action of guanosine 3',5'-monophosphate on thrombin-induced phosphatidylinositol turnover and protein phosphorylation in human platelets. *Biochem. Biophys. Res. Commun.* **101,** 61–67.

Tamura, N., Itoh, H., Ogawa, Y., Nakagawa, O., Harada, M., Chun, T.-W., Suga, S., Yoshimasa, T., and Nakao, K. (1996). cDNA cloning and gene expression of human TypeIα cGMP-dependent protein kinase. *Hypertension* **27,** 552–557.

Tegge, W., Frank, R., Hofmann, F., and Dostmann, W. R. (1995). Determination of cyclic nucleotide-dependent protein kinase substrate specificity by the use of peptide libraries on cellulose paper. *Biochemistry* **34,** 10569–10577.

Thornbury, K. D., Ward, S. M., Dalziel, H. H., Carl, A., Westfall, D. P., and Sanders, K. M. (1991). Nitric oxide and nitrocysteine mimic nonadrenergic noncholinergic hyperpolarization in canine proximal colon. *Am. J. Physiol.* **261,** G553–G557.

Tseng-Crank, J., Foster, C. D., Krause, J. D., Mertz, R., Godinot, N., DiChiara, T. J., and Reinhart, P. H. (1994). Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^+ channel isoforms from human brain. *Neuron* **13,** 1315–1330.

Tsou, K., Snyder, G. L., and Greengard, P. (1993). Nitric oxide/cGMP pathway stimulates phosphorylation of DARPP-32, a dopamine and cAMP-regulated phosphoprotein, in the substantia nigra. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3462–3465.

Uehata, M., Ishizaki, T., Satoh, H., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagumi, K., Inui, J., Maekawa, M., and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389,** 990–994.

Uhler, M. D. (1993). Cloning and expression of a novel cyclic GMP-dependent protein kinase from mouse brain. *J. Biol. Chem.* **268,** 13586–13591.

Vaandrager, A. B., Tilly, B. C., Smolenski, A., Schneider-Rasp, S., Bot, A. G. M., Edixhoven, M., Scholte, B. J., Jarchau, T., Walter, U., Lohmann, S. M., Poller, W. C., and DeJonge, H. R. (1997). cGMP-stimulation of cystic fibrosis transmembrane conductance regulator Cl^- channels co-expressed with cGMP-dependent protein kinase type II but not type Iβ. *J. Biol. Chem.* **272,** 4195–4200.

van der Leyen, H. E., Gibbons, G. H., Morishita, R., Lewis, N. P., Zhang, L., Nakayima, M., Kaneda, Y., Cooke, J. P., and Dzau, V. J. (1995). Gene therapy inhibits neointimal vascular lesion: In vivo transfer of endothelial nitric oxide synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1137–1141.

Vrolix, M., Raeymaekers, L., Wuytack, F., and Hofmann, F. (1988). Cyclic GMP-dependent protein kinase stimulates the plasmalemmal Ca^{2+} pump of smooth muscle via phosphorylation of phosphatidylinositol. *Biochem. J.* **255,** 855–863.

Wahler, G. M., Rusch, N. J., and Sperelakis, N. (1990). 8-Bromo-cyclic GMP inhibits the calcium channel current in embryonic chick ventricular myocytes. *Can. J. Physiol Pharmacol.* **68,** 531–534.

Weisbrod, R. M., Griswold, M. C., Yaghoubi, M., Komalavilas, P., Lincoln, T. M., and Cohen, R. A. (1998). Evidence that additional mechanisms to cyclic GMP mediate the decrease in intracellular calcium and relaxation of rabbit aortic smooth muscle to nitric oxide. *Br. J. Pharmacol.* **125,** 1695–1707.

Wellman, G. C., Eckman, D. M., Santana, L. F., Heppner, T. J., Kranias, E. G., and Nelson, M. T. (1999). Protein kinase A increases Ca^{2+} spark frequency both through effects on phospholamban and on ryanodine receptors (RyR) in intact mouse cerebral arteries. *FASEB J.* **13,** A1034.

Wen, W., Harootunian, A. T., Adams, S. R., Feramisco, J., Tsien, R. Y., Meinkoth, J. L., and Taylor, S. R. (1994). Heat stable inhibitors of cAMP-dependent protein kinase carry a nuclear export signal. *J. Biol. Chem.* **269,** 32214–32220.

Wernet, W., Flockerzi, V., and Hofmann, F. (1989). The cDNA of the two isoforms of bovine cGMP-dependent protein kinase. *FEBS Lett.* **264,** 191–196.

White, R. E., Lee, A. B., Shcherbatko, A. D., Lincoln, T. M., Schonbrunn, A., and Armstrong, D. L. (1993). Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation. *Nature* **361,** 263–266.

Wong, G., Zhu, Y., Halushka, P. V., Lincoln, T. M., and Mendelsohn, M. A. (1998). Mechanism of platelet inhibition by nitric oxide: In vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 4888–4893.

Word, R. A., and Cornwell, T. L. (1998). Regulation of cGMP-induced relaxation and cGMP-dependent protein kinase in rat myometrium during pregnancy. *Am. J. Physiol.* **274,** C748–C756.

Word, R. A., Casey, M. L., Kamm, K. E., and Stull, J. T. (1991). Effects of cGMP on $[Ca^{2+}]_i$, myosin light chain phosphorylation, and contraction in human myometrium. *Am. J. Physiol.* **260,** C861–C867.

Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993). Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science* **262,** 988–990.

Wu, X., Somlyo, A. V., and Somlyo, A. P. (1996). Cyclic GMP-dependent stimulation reverses G-protein-coupled inhibition of smooth muscle myosin light chain phosphatase. *Biochem. Biophys. Res. Commun.* **220,** 658–663.

Wu, X. Q., Haystead, T. A. J., Nakamoto, R. K., Somlyo, A. V., and Somlyo, A. P. (1999). Acceleration of myosin light chain dephosphorylation and relaxation of smooth muscle by telokin: Synergism with cyclic nucleotide-activated kinase. *J. Biol. Chem.* **273,** 11362–11369.

Wyatt, T. A., Pryzwansky, K. B., and Lincoln, T. M. (1991). KT 5823 activates human neutrophils and fails to inhibit cGMP-dependent protein kinase phosphorylation of vimentin. *Res. Commun. Chem. Pathol. Pharmacol.* **74,** 3–14.

Wyatt, T. A., Lincoln, T. M., and Pryzwansky, K. B. (1993). Regulation of human neutrophil degranulation by LY-83583 and L-arginine: Role of cGMP-dependent protein kinase. *Am. J. Physiol.* **265,** C201–C211.

Wyatt, T. A., Naftilan, A. J., Francis, S. H., and Corbin, J. C. (1998). ANF elicits phosphorylation of the cGMP phosphodiesterase (PDE5) in vascular smooth muscle cells. *Am. J. Physiol.* **274,** H448–H455.

Yallampalli, C., Garfield, R. E., and Byam-Smith, M. (1993). Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. *Endocrinology* **133,** 1899–1902.

Yallampalli, C., Izumi, H., Byam-Smith, M., and Garfield, R. E. (1994). An -arginine–nitric oxide–cyclic guanosine monophosphate system exists

in the uterus and inhibits contractility during pregnancy. *Am. J. Obstet. Gynecol.* **170,** 175–185.

Yan, Z. Q., and Hansson, G. K. (1998). Overexpression of inducible nitric oxide synthase by neointimal smooth muscle cells. *Circ. Res.* **82,** 21–29.

Yee, W. M., and Worley, P. F. (1997). Rheb interacts with Raf-1 kinase and may function to integrate growth factor and protein kinase A-dependent signals. *Mol. Cell. Biol.* **17,** 921–933.

Yu, S. M., Hung, L. M., and Lin, C. C. (1997). cGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signaling pathway. *Circulation* **95,** 1269–1277.

Zhou, S. B., Schlossmann, J., Hofmann, F., Ruth, P., and Korth, M. (1998). Regulation of stably expressed and native BK channels from human myometrium by cGMP- and cAMP-dependent protein kinase. *Pfluegers Arch.* **436,** 725–734.

Zhou, X.-B., Ruth, P., Schlossmann, J., Hofmann, F., and Korth, M. (1996). Protein phosphatase 2A is essential for the activation of Ca^{2+}-activated K^+ currents by cGMP-dependent protein kinase in trachael smooth muscle and Chinese hamster ovary cells. *J. Biol. Chem.* **271,** 19760–19767.

Zong, X., Schreieck, J., Mehrke, G., Welling, A., Schuster, A., Bosse, E., Flockerzi, V., and Hofmann, F. (1995). On the regulation of the expressed L-type calcium channel by cAMP-dependent phosphorylation. *Pfluegers Arch.* **430,** 340–347.

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SECTION III

Principles of Pathobiology

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CHAPTER 27

Influence of Nitric Oxide on Neuroendocrine Function and Behavior

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NITRIC OXIDE (NO) FUNCTIONS AS A NEUROTRANSMITTER AND NEUROMODULATOR IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS. THE EFFECTS OF NO ON BLOOD VESSEL TONE AND NEURONAL FUNCTION FORM THE BASIS FOR AN IMPORTANT ROLE OF NO ON NEUROENDOCRINE FUNCTION AND BEHAVIOR. NO MEDIATES HYPOTHALAMIC PORTAL BLOOD FLOW, AND THUS AFFECTS NEUROPEPTIDE SECRETION, AS WELL AS MEDIATES NEUROENDOCRINE FUNCTION IN THE HYPOTHALAMUS–PITUITARY–ADRENAL AND THE HYPOTHALAMUS–PITUITARY–GONADAL AXES. NO INFLUENCES SEVERAL MOTIVATED BEHAVIORS INCLUDING SEXUAL, AGGRESSIVE, AND INGESTIVE BEHAVIORS. LEARNING AND MEMORY FUNCTIONS ARE ALSO INFLUENCED BY NO. TAKEN TOGETHER, NO IS EMERGING AS AN IMPORTANT CHEMICAL MEDIATOR OF NEUROENDOCRINE FUNCTION AND BEHAVIOR.

Introduction

As described in earlier chapters, the first biological function of nitric oxide (NO) was discovered in the circulatory system. Subsequently, NO was discovered to play a role in neuronal processes and in immune responses. In general, NO used in these three different roles is produced by three different isoforms of the nitric oxide synthase (NOS) enzyme. NO produced by endothelial NOS (eNOS) causes vasodilation and an increase in blood flow, whereas NO produced by inducible NOS (iNOS) helps macrophages to fight a pathogen. In the brain and other parts of the nervous system, NO derived from neuronal NOS (nNOS) activates signaling pathways and, in many ways, acts as a traditional neurotransmitter. As a signaling molecule, though, NO also has unique properties that are outlined later. NO derived from eNOS and nNOS are both important in regulating several neuroendocrine and behavioral functions.

NO acts on neuroendocrine and behavioral processes generally in one of three ways: (1) indirectly, as NO derived from eNOS mediates blood flow, and differential blood flow can affect neuroendocrine secretion rates; (2) directly, as NO derived from nNOS affects neuronal functioning within the brain; and (3) hormonally, as NO derived from nNOS in endocrine glands or reproductive organs can regulate hormone release. In neuronal processes, glutamate is a common, but not the only, activator of NO release. Because elevated calcium, acting in conjunction with calmodulin, activates nNOS, normal presynaptic activity can cause NO release. As a signaling molecule, NO is unusual as it is a labile molecule at body temperature capable of easily crossing cell membranes. NO does not require a traditional postsynaptic

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membrane-bound receptor. For NO to have a biological effect, however, a response cell must contain a ''receptor.'' The most common target of NO is the soluble guanylate cyclase (sGC) that, once activated, produces the second messenger, cGMP. cGMP can alter the excitability of a neuron by either directly acting on an ion channel, activating a cGMP-dependent protein kinase (PKG) that then can phosphorylate ion channels, or activating a phosphodiesterase that can alter cAMP levels. Depending on the target cell, NO can either increase or decrease the excitability of the target cell. NO was first shown to act as an anterograde messenger in the sea slug *Aplysia,* but it is also capable of acting as a retrograde messenger. Work on the pond snail *Lymnaea* has shown that a presynaptic neuron releasing NO can cause depolarizing potentials in a ''follower'' neuron at distances up to 50 μm, indicating that some NO-releasing and NO-responding cells can be separated by much larger distances than traditional pre- and postsynaptic cells.

The use of pharmacological agents that block synthesis of NO has been important in establishing a role for NO in neuroendocrine and behavioral pathways. A number of studies on the biological actions of NO have involved the use of nonspecific inhibitors of NOS, such as *N*-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (NMMA), or N^G-nitro-L-arginine (L-NNA). A major drawback of these studies is that it remains unclear whether a certain outcome results from the inhibition of nNOS, eNOS, iNOS or a combination of these enzymes, or from alterations in other nonspecific, nitrogen-dependent processes. Specific inhibitors of NOS have been developed, such as 7-nitroindazole (7-NI), that more specifically inhibit nNOS than eNOS. 7-NI also has its limitations because more recent work suggests it can affect dopamine signaling. For any work examining the role of NO in biological pathways, converging evidence using different research approaches will be required. In this chapter, we describe how the use of pharmacological agents that block synthesis of NO, the use of knockout mice that lack one of the various isoforms of NOS, and neuroanatomical studies have revealed that NO appears to play an important role in the control of several neuroendocrine pathways and behaviors.

NO and Neuroendocrine Function

Hypothalamic–Pituitary–Adrenal Axis

The stress response in vertebrates involves the secretion of a group of steroids, the glucocorticoids (e.g., cortisol, in humans), from the cortex of the adrenal gland that act both to increase the mobilization of energy and to suppress nonessential functions, such as reproduction, growth, digestion, and the immune system. The release of glucocorticoids is controlled by the brain, and NO appears to play an important role in activating the hypothalamic–pituitary–adrenal (HPA) axis. A wide range of stressors can activate the HPA axis, and these include a lack of food (starvation), an immediate threat of attack, and the immune system (specifically, high levels of cytokines). Regardless of the stressor, a common feature of the activation of the HPA axis is the release within the hypothalamus of the peptide corticotropin-releasing factor (CRF), which then activates cells in the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the circulatory system. ACTH acts on the adrenal glands to trigger the release of glucocorticoids.

NO release is linked to the activation of the HPA axis and CRF release because NO donors injected into the brain trigger the release of CRF from the hypothalamus. Although, the specific neural pathways by which stressors, such as starvation or the threat of attack, activate CRF release is not known, some of the signaling molecules that alter CRF release are known. These activators of CRF release include the catecholamines, prostaglandins, and neuropeptides. Two neuropeptides, oxytocin and vasopressin, both activate CRF release when injected centrally into the brains of rodents. The actions of both neuropeptides are suppressed by treatment with the inhibitor of NO production L-NAME, suggesting that both of these peptides depend on NO release to trigger CRF release. The most likely targets of oxytocin and vasopressin are CRF-containing cells in the paraventricular nucleus (PVN) of the hypothalamus.

Other activators of CRF include morphine and norepinephrine (NE). Morphine triggers the release both of NO within the median eminence of the hypothalamus and the release of CRF. This release of CRF is NO-dependent, providing another link between NO release and CRF release. Interestingly, the neurotransmitter NE triggers CRF release, but this is not dependent on NO release.

As mentioned, cytokines, including interleukin-1 (IL-1), IL-1β, IL-2, and IL-2β, can trigger the release of CRF from the central nervous system (CNS). The specific pathways by which the interleukins activate CRF release is not known. In the case of IL-1β and IL-2, however, the triggering of CRF release is attenuated by NOS inhibitors, such as NMMA, suggesting the activation of CRF release by these cytokines is NO dependent. It is possible that cytokine actions on CRF release involve the indirect action of NO. For example, some cytokines may trigger increases in NO that then activate the release of NE, prostaglandins, acetylcholine, or neurohormones that in turn elicit the release of CRF. IL-2 and acetylcholine both trigger CRF release from hypothalamus, and both actions are inhibited by NOS inhibitors. Furthermore, it is possible that IL-2 actions are dependent on acetylcholine release.

Neuronal NOS is localized in discrete areas of the brain, including the PVN and supraoptic (SON) nuclei of the hypothalamus, both of which represent the major source of neurons containing CRF. Anatomically, then, NO production and CRF appear to be closely adjoined, but future work will be required to determine the multiple ways that NO release may be linked to CRF release. Taken together, it appears that multiple pathways exist for activating the release of CRF, some of which are NO dependent and some of which are not. A summary of some of the NO-dependent and NO-

independent pathways by which CRF is released is shown in Fig. 1.

Another mechanism for controlling NO and subsequent CRF release could involve the up- or downregulation of the nNOS protein. Within the hypothalamus, IL-1β and CRF, itself, appear to trigger increases in nNOS mRNA. The functional significance of these changes are not known. Much of the research presented in this chapter indicates that NO often plays an excitatory role in causing the release of a signaling molecule. Interestingly, in the adrenal gland, where the glucocorticoids are made and released, nNOS expression is upregulated by high levels of stress. Additionally, in the adrenal gland NO acts to inhibit glucocorticoid release. The ability to increase levels of nNOS in the adrenal gland under stressful conditions in conjunction with NO acting as an inhibitor of glucocorticoid release in the adrenal glands may have developed as a useful mechanism for regulating the activity of the HPA axis.

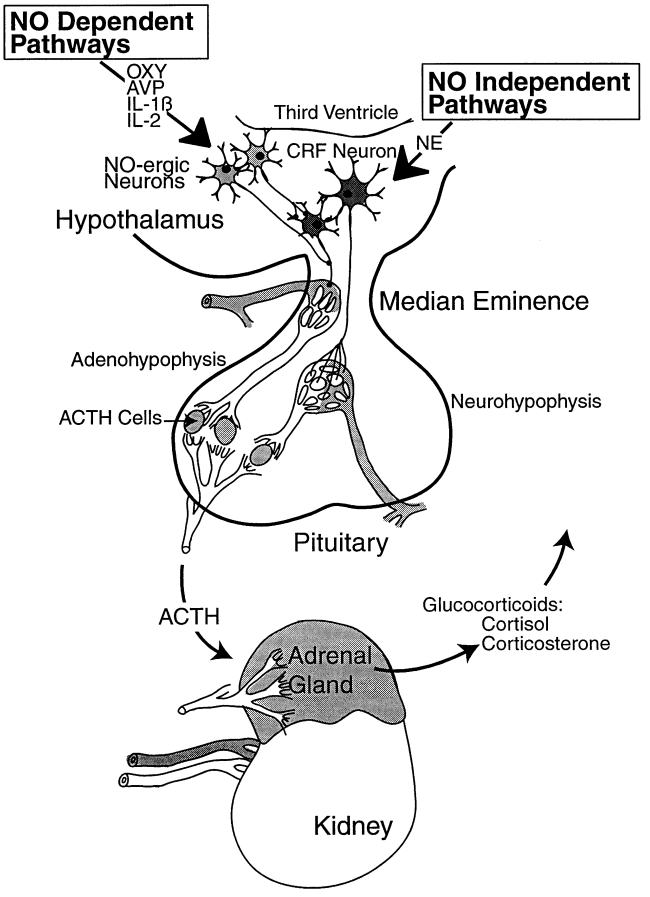


Figure 1 Schematic representation of how NO interacts with the HPA axis. Oxytocin (OXY), vasopressin (AVP), interleukin-1β (IL-1β), and IL-2 all can trigger the release of corticotropin-releasing factor (CRF), but this action is impaired by NOS inhibitors. Norepinephrine (NE) triggers CRF release, but this action is not disrupted by NOS inhibitors. Once released, CRF triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the blood, and ACTH stimulates the release of the glucorticoids (or stress hormones) from the adrenal glands.

Hypothalamic–Pituitary–Gonadal Axis

Neuroendocrine regulation of reproduction in vertebrates is complex and includes the control by the brain of the synthesis and release of sex steroid hormones from the reproductive organs. Of course, the sex steroid hormones themselves act on targets throughout the body, including the brain. At the center of this control over reproduction in males and females is the release of the peptide hormone gonadotropin-releasing hormone (GnRH) from the hypothalamus. NO appears to play an important role in regulating GnRH release. In mammalian species, GnRH neurons are widely distributed and form a loose continuum from the telencephalic diagonal band of Broca, and more dorsal septal areas, to the bed nucleus of the stria terminalis, and diencephalic areas (including subregions of the hypothalamus). GnRH neurons send axonal projections primarily to the median eminence to regulate anterior pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), collectively known as the gonadotropins. In turn, the gonadotropins act on the gonads to regulate steroidogenesis and gametogenesis (LH and FSH, respectively). The activation of the hypothalamic–pituitary–gonadal (HPG) axis enables an animal to reproduce sexually. For GnRH to achieve its maximal effect on the release of LH and FSH, it must be released in a pulsatile manner.

Substantial evidence indicates that the release of GnRH is activated and modulated by the upstream release of NO. Because the release of GnRH is at the center of the control of reproduction, the control of the release of GnRH is the primary mechanism for activating or inhibiting reproduction. Anatomically, nNOS does not colocalize with GnRH neurons, but neurons with nNOS are found to closely adjoin GnRH cells in the hypothalamus and the median eminence. It is not clear whether NO directly acts on GnRH cells, but some evidence suggests that this is a possible pathway. The most common, but not the only, target of NO is the sGC that once activated triggers the production of cGMP. cGMP can then alter cell excitability and neurosecretion by acting through a variety of pathways. sGC was identified in the terminals of GnRH cells in the median eminence, suggesting that NO can directly act on GnRH cells. Additionally, NO application to the median eminence activates a sGC that triggers the release of GnRH. It remains possible that the actions of NO on GnRH cells can be indirect, and these possibilities will need to be explored with future research. Interestingly, immortalized GnRH cells show both nNOS mRNA and the ability to release GnRH when stimulated by NO. The cells also show increasing levels of secretory activity with increasing levels of cGMP, suggesting the use of the NO-activated sGC signaling pathway.

A number of signaling pathways activate NO release and directly, or indirectly trigger GnRH release. Both *in vivo* and *in vitro,* glutamate administration elevates GnRH and LH concentrations. Glutamate often acts on the NMDA receptor (which activates NOS through the influx of Ca^{2+}), and glutamate may act on this subtype of glutamate receptor to reg-

ulate GnRH and gonadotropin secretion. Intracerebroventricular (icv) injections of NMDA lead to increased LH secretion in gonadectomized male and female rats, and NMDA receptor antagonists lead to reductions in GnRH and LH levels. Glutamate may also act through an alternate route to trigger GnRH release. Both glutamate and oxytocin activate the release of NE from the medial basal hypothalamus (MBH), and NE then appears to act on α_1-adrenergic receptors to trigger the release of NO and GnRH. Incubation of hypothalamic explants with NE increases NOS activity; this increased NOS activity is blocked by the addition of prazosine, an α_1-adrenergic receptor antagonist. Likewise, the actions of oxytocin and glutamic acid are blocked by phentolamine, a different inhibitor of the α_1-adrenergic receptor, indicating that the actions of oxytocin and glutamic acid can be indirect. In contrast, NO donors such as sodium nitroprusside (SNP) activate GnRH release in a dose-dependent manner, and this action is not blocked by phentolamine, indicating that NO release is downstream of NE release. Morphine, anandamide, and leptin are other signaling molecules that can trigger increases in NO and GnRH release in the median eminence. For anandamide and morphine, these actions are inhibited by NOS inhibitors, suggesting that in these cases the induction of GnRH release is dependent on NO release.

Cytokines can both inhibit and activate GnRH release. Evidence indicates that IL-2 increases acetylcholine secretion, which triggers NO and GnRH release. Granulocyte macrophage colony-stimulting factor (GMCSF) and IL-1 inhibit GnRH release. Interestingly, GMCSF blocks the ability of NO to trigger the release of GnRH. This block likely results from the GMCSF activating the release of the signaling molecule γ-aminobutyric acid (GABA). The actions of GABA are complex. In some studies GABA has been found to inhibit GnRH cells directly and prevent their activation by NO, whereas other studies report that it stimulates GnRH release. Interestingly, NO can also stimulate the release of GABA; SNP increases GABA release in cortical neurons and MBH explants *in vitro*. Exactly how NO and GABA interact in controlling GnRH release will need to be clarified in future research. A summary of some of the pathways by which NO interacts with GnRH release is shown in Fig. 2.

The immediate targets of GnRH are the gonadotropes in the anterior pituitary that release LH and FSH. NO may also play a role in the activity of the gonadotropes. At proestrus in rodents, levels of nNOS mRNA increase in the gonadotropes, and this increase appears to be activated by GnRH because GnRH antagonists block this increase. It is possible, then, that the increases in nNOS protein cause an increase in NO release within the gonadotropes that triggers increased amounts of LH and FSH release.

In addition to the effects of NO on GnRH secretion, NO is also involved directly in ovulatory processes. Treatment with L-NAME inhibits ovulation in rats. Fertility is reduced in female mice lacking the gene encoding the neuronal isoform of NOS (nNOS$-/-$). To test for physiological defects

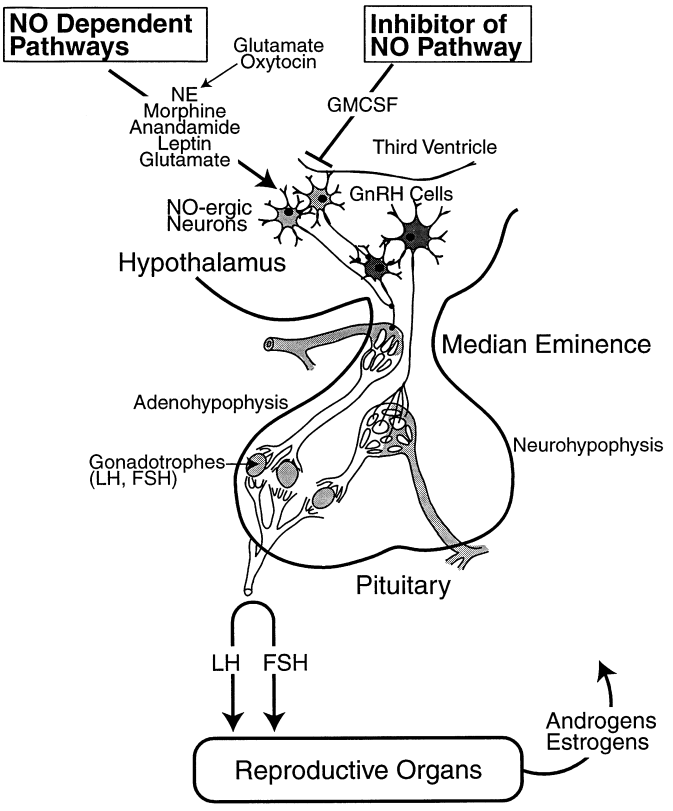


Figure 2 Schematic representation of how NO interacts with the HPG axis. NE, morphine, anandamide, leptin, and glutamate all can trigger GnRH release, but this action is disrupted by NOS inhibitors. Additionally, glutamate and oxytocin can trigger NE release that then activates the release of GnRH through an NO-dependent pathway. Granulocyte macrophage colony-stimulating factor (GMCSF) can prevent NO from stimulating GnRH release. Once released, GnRH triggers the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. LH and FSH, released into the blood, trigger the production and release of androgens and estrogens from the reproductive glands.

that could account for reduced fertility, the rate of ovulation as determined by the number of rupture sites on the ovaries, as well as the number of oocytes recovered from the oviducts, was evaluated. Female wild-type (WT) and nNOS$-/-$ mice did not differ in these parameters after mating with a WT male. Female nNOS$-/-$ mice had fewer ovulation rupture sites than WT females after GnRH treatment. Despite equivalent numbers of ovulation rupture sites after a superovulating dose of pregnant mare serum gonadotropin, fewer oocytes were recovered from the oviducts of nNOS$-/-$ mice as compared to WT mice. Thus, nNOS$-/-$ female mice display normal ovulation during natural estrus or after estrus induction with GnRH injections, suggesting that the central mechanisms of ovulation are intact. However, ova capture by the fallopian tubes was impaired during superovulation. Taken together, these results indicate that nNOS contributes to female fertility. Female eNOS$-/-$ mice display normal estrous cycles and essentially normal ovulatory mechanisms.

Behavioral Effects of NO

Circadian Organization

Behavior is organized on a temporal basis. It is critical for animals to breed, eat, sleep, socialize, drink, and locomote at the appropriate time of day or year. Several studies, using pharmacological agents, have indicated that NO is an important mediator of mammalian biological rhythms. In mammals, synchronization (entrainment) of circadian rhythms to the environmental light–dark cycle is mediated by the transmission of photic information from retinal ganglion cells to the suprachiasmatic nuclei (SCN) of the hypothalamus, which functions as a self-sustained circadian clock. Glutamate is involved in this light transmission process, and it appears to act on NMDA receptors in the SCN. Glutamate binding can activate NOS. Several lines of evidence implicates NO in light transmission from the eye to the SCN. Administration of L-NAME blocks the effects of light on biological rhythms in locomotor activity or physiological processes in the SCN. L-NAME has many nonspecific effects on blood flow and pressure in the brain because it inhibits both eNOS and nNOS. Consequently, L-NAME also affects many other nitrogen-dependent physiological processes.

Despite the pharmacological evidence that NO is crucial in mediating circadian rhythms, work with transgenic mice suggests otherwise. Both nNOS−/− and eNOS−/− mice show normal entrainment to daily light–dark cycles, and they showed normal phase shifts of locomotor activity in response to light pulses. Further research is necessary to clarify the contribution of NO to circadian organization of behavior and physiology.

One study suggests that NO plays some role in the temporal organization of behavior. Neuronal NOS−/− mice have no obvious defects in locomotor activity, breeding, long-term depression in the cerebellum, long-term potentiation in the hippocampus, or overall sensorimotor function. Because the cerebellum possesses the greatest numbers of nNOS neurons in the brain, it was surprising that presumed cerebellar functions such as balance and coordination were generally normal in nNOS−/− mice. However, this conclusion was based on studies that were conducted during the day (between 1400 and 1600 hours, lights on at 0700 hours) when the animals are typically inactive. When the animals were tested during their nocturnal, active period, dramatic abnormalities in balance and motor coordination were evident in nNOS−/− mice.

Reproductive Behaviors

NO affects reproductive behavior. There have been reports that both sexual motivation and performance are influenced by NO, though much of the effects of NO on male sexual performance reflect the direct consequences of the well-established effects of NO on penile erectile function. Generally, sexual motivation in male rats is not affected by manipulations of NO levels. Treatment of male rats with NOS inhibitors (i.e., L-NAME) did not reduce the latency to first mount or the number of mounting attempts of estrous females by male rats. Male nNOS−/− mice displayed normal sexual motivation to mount with estrous females; however, male nNOS−/− mice exhibited inappropriate persistent mounting attempts of anestrous females.

Administration (intraperitoneal) of L-arginine, the natural substrate for NOS, increased the percentage of naive male rats copulating and improved sexual performance in sexually experienced males. These behavioral effects were reversed in rats receiving systemic injections of L-NAME. Administration (icv) of L-arginine did not affect reproductive behavior. L-NAME icv infusion prevented ejaculation of naive rats (100 μg/rat), but did not affect ejaculation of experienced male rats at doses up to 300 μg/rat. Systemic treatment of sexually naive male rats with 30 or 60 mg/kg of L-NAME reduced the number of ejaculations by 43 and 86%, respectively. In both experimental groups, the number of intromissions was reduced, although mounting behaviors were elevated; increased number of mounts is common with penile erectile dysfunction in rats. The number of ultrasonic vocalizations increased; these vocalizations are an important component of sexual behavior in rats. Systemic L-NAME administration impaired copulation, reduced the number of *ex copula* penile erections, increased the number of *ex copula* seminal emissions, and decreased the latency of first seminal emission of male rats. Treatment with L-arginine increased the number of penile reflexes but had no other effects on male rat reproductive behavior. There is no obvious explanation for the conflicting reports on the behavioral effects of L-arginine treatment in rats. Male mice with targeted deletion of the gene encoding nNOS exhibited equivalent penile intromissions and ejaculations as WT males when paired with estrous females. This outcome was surprising because it had been firmly established using L-NAME and other NOS inhibitors that NO mediated penile erectile function. However, it appears that eNOS is upregulated in nNOS−/− animals, and that eNOS, not nNOS, mediates penile erections.

NO functions in the brain to affect sexual behavior. Expression of nNOS mRNA in the PVN of the hypothalamus was nearly doubled in sexually potent male rats as compared to impotent males. Reproductive performance in male golden hamsters (*Mesocricetus auratus*) is dependent on chemosensory and endocrine stimuli processed in limbic neural circuits. Neurons expressing nNOS in hamster brains were discovered in the medial amygdala nucleus (Me), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA). nNOS-positive fibers were observed in the stria terminalis and the ventral amygdalo–fugal pathway; this pathway connects the Me with the BNST and MPOA. Several of the NOS-positive neurons in the ME and the medial preotic nucleus (MPN) also contained androgen receptors. Castration decreased the number of NOS-positive neurons in the MPN, suggesting that NOS may be regulated by gonadal steroids.

The involvement of NO in male reproductive behavior appears to be conserved throughout vertebrate evolution. NO plays an important role during courtship of urodele crested newts (*Triturus carnifex*). Courtship is complex in this species with four distinct stages of courtship. Females that are receptive remain relatively immobile and close to the male during his approach, fanning, lashing, and spermatophore deposition, whereas nonreceptive females move away from courting males. Some males do not court in the presence of receptive females. Brain NOS was measured in receptive and nonreceptive females, in males at various points during courtship, and in noncourting males. Mating success of male newts was characterized by high brain NOS activity that progressively increases during each of the courtship phases and returned to baseline levels after spermatophore deposition. Inactive males exhibited the lowest brain NOS activity. There was no difference among female newts regardless of their receptive state. Thus, NO appears important in male, but not female, newt reproductive behavior.

NO appears to mediate female sexual behavior in rats. Receptive behavior can be elicited in estrogen-primed, ovariectomized rats with progesterone treatment. Receptive behavior is usually indicated by the display of the mating posture, lordosis, in response to male mounting behavior. When N^G-monomethyl-L-arginine was microinjected icv into the third cerebral ventricle of awake and freely moving female rats, lordosis was not observed after progesterone administration. Microinjections (icv) of N^G-monomethyl-D-arginine, a compound that does not inhibit NO production, did not inhibit lordosis after progesterone administration. Microinjections (icv) of SNP, a substance that spontaneously releases NO, promoted the display of lordosis of estrogen-primed rats in the absence of progesterone. Lordosis that was evoked in estrogen-primed female rats by either progesterone or SNP could be blocked by microinjections (icv) of GnRH antiserum. Thus, it appears that progesterone normally causes the release of NO that stimulates GnRH secretion from the hypothalamus, which facilitates lordosis in rats.

In contrast, mating behavior appeared normal in nNOS−/− female mice, though fertility rates are lower than for WT mice. nNOS−/− females did not differ from WT females in the latency to first lordosis posture or in the number of lordosis postures during a 30-min mating test. If impregnated, then nNOS−/− and WT females had equivalent litter sizes at birth and at weaning, and their litters displayed normal sex ratios. As noted above, NO appears to be involved in the mechanisms of ovulation, and the reduction of fertility among nNOS−/− females might reflect this physiological defect.

Aggressive Behaviors

There are no published reports that L-NAME or other nonspecific NOS inhibitors affect aggression. However, nNOS−/− males are dramatically more aggressive and sexually persistent than WT mice. When examined in an intruder–resident test of aggression, these nNOS−/− mice engaged in three to four times more aggressive encounters than WT mice. Nearly 90% of the aggressive encounters were initiated by the nNOS−/− animals. Similar results were obtained in dyadic or group encounters in neutral arenas. In all test situations, male nNOS−/− mice rarely displayed submissive behaviors.

Behavioral studies of mice with targeted deletion of specific genes suffer from the criticism that the gene product is not only missing during the testing period, but missing throughout development when critical ontogenetic processes, including activation of compensatory mechanisms, may be affected. To address this criticism, mice were treated with 7-NI, a specific inhibitor of nNOS formation *in vivo.* Mice treated with 7-NI displayed substantially increased aggression in two different tests of aggressiveness as compared to control animals. Drug treatment did not affect nonspecific locomotor activities. Importantly, NOS activity in brain homogenates was reduced >90% in 7-NI-treated mice. Similarly, immunohistochemical staining for citrulline revealed a dramatic reduction in 7-NI-treated animals. Taken together, 7-NI inhibited NOS staining in the limbic system and reduced citrulline immunohistochemical staining, suggesting that NO formation was virtually eliminated in these experimental animals. Because 7-NI treatment in WT mice caused aggression to be elevated to the levels displayed by nNOS−/− mice, it appears that nNOS is an important mediator of aggression.

Plasma androgen concentrations can affect the display of aggressive behavior. There were no differences between nNOS−/− and WT mice in blood testosterone concentrations either before or after agonistic encounters. However, more recent data on castrated nNOS−/− males suggest that testosterone is necessary, but not sufficient to promote increased aggression. Castrated nNOS−/− mice displayed low levels of aggression that were equivalent to the aggression observed among castrated WT males. Androgen-replacement therapy restored the elevated levels of aggression in nNOS−/− mice. Importantly, inappropriate aggressiveness was never observed among female nNOS−/− mice; however, aggressive behavior was not examined in female nNOS−/− mice in the context of maternal aggression, during which WT females are highly aggressive toward an intruder. There were no sensorimotor deficits among the mutant mice to account for the increased aggression.

It is interesting to note that males lacking the gene encoding the endothelial isoform of NOS are virtually never aggressive. Although eNOS was initially reported to be localized in neural tissue, work using *in situ* hybridization failed to locate eNOS in neurons; eNOS is found only in moderate to large blood vessels in the brain. Why these mice are so docile is not known. Blood concentrations of testosterone are normal. eNOS−/− males have elevated blood pressure, but these mice remain very docile even when the hypertension is treated. Perhaps NO from endothelial sources in the brain relax the blood vessels and allow more blood flow and, subsequently, more neuroendocrine products

to be delivered. A summary of the reproductive and aggressive behaviors of the nNOS−/− and eNOS−/− mice is shown in Fig. 3.

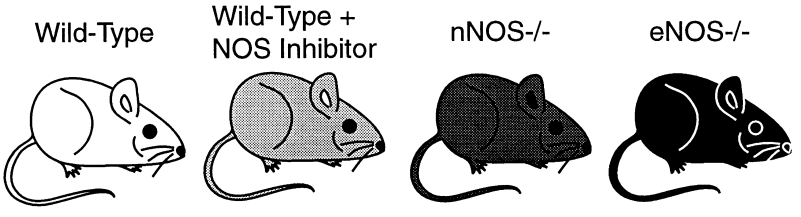
Female mice typically only display aggression when defending a nest of newborn pups. The onset of maternal aggressive behavior is dependent on the hormones released during pregnancy and lactation, whereas maintenance of maternal aggression depends on sensory feedback from the nursing pups. Using antibodies to citrulline, an indirect indicator of NO, it was determined that a small group of neurons in the hypothalamus was involved in the regulation of maternal aggression. Furthermore, nNOS−/− mothers showed normal maternal care, but no maternal aggression in response to a male intruder. Mothers missing the gene encoding eNOS display normal maternal aggression. These results suggest that female mice release NO from neurons within specific regions of the hypothalamus to trigger maternal aggression. These results also indicate that males and females use fundamentally different mechanisms to regulate aggression.

Regulation of Feeding

As with many behaviors, feeding behavior can be triggered by a number of stimuli. For example, the sight and smell of food, hunger, and even the time of day can all help to trigger feeding behavior. Feeding behavior itself is complex and includes sniffing, chewing, swallowing, and digesting. The neural pathways underlying feeding are largely unknown, but it is likely that the stimulation of olfactory and visual pathways helps to activate the neural circuitry underlying this behavior. A range of evidence suggests that NO, acting as a neuromodulator, is released in the brain during feeding behavior.

NOS blockers consistently decrease feeding in mice, rats, and chickens, suggesting that NO is used to activate feeding behavior. For example, in food-deprived rats and mice, the NOS inhibitor L-NNA decreases food intake even though the animals should be highly motivated to eat. This response, however, can be reversed by L-arginine, suggesting that feeding can be reinitiated by the production of NO. Additional evidence that NO activates feeding comes from research on ''fat'' rats and mice. In the obese rats, the NOS inhibitor L-NAME decreases feeding. As one might expect, the obese mice exhibit increased levels of nNOS in the brain relative to WT, indicating that the obesity may result from the overproduction of NO and overstimulation of the feeding circuits.

NO may activate feeding behavior, but the site of its actions within the feeding neural circuit is unknown. Central NE administration increases food intake in rats, and this increase is reduced by coadministration of α_2-receptor antagonists, suggesting that NE acts on α_2-receptors to modulate feeding. In chickens, a specific α_2-agonist, clonidine, increases food intake, but coadministration of L-NNA attenuates this increased food intake in a dose-dependent manner, indicating that NO release may be downstream of NE action. NOS has been localized to areas known to be involved in the control of feeding, particularly in the PVN of the hypothalamus. Food deprivation studies have documented a decrease in the levels of nNOS in the PVN and SON of the hypothal-



Reproductive Behavior				
Mounting estrous females	Normal	Normal (L-NAME)	Normal	Normal
Mounting anestrous females	Normal		Inappropriate Mounting	Normal
Lordosis in Females	Normal		Normal	Normal
Fertility in Females	Normal	Decreased (L-NAME)	Decreased	Decreased
Aggressive Behavior				
Latency to First Attack	Normal		Decreased	Increased
Number of Attacks	Normal	Increased (7-NI)	Increased	Decreased
Duration of Attacks	Normal	Increased (7-NI)	Increased	Decreased

Figure 3 Summary of reproductive and aggressive phenotypes of wild-type mice, wild-type mice exposed to pharmacological inhibitors of NOS, and knockout mice lacking nNOS and eNOS. The aggressive behavior is specific to male mice only. The use of the specific nNOS inhibitor 7-NI produced a behavioral phenotype that matches the nNOS−/− mice but not the eNOS−/− mice.

amus of rats. The PVN (and also the SON) play a role in reproductive behaviors, and consequently, it is possible that decreases in nNOS and NO production in these nuclei help to shut down reproductive behaviors in times of low food. Interestingly, nNOS levels are increased in the PVN and SON during water deprivation, indicating that NO release may be involved in drinking behavior.

The relative amount of food intake decreases as animals, including humans, age. One possibility for this observation is that diminished production of NO accounts for decreased food intake during aging. In mice, the NOS inhibitor L-NAME shows greater efficacy in reducing feeding in older animals, indicating that mice may rely increasingly on NO to activate feeding as they age. Whether decreases in food intake during aging in rodents, or humans, results from changes in the production or use of NO remains to be determined.

As with the activation of any neural circuit underlying a behavior, the release of a given neurotransmitter, or neuromodulator, such as NO, not only results from the release of a upstream signaling molecule, but also triggers the release of a downstream signaling molecule. Which cells trigger NO release, which cells release NO, and which cells respond to NO in feeding behavior remain unspecified. The peptide hormone leptin is involved in controlling body weight of rodents, and increased leptin concentrations result in decreased food intake. Interestingly, central injection of leptin causes a decrease in NO production, and it is possible that leptin reduces feeding by inhibiting NO release. The neuromodulator serotonin (5-HT) may also play a role in controlling feeding, but whether or how serotonin and NO interact remains to be determined. Food deprivation leads to a reduction in brain 5-HT levels in rats. L-NNA reduces food intake in food-deprived animals but increases 5-HT levels in the diencephalon of rats. This inverse relationship between 5-HT and NOS levels suggests that NO may regulate feeding through a 5-HT mechanism.

Invertebrate Feeding Behavior

In the pond snail *Lymnaea stagnalis,* feeding behavior is simpler than that in vertebrates and involves a stereotyped pattern of muscle contractions that is driven by a stereotyped firing activity of motoneurons in the CNS. The production of snail feeding behavior is extremely sensitive to sensory input, and application of sugar water to the lips alone will activate the feeding behavior. An advantage of studying invertebrate behaviors is that the CNS can be isolated from the body and still can produce a behavioral motor output. In the case of *Lymnaea,* the lips and the CNS (still attached to one another) can be isolated, and application of sugar water to the lips activates a fictive feeding behavior from the CNS. As in vertebrates, NO plays a role in activating the feeding circuit. The sensory cells in the snail lips contain high levels of NOS, and application of an NO donor activates feeding behavior, even in the absence of sugar water. Likewise, use of hemoglobin, an NO scavenger, disrupts the ability of sugar water applied to the lips to activate the feeding motor output, indicating that NO is normally released to trigger feeding.

As mentioned, feeding behavior in *Lymnaea* involves a stereotyped output of motoneuron firing. This pattern of firing is driven by a central pattern generator, and within the CNS the electrical stimulation of individual modulatory interneurons can also trigger the stereotyped fictive feeding behavior. Interestingly, NO release only appears to be involved in the activation of modulatory interneurons by sensory neurons because NO scavengers inhibit the ability of sucrose to stimulate these interneurons and the behavior, but they do not disrupt the ability of the modulatory interneurons to trigger the behavior. Consequently, the release of NO by sensory neurons as a result of sugar water activation is the first step and possibly the only role NO release plays in activating snail feeding. Among vertebrates, whether NO acts only at one level and where in the pathway NO release occurs in stimulating feeding remain unknown. An additional complication of feeding in both vertebrates and invertebrates is whether NO plays a role in activating movement and locomotory behaviors involved in the searching for food.

Learning and Memory

Inhibition of NOS impairs spatial learning in mice and rats. It appears that NO is important during initial acquisition, but according to many researchers the role of NO on the mechanisms of spatial learning remains unspecified. NOS inhibitors might affect learning by affecting blood pressure or other nitrogen-dependent processes. NOS inhibition has also been reported to impair social and olfactory memory as well as performance in some inhibitory avoidance learning paradigms in rats. Other laboratories have reported no effect of NOS inhibition on passive avoidance. Inhibition of NOS does not impair visual or spatial discrimination, nor does it affect sensorimotor or motivational processes. Sensorimotor skills are not impaired in nNOS−/− mice.

The role of NO in learning and memory appears to have been conserved throughout the animal kingdom. NOS inhibition has been reported to impair learning and memory processes in honeybees (*Apis mellifera*), crickets (*Pteronemobius* spp), octopuses (*Octopus vulgaris*), and goldfish (*Carassius auratus*).

Many reports suggest that NO is part of the mechanism underlying learning and memory. Long-term potentiation (LTP) and long-term depression (LTD) are stable and enduring increases or decreases, respectively, in the magnitude of neuronal responses after afferent cells to the region have been stimulated with bursts of electrical stimulation of relatively high or low frequency, respectively. Because afferent neural activity affects firing patterns, the existence of retrograde messengers has been proposed. NO is a candidate molecule to serve as the retrograde messenger in LTP and LTD, although there have been conflicting reports about the role of NO in the mediation of LTP and LTD.

Again, LTP and LTD are forms of synaptic plasticity that are thought to be involved with learning and memory. Calcium influx through postsynaptic NMDA receptor channels and enhancement of glutamate release by Schaeffer collaterals is thought to be required for induction of LTP in the CA1 region of hippocampal slices; further, it has been hypothesized that LTP requires release of a retrograde messenger from postsynaptic CA1 pyramidal cells. NO may act as a retrograde messenger because NO produces an increase in the frequency of spontaneous miniature excitatory postsynaptic potentials and observations that direct application of NO elicits LTP. The establishment of LTP is blocked by NOS inhibitors and hemoglobin that binds NO blocks LTP. Furthermore, NOS inhibitors directly injected into CA1 pyramidal neurons block LTP. Support for the contention that postsynaptically generated NO acts directly on the postsynaptic neuron to produce LTP is the observation that intracellular application of an NO scavenger or use of a UV-sensitive NO donor elicits LTP. Similar evidence exists for a role of NO in LTD. Genetic knockout of nNOS or eNOS failed to support the role of NO in LTP. LTP in nNOS null or eNOS null mice appeared normal and can still be blocked by NOS inhibition. Kandel and colleagues generated double knockout mice lacking both nNOS and eNOS expression and reported that hippocampal LTP in the stratum radiatum of CA1 was markedly reduced. These mutant mice showed normal LTP in the stratum oriens of hippocampal CA1. Thus, there is both NO-dependent and NO-independent LTP. LTP in wild-type mice may be mediated through eNOS as adenovirus-mediated disruption of eNOS activity in wild-type hippocampal slices eliminates NO-dependent LTP. Some studies suggest that NO might help regulate the threshold for LTP induction. An overview of the possible site of action of NO in mediating LTP in the hippocampus is shown in Fig. 4.

Other studies also support a role for NO in synaptic plasticity. Calcium-dependent activation of nNOS and NO generation via stimulation of the NMDA receptor in cultured cortical neurons activates the Ras–ERK (extracellular signal receptor kinase) pathway. NMDA-stimulated phosphorylation of the cyclic AMP-response element binding protein (CREB), a downstream effector of ERK, is also NO dependent. Activation of the Ras–ERK pathway by NO may be mediated by direct activation of Ras GTPase activity, presumably by nitrosylation of cysteine through a redox-sensitive interaction. Because calcium-dependent activation of the Ras–MAPK pathway is considered to be a major pathway of neural activity-dependent long-term changes in the nervous system, NO may be a key mediator linking activity to gene expression and long-term plasticity.

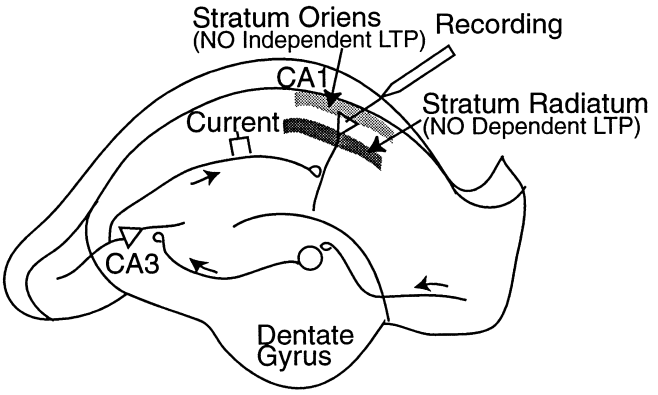


Figure 4 Schematic diagram of the hippocampus and the subregions where NO-dependent and NO-independent long-term potentiation (LTP) may occur. Information in the hippocampus typically flows from the dendate gyrus to the CA3 region and on to the CA1 region. LTP can be recorded from the CA1 region. In one subregion of the CA1, the stratum radiatum, LTP is impaired in mice lacking eNOS and nNOS, but in a second subregion, the stratum oriens, LTP is normal, suggesting that NO-dependent and NO-independent forms of LTP exist.

Conclusions

The release of NO plays an integral role in several neuroendocrine pathways and is a well-established mediator of a number of behaviors. Neuroendocrine pathways and behaviors are often associated with one another, and NO may play a role in maintaining this link. Because NO can regulate blood flow and act as a neuromodulator, it possesses two main mechanisms for controlling neurosecretion and behavior. Significant progress has been made in understanding how the release of NO is controlled, where NO is released, and how NO acts on its targets. With the advent of pharmacological inhibitors of specific isoforms of NOS as well as spatial and temporal gene activation and inactivation, it is likely that additional neuroendocrine and behavioral roles of NO will be discovered.

Additional Reading

Amir, S. (1995). Nitric oxide signaling in the hypothalamus. *In* ''Nitric Oxide in the Nervous System'' (S. Vincent, ed.), pp. 151–162. Academic Press, New York.

Arancio, O., Kiebler, M., Lee, C. J., Lev-Ram, V., Tsien, R. Y., Kandel, E. R., and Hawkins, R. D. (1996). Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons. *Cell* **87,** 1025–1035.

Berabeu, R., de Stein, M. L., Fin, C., Izquierdo, I., and Medina, J. H. (1995). Role of hippocampal NO in the acquisition and consolidation of inhibitory avoidance learning. *Neuroreport* **6,** 1498–1500.

Bialy, M., Beck, J., Abramczyk, P., Trzebski, A., and Przbylski, J. (1996). Sexual behavior in male rats after nitric oxide synthase inhibition. *Physiol. Behav.* **60,** 139–143.

Bliss, T. V. P., and Collingridge, G. L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature* **361,** 31–39.

Burnett, A. L., Lowenstein, C. J., Bredt, D. S., Chang, T. S., and Snyder, S. H. (1992). Nitric oxide: A physiologic mediator of penile erection. *Science* **257,** 401–403.

Burnett, A. L., Nelson, R. J., Calvin, D. C., Liu, J.-X., Demas, G. E., Klein, S. L., Kriegsfeld, L. J., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1996). Nitric oxide dependent penile erection in mice lacking neuronal nitric oxide synthase. *Mol. Med.* **2,** 288–296.

Canteros, G., Rettori, V., Genaro, A., Suburo, A., Gimeno, M., and McCann, S. M. (1996). Nitric oxide synthase content of hypothalamic ex-

plants: Increased by norepinephrine and inactivated by NO and cGMP. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4246–4250.

Ceccatelli, S., Lundberg, J. M., Fahrenkrug, J., Bredt, D. S., Snyder, S. H., and Hokfelt, T. (1992). Evidence for involvement of nitric oxide in the regulation of hypothalamic portal blood flow. *Neuroscience* **51,** 769–772.

Dawson, T. M., and Dawson, V. L. (1996). Nitric oxide synthase: Role as a transmitter/mediator in the brain and endocrine system. *Annu. Rev. Med.* **47,** 219–227.

Dawson, T. M., and Snyder, S. H. (1994). Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. *J. Neurosci.* **14,** 5147–5159.

Demas, G. E., Eliasson, M. J. L., Dawson, T. M., Dawson, V. L., Kriegsfeld, L. J., Nelson, R. J., and Snyder, S. H. (1997). Inhibition of neuronal nitric oxide synthase increases aggressive behavior in mice. *Mol. Med.* **3,** 611–617.

Ding, J. M., Chen, D., Weber, E. T., Faiman, L. E., Rea, M. A., and Gillette, M. U. (1994). Resetting the biological clock: Mediation of nocturnal circadian shifts by glutamate and NO. *Science* **266,** 1713–1717.

Elphick, M. R., Kemenes, G., Staras, K., and O'Shea, M. (1995). Behavioral role for nitric oxide in chemosensory activation of feeding in a mollusc. *J. Neurosci.* **15,** 7653–7664.

Grossman, A. B., Rossansmith, W. G., Kabigting, E. B., Cadd, G., Clifton, D., and Steiner, R. A. (1994). The distribution of hypothalamic nitric oxide synthase mRNA in relation to gonadotropin-releasing hormone neurons. *J. Endocrinol.* **140,** 163–168.

Herbison, A. E., Simonian, S. X., Norris, P. J., and Emson, P. C. (1996). Relationship of neuronal nitric oxide synthase immunoreactivity to GnRH neurons in the ovariectomized and intact female rat. *J. Neuroendocrinol.* **8,** 73–82.

Klein, S. L., Carnovale, D. M., Burnett, A. L., Wallach, E. E., Zacur, H., Crone, J. K., Dawson, V. L., Nelson, R. J., and Dawson, T. M. (1998). Impaired ovulation in mice with targeted deletion of the neuronal isoform of nitric oxide synthase. *Mol. Med.* **4,** 658–664.

Kriegsfeld, L. J., Dawson, T. M., Dawson, V. L., Nelson, R. J., and Snyder, S. H. (1997). Aggressive behavior in male mice lacking the gene for nNOS is testosterone-dependent. *Brain Res.* **769,** 66–70.

Linden, D. J., and Connor, J. A. (1995). Long-term synaptic depression. *Annu. Rev. Neurosci.* **18,** 319–357.

McCann, S. M., and Rettori, V. (1996). The role of nitric oxide in reproduction. *Proc. Soc. Exp. Biol. Med.* **211,** 7–15.

McCann, S. M., Kimura, M., Karanth, S., Yu, W. H., and Rettori, V. (1997). Nitric oxide controls the hypothalamic–pituitary response to cytokines. *Neuroimmunomodulation* **4,** 98–106.

Malen, P. L., and Chapman, P. F. (1997). Nitric oxide facilitates long-term potentiation, but not long-term depression. *J. Neurosci.* **17,** 2645–2651.

Mani, S. K., Allen, J. M., Rettori, V., McCann, S. M., O'Malley, B. W., and Clark, J. H. (1994). Nitric oxide mediates sexual behavior in female rats. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6468–6472.

Nelson, R. J. (1997). The use of genetic ''knock-out'' mice in behavioral endocrinology research. *Horm. Behav.* **31,** 188–196.

Nelson, R. J., and Young, K. A. (1998). Behavioral effects of targeted disruption of specific genes. *Neurosci. Biobehav. Rev.* **22,** 453–462.

Nelson, R. J., Demas, G. E., Huang, P., Fishman, M. C., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1995). Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature* **378,** 383–386.

Nelson, R. J., Kriegsfeld, L. J., Dawson, V. L., and Dawson, T. M. (1997). Role of nitric oxide in neuroendocrine regulation of physiology and behavior. *Front. Neuroendocrinol.* **18,** 463–491.

O'Dell, T. J., Hawkins, R. D., Kandel, E. R., and Arancio, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: Evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 11285–11289.

Park, J.-H., Straub, V. A., and O'Shea, M. (1998). Anterograde signaling by nitric oxide: Characterization and *in vitro* reconstitution of an identified nitrergic synapse. *J. Neurosci.* **18,** 5463–5476.

Rettori, V., Belova, N., Dees, W. L., Nyberg, C. L., Gimeno, M., and McCann, S. M. (1993). Role of nitric oxide in the control of luteinizing hormone-releasing hormone release *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10130–10134.

Seilicovich, A., Duvilanski, B. H., Pisera, D., Theas, S., Gimeno, M., Rettori, V., and McCann, S. M. (1995). Nitric oxide inhibits hypothalamic luteinizing hormone–releasing hormone release by releasing γ-aminobutyric acid. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 3421–3424.

Son, H., Hawkins, R. D., Martin, K., Kiebler, M., Huang, P. L., Fishman, M. C., and Kandel, E. R. (1996). Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* **87,** 1015–1023.

Zerani, M., and Gobbetti, A. (1996). NO sexual behaviour in newts. *Nature* **382,** 31.

CHAPTER 28

Nitric Oxide and Cerebral Ischemia

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IN THIS CHAPTER, WE REVIEW THE ROLE OF NO IN REGULATION OF THE CEREBRAL CIRCULATION AND AS A MECHANISM OF BRAIN INJURY DURING EXPERIMENTAL STROKE AND CEREBRAL ISCHEMIA. IT IS CLEAR THAT THERE ARE A VARIETY OF NO-LINKED MECHANISMS THAT PROTECT VASCULAR FUNCTION DURING ISCHEMIA BUT THAT ALSO POSE A THREAT TO NEURONS, PARTICULARLY GLUTAMATERGIC NEURONS. PRO-OXIDANT MECHANISMS INVOLVING THE CHEMICAL INTERACTION OF NO AND SUPEROXIDE AND FORMATION OF PEROXYNITRITE LEAD TO MITOCHONDRIAL DYSFUNCTION AND DNA DAMAGE. SUCH PATHOLOGY ENTRAINS DOWNSTREAM MECHANISMS LINKED TO PARP ACTIVATION AND FURTHER ENERGY DEPLETION. THE ESSENTIAL PRINCIPLE TO BE GLEANED IS THAT THE OVERALL ROLE OF NO IN CEREBRAL ISCHEMIA IS DEPENDENT ON A DYNAMIC BALANCE BETWEEN THESE TWO STATES: DIRECT NEUROTOXICITY DUE TO NO OVERLOAD AND VASCULAR DEFECTS DUE TO NO DEFICIENCY. SIMILARLY, THE THERAPEUTIC VALUE OF INHIBITING NOS AND NO FORMATION DEPENDS ON OUR ABILITY TO MANIPULATE THIS BALANCE EFFECTIVELY.

Introduction

Cerebral ischemia is defined as a reduction in cerebral blood flow (CBF). Cerebral ischemia may occur as a result of a variety of different pathological mechanisms, and it may result in devastating neurological sequalae. Ischemia may be complete and global, in which case CBF ceases (e.g., cardiac arrest, severe increase in intracranial pressure); it may be incomplete and global, in which case CBF is reduced throughout the brain (e.g., severe hypotension, moderate increase in intracranial pressure); or it may be focal, in which case there is a gradient of CBF reduction, with the greatest reduction occurring in the core of the lesion and less reduction in regions more distant from the core (e.g., ischemic stroke). In this chapter we provide an overview of basic principles regarding the role of nitric oxide (NO) in control of baseline CBF and blood flow in important peri-ischemic states such as hypoglycemia and seizures; we also discuss the role that NO plays in the evolution of experimental stroke and ischemia as well as the evidence for the importance of interrupting NO overproduction and downstream mechanisms of injury.

Because NO is highly reactive and difficult to measure, much of our understanding of this molecule in the cerebral circulation and in brain injury comes from the study of its synthetic enzyme family, the nitric oxide synthases (NOSs). At present, three distinct isoforms have been characterized; each one is the product of a different gene. All known isoforms utilize arginine and NADPH as substrates and produce NO as a coproduct with citrulline. The characterization of NOS was first accomplished in resting endothelium; therefore it was considered to be constitutive and labeled as eNOS (type III by later nomenclature). In activated macrophages and cells such as glia, NOS was found to be calcium independent and to be induced by combinations of cytokines; consequently, it was referred to as inducible NOS (iNOS or type II NOS). A third general category of NOSs, neuronal NOS (nNOS or type I), is particularly important in brain because of its abundance relative to other isoforms. Like eNOS, nNOS is calcium dependent and historically con-

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sidered to be constitutive, although it is increasingly clear that this ''constitutive'' enzyme is actually subject to expressional control by transcript diversity and by several brain conditions, including cerebral ischemia. Consequently, nNOS is not static, and cell-type-specific transcription and splicing factors may increase or decrease its expression in naive or injured tissue (for review, see Boissel *et al.,* 1998).

All of the NOS isoforms have been implicated in the pathophysiology of cerebral ischemia and in regulation of CBF. In addition, accumulating evidence suggests diverse expression of NOS isoforms in tissue other than endothelial cells, neurons, glia, and inflammatory cells. For example, both iNOS and nNOS are expressed and functional in vascular smooth muscle under a variety of physiological and disease-related states (Gunnett *et al.,* 1998; Papadaki *et al.,* 1998; Boulanger *et al.,* 1998; Meng *et al.,* 1998; Hecker *et al.,* 1999). Further, there can be crossover in expression of NOS isoforms within the normal brain, for example, expression of eNOS in hippocampal neurons (Dinerman *et al.,* 1994). Vascular behavior and ischemic outcomes in the intact animal must therefore be analyzed with these complexities in mind.

Last, much of our understanding of NO-mediated ischemic injury has been based on data with enzyme inhibitors of limited specificity among NOS isoforms. Specific eNOS inhibitors are not yet available as pharmacological probes, although the crystal structure of the enzyme has been resolved (Fischmann *et al.,* 1999). Selective nNOS inhibitors have been developed, but their utility, *in vivo,* has been limited by poor solubility in physiological solutions, poor blood–brain barrier permeability characteristics, and only moderate specificity for the nNOS isoform. When comparing results from different laboratories, it is important to have an accurate quantitation of the NOS inhibition that is produced by the particular pharmacological probe in each study. The activity of the enzyme is most frequently assessed by measuring labeled citrulline production in brain biopsy samples post mortem (Traystman *et al.,* 1995) or in *in vivo* microdialysates (Bhardwaj *et al.,* 1995, 1997a,b), or activity is assessed by a more indirect approach such as measuring a physiological response to an intervention known to be mediated through NO. One conclusion drawn from these numerous studies is that even in the presence of a standard dose of inhibitor, the resulting degree of NOS inhibition in brain is variable and depends on the route of administration and the species and strain under investigation (Traystman *et al.,* 1995). In addition, current techniques for confirming functional enzyme inhibition can only provide a measurement of whole-tissue NOS activity, that is, the combined activity of all isoforms without distinction among nNOS, eNOS, or iNOS.

NO and Basal Cerebral Blood Flow

Both cerebral endothelium and, less prominently, nitroxidergic neural activity supply NO to vascular smooth muscle, activating soluble guanylyl cyclase. This results in conversion of guanosine triphosphate to 3',5'-cyclic guanosine monophosphate (cGMP) and in vascular relaxation (Ignarro *et al.,* 1982, 1987). In addition to direct vasodilation, NO can inhibit vasoconstriction produced by other agonists (e.g., endothelin 1) (Chen *et al.,* 1999). Less well studied is nitroxidergic vasodilation within brain. Cerebral arteries, including the anterior and middle cerebral arteries, caudal circle of Willis, and some pial vessels, are innervated by NOS-containing nerves that originate largely via the sphenopalatine ganglia (Nozaki *et al.,* 1993). Electrical stimulation of porcine cerebral vessels results in NO liberation and vessel relaxation (Toda and Okamura, 1992; Tanaka *et al.,* 1999).

In general, intravenous administration of a nonspecific NOS inhibitor decreases CBF (Greenberg *et al.,* 1994). Regions with poor blood–brain barrier function and with high NOS activity exhibit the largest reductions in CBF after administration of a nonspecific inhibitor. The decrease in CBF is not associated with a change in cerebral oxygen consumption; therefore, the mechanism is not related to a decrease in oxygen utilization (Greenberg *et al.,* 1994). As would be expected, CBF reduction in response to competitive inhibitors can be reversed by subsequent administration of substrate, namely, L-arginine. Brain surface pial arterioles dilate *in vivo* to topically applied L-arginine (which presumably increases NO generation) and constrict in response to topical nonspecific NOS inhibitors (Rosenblum *et al.,* 1990). In contrast, systemic administration of a fairly selective nNOS inhibitor has little effect on resting CBF (Harukuni *et al.,* 1999; Z. G. Zhang *et al.,* 1996), suggesting that neuronal NO is not important in regulating basal blood flow. In summary, vascularly derived NO appears to be an important mediator of resting cerebrovascular tone *in vivo.*

NO and Cerebrovascular Reactivity

NO has also been considered to be an important mediator of cerebrovasodilation resulting from hypercapnia, administration of inhaled anesthetics, hypoglycemia, and seizures. We will briefly provide the evidence in these states because each is important to understanding the importance of NO in mechanisms of cerebrovascular control, and each may coexist with experimental or clinical ischemia. We emphasize the evidence for a role of NO in CBF hyperemia resulting from inhalational anesthesia because many experimental data have been acquired in animals anesthetized with these agents. Therefore, if NO is to be proved as an important mechanism in ischemic pathophysiology, one must also consider the potential effects of NO produced by inhalational anesthetics on brain injury.

NO and Carbon Dioxide Reactivity

Cerebrovascular dilation in response to hypercapnia and constriction in response to hypocapnia are a universal finding in mammals. Toda *et al.* (1993) demonstrated that removal of endothelium in large cerebral blood vessels isolated from dog and monkey does not affect the cerebral vas-

cular response to hypercapnia (Toda *et al.,* 1993). These data suggested that NO derived from endothelium (or other endothelium-derived relaxation factors) are not important in the mechanism of cerebral vascular dilation during hypercapnia. However, because the level of PCO_2 (110–120 mmHg) was potentially high enough to cause nonspecific direct relaxation of vascular smooth muscle, it remains difficult to extrapolate these results to more physiologically relevant situations. Other investigators (Bertalanffy *et al.,* 1993) have found that *in situ* ablation of pial vessel endothelium with light at visible wavelengths did not affect pial arteriolar dilation to hypercapnia. Together these *in vitro* data (Toda *et al.,* 1993; Bertalanffy *et al.,* 1993) suggest that vascular endothelium is not important in hypercapnia-induced cerebral vasodilation in monkey, dog, or cat.

However, the role of NO in the mechanism of cerebrovasodilation to hypercapnia *in vivo* is controversial. In the cat, one group of investigators has reported a significant role of NO in mediation of regional blood flow during hypercapnia (arterial PCO_2 55–65 mmHg) in brain and spinal cord (Sandor *et al.,* 1994). However, others have demonstrated that NOS inhibition does not alter hypercapnia-induced dilation when surface pial vessels are directly visualized. In another study, administration of nonspecific NOS inhibitors substantially reduced CBF hyperemia with hypercapnia in rat (Wang *et al.,* 1992). However, it is difficult to interpret these data given that NOS inhibition decreased baseline CBF and increased blood pressure, with the net result of increased baseline cerebrovascular resistance. Therefore, although the increase in CBF during hypercapnia was smaller after the NOS inhibitor as compared to before inhibitor administration, this result is not straightforward. Because baseline cerebrovascular resistance was high, the absolute reduction in cerebrovascular resistance during hypercapnia was not different from that observed prior to NOS inhibition. Thus, it could be concluded that the overall cerebrovascular response to hypercapnia is not affected by NOS inhibition. We have evaluated the effect of NOS inhibition on the cerebral vascular response to hypercapnia in isoflurane-anesthetized monkeys (McPherson *et al.,* 1995), carefully controlling blood pressure and brain temperature. During hypercapnia, arterial PCO_2 was increased from 40 to 50–70 mmHg. Despite confirmation of complete NOS inhibition in brain homogenates from these animals, we found little effect on vascular reactivity during hypercapnia in most brain vascular beds. In cortex, a modest 30% attenuation of the hypercapnic response was observed. In summary, NO is not a major mediator of cerebral vascular dilation in response to hypercapnia.

Role of NO in Cerebrovasodilation Produced by Inhalational Anesthetics

Inhalational anesthetics cause an increase in CBF *in vivo* and vasodilation of cerebral blood vessels *in vitro.* The cerebral hyperemic response to inhalational anesthetics is only transient in subprimate mammals (Brian *et al.,* 1990), but it is sustained in primates (McPherson *et al.,* 1994). Many different mechanisms have been considered in these vascular actions, including alterations in cellular calcium homeostasis, excitatory amino acids, free radicals, and prostanoids. Whether NO mediates the hyperemia induced by inhalational anesthetics has been evaluated both *in vivo* and *in vitro,* and the resulting data are again based predominately on inhibitor studies. Exposure of middle cerebral artery rings (Flynn *et al.,* 1992) and basilar artery (Jensen *et al.,* 1992) to inhalational anesthetics produced a dose-dependent vasodilation that was not affected by inhibition of NOS or removal of endothelium. Inhalational anesthetics have also been demonstrated to attenuate vasodilator mechanisms that depend on NO production (Uggeri *et al.,* 1992). Although *in vitro* data suggest that neither endothelium, NO, nor cGMP play an important role in vasodilation from inhalational anesthetics, *in vivo* experiments support a role for NO. Halothane has been shown to increase diameter in canine middle cerebral arterial rings with intact endothelium, via a mechanism that involves either stimulation of particulate, but not soluble, guanylate cyclase or inhibition of cyclic GMP phosphodiesterase (Eskinder *et al.,* 1992). The mechanism for activation of particulate guanylate cyclase may involve halothane-mediated increases in membrane fluidity.

An interesting mechanistic clue is present in the observation that halothane caused cGMP-dependent vasodilation in endothelium-denuded rat aortas, and the effect was inhibited by methylene blue (a known inhibitor of guanylate cyclase) (Nakamura *et al.,* 1991). This finding suggests that the mechanism of vasodilation by halothane involves production of NO within vessels from a source other than endothelium. For example, prolonged exposure to inhalational anesthetics has been demonstrated to upregulate iNOS from macrophages along with eNOS from pulmonary endothelial cells (Zuo and Johns, 1997). NOS inhibition, dose dependently and reversibly, reduced the threshold for halothane anesthesia in the rat (Johns *et al.,* 1992), again suggesting a role for NO in inhalational anesthetic mechanisms. In addition, halothane increased cortical cGMP but not cGMP in the cerebellum (Nahrwold *et al.,* 1977), which is a region of high baseline NOS activity. We found that NOS inhibition prevented cerebral hyperemia to halothane, isoflurane, and N_2O in dogs under baseline pentobarbital anesthesia (McPherson *et al.,* 1993). A subsequent study showed that these inhibitory effects were reversible with L-arginine, supporting a direct role for NO in the mechanism of isoflurane-induced cerebral hyperemia (Moore *et al.,* 1994). Similar NO linkages have been reported for halothane-induced vasodilation in rats, using a pial vessel preparation (Koenig *et al.,* 1992). The discrepancy between *in vitro* data showing little or no role for NO in anesthetic induced hyperemia and the results *in vivo* that implicate NO is likely explained by the presence of nonvascular sources of NO in intact brain. In summary, NO is an important mediator of inhalational agent-induced cerebral vasodilation *in vivo.* The source of NO production likely involves several tissue types, including perivascular nerves, astrocytes, and/or parenchymal neurons.

Role of NO in Cerebrovasodilation from Hypoglycemia

Hypoglycemia causes cerebral vasodilation, which begins with the onset of EEG slowing and is maximal at the onset of isoelectric EEG. The mechanism of hypoglycemia-induced cerebral vasodilation is unknown; however, tissue acidosis, potassium efflux, and prostaglandin production have been excluded as primary mechanisms. We have tested the role of NO in hypoglycemia-induced cerebral hyperemia (Ichord *et al.,* 1994) and found that NOS inhibition attenuated the vascular response to systemic hypoglycemia in a regionally selective manner. During hypoglycemia, CBF increased maximally at the onset of EEG silence, and this was associated with an increase in cerebral oxygen utilization. However, administration of a nonspecific NOS inhibitor accelerated the onset of EEG dysfunction during hypoglycemia and blocked the rise in oxygen utilization that accompanied an isoelectric EEG. Administration of the same inhibitor dose did not alter the increase in CBF which ordinarily occurs during hypoxia, suggesting that the role of NO was specific for hypoglycemia-induced hyperemia. In summary, NO is an important controller of CBF during systemic hypoglycemia and facilitates coupling of CBF to the high energy needs of the brain.

NO and Control of Cerebral Blood Flow during Seizures

Seizures are relatively common sequelae of ischemia. Although NO is clearly an important mediator of the increased CBF that accompanies seizures (Faraci *et al.,* 1993), the role of NO in the genesis or propagation of seizure activity is controversial. Some studies have demonstrated that systemic administration of nonspecific or specific inhibitors of nNOS reduced the intensity of pharmacologically induced seizures in animals (Mulsch *et al.,* 1994). However, others have demonstrated that NOS inhibition worsened seizure intensity, suggesting a protective role for NO, at least for pharmacologically induced epilepsy (Maggio *et al.,* 1995). Some of the confusion in the literature has been resolved by the demonstration that although generalized NOS inhibition accentuated pharmacologically induced seizures, this was not observed when a specific nNOS inhibitor was administered (Penix *et al.,* 1994). Thus, it is speculated that inhibition of eNOS must limit blood flow during seizures and worsen seizure intensity, whereas limiting NO production from neurons has some therapeutic value.

NO and Cerebral Ischemia

In normal brain, NO is a nontoxic mediator of cerebral vasodilation and a neurotransmitter. However, converging evidence from many laboratories and experimental preparations indicates that NO is neurotoxic when present in abnormally high concentrations and is a significant threat by pro-oxidant mechanisms to neuronal integrity and function. In contrast, diminished endothelial production of NO and the resulting loss of vasodilatory capacity and microvascular patency serve only to compromise perfusion within partially injured and recoverable tissue regions. Therefore, the net effect of NO during cerebral ischemia is dependent on a dynamic balance: direct neurotoxicity due to NO overload versus vascular defects due to NO deficiency. Similarly, the experimental and therapeutic value of inhibiting NOS and NO formation depends on this same balance. Using this simplistic framework, the role of NO in cerebral ischemic mechanisms and potential therapeutic implications are examined.

NO Production in Brain during Cerebral Ischemia

Increased NO production in association with global ischemia and reperfusion was first documented at micromolar concentrations with an NO electrochemical probe (Malinski *et al.,* 1993). More recently, we have used a microdialysis technique in conjunction with middle cerebral artery occlusion (MCAO) to estimate *in vivo* NO production in ischemic and reperfused brain. The brain region of interest (striatum) is loaded with L-[^{3}H]arginine administered by microdialysis probe prior to MCAO, then the resulting [^{3}H]citrulline is collected during ischemia and measured by ion-exchange chromatography (Bhardwaj *et al.* 1995, 1997a,b). The rate of [^{3}H] citrulline production provides an estimate of NO production. Using this technique, we have shown increases in NO during MCAO and its persistence during early reperfusion (Toung *et al.,* 1999). Others have demonstrated an accumulation of stable oxidative NO metabolites (NO_2^- and NO_3^-) within minutes of reperfusion that lasts for several hours in tissue extracts (Rao *et al.,* 1998) and in microdialysis samples (Togashi *et al.,* 1998). Thus measurements of accumulated, stable oxidative NO metabolites serve as a reasonable indicator of NOS activity over time. In focal ischemia, NO production can be limited in central ischemic regions due to the reduced availability of cofactors required for optimal NOS function (Ashwal *et al.,* 1998). Persistent changes in NOS activity have also been demonstrated in the rat fetal brain exposed to transient hypoxia–ischemia. After an initial increase in cerebral NOS activity, a substantial reduction in enzyme activity is observed that may last for days (Cai *et al.,* 1998). It must be emphasized that whereas each of these studies demonstrated that NO production increases during ischemia–reperfusion, the source of the NO was not determined. The presumption is that a large pat of the NO ''signal'' in brain arises from neurons, via activation of nNOS. However, measurements of NO production over time likely contain a significant contribution from iNOS as well.

In addition to rapid increases in NO production, cerebral ischemia leads to an upregulation of nNOS (NOS I). Upregulation of nNOS mRNA may be a generalized response to neuronal stress and injury, as numerous chemical and biological agents result in enhanced nNOS expression. These include diverse stimuli such as pain, heat stress, axonal transection, colchicine treatment, immobilization stress, as well as hypoxia and ischemia. Enhanced nNOS expression

is frequently accompanied by induction of transcription factors such as c-*jun* and c-*fos*. Increases in mRNA for nNOS have been demonstrated after MCAO in the rat (Zhang *et al.,* 1994; Samdani *et al.,* 1997). Further, increased nNOS expression was demonstrated by 6–18 hours of reperfusion in dogs exposed to hypothermic circulatory arrest (Brock *et al.,* 1996).

Overproduction of local NO is also the consequence of marked iNOS induction in glia and inflammatory cells recruited to areas of injury. Increased expression of eNOS in brain vessels has also been reported within hours of global cerebral ischemia (Gajkowska and Mossakowski, 1997; Beasley *et al.,* 1998) and of traumatic brain injury in rat (Cobbs *et al.,* 1997). However, it is unclear if the protein is functional, because vasodilatory capacity in postischemic pial vessels is markedly impaired in many species. Further, CBF abnormalities after ischemic insult are well documented, including delayed hypoperfusion and impaired microvascular flow.

Mechanism of NO-Induced Toxicity

With the onset of ischemia and anoxic depolarization, excitatory neurotransmitter levels rise with overstimulation of postsynaptic receptors and associated ionotropic channels. It is hypothesized that, once released, excitatory amino acids cause an increase in postsynaptic intracellular calcium, which then causes an increase in NOS activity. NMDA glutamate receptors have been widely implicated as regulators of NO synthesis in this circumstance. Throughout neocortex, striatum, and hippocampus, nNOS immunoreactive neurons colabel for NMDAR1 receptor subunit protein and have a distinct NMDA receptor phenotype (Weiss *et al.,* 1998). NO release may further disrupt calcium homeostasis (Brorson and Zhang, 1997; Ohta *et al.,* 1997), and it may amplify persistent release of excitatory amino acids and spreading depression-like waves of depolarization. These spreading depression-like waves of depolarization and glutamate release correlate well with infarction size (Shimizu-Sasamata *et al.,* 1998). An essential component of NO toxicity is due to the propensity of the molecule to become highly reactive and toxic on conversion to more strongly oxidizing species. During reperfusion, oxygen radicals may be produced through mechanisms involving metabolism of free fatty acids, xanthine (Ratych *et al.,* 1987), and neutrophils (Hallenbeck *et al.,* 1986; Fabian and Kent, 1999; Yenari *et al.,* 1998; Soriano *et al.,* 1999). Although oxygen radicals themselves cause significant injury following transient cerebral ischemia (Fabian and Kent, 1999; Yenari *et al.,* 1998; Soriano *et al.,* 1999; Fujimura *et al.,* 1999; Li *et al.,* 1998; Kamii *et al.,* 1996; Yang *et al.,* 1994; Kinouchi *et al.,* 1991; Imaizumi *et al.,* 1990; Matsumiya *et al.,* 1991; Liu *et al.,* 1989), toxicity is greatly accentuated when they react with NO.

NO in high concentration likely reacts with the oxygen radical superoxide, forming peroxynitrite. Once produced, peroxynitrite causes increased membrane permeability to sodium and further neuronal leakage of excitatory amino acids (Moro *et al.,* 1998). As discussed later in this chapter, peroxynitrite and protein nitration play important mechanistic roles in NO neurotoxicity.

NO and Postischemic Vascular Defects

If overproduction of NO and its pro-oxidant metabolites cause cell damage, then vascular sources of NO may participate in causing of endothelial damage and dysfunction after cerebral ischemia. However, as endothelial damage matures, NO production via eNOS may be compromised. Deficiency in NO production has been hypothesized to have significant effects on the ischemic and postischemic cerebral circulation. Altered NO has been implicated in the mechanism of postischemic transient hyperemia and delayed hypoperfusion (Greenberg *et al.,* 1995). Moskowitz *et al.* (1989) have demonstrated that postischemic hyperemia is attenuated by chronic trigeminal ganglionectomy, and they suggested that the mechanism of hyperemia is linked to transient neural stimulation of NOS and increased release of NO. More recently, NO has been more directly linked as a mechanism of postischemic hyperemia (Greenberg *et al.,* 1995; Humphreys and Koss, 1998).

During reperfusion from ischemia, particularly global cerebral ischemia, a period of delayed hypoperfusion is frequently observed in animal models. The phenomenon is characterized by depression of cerebral oxygen utilization and reduced blood flow to below preischemic values. The significance and mechanism for hypoperfusion are unclear, but it is likely that damage to vascular endothelium and consequent reduced vasodilatory capacity are important factors. Oxygen radicals such as superoxide anion participate in vascular injury, impairing NO synthesis. Further, superoxide anion avidly reacts with NO, resulting in its deactivation and loss of NO-mediated vasodilation during reperfusion. Therefore, two potential mechanisms for postischemic hypoperfusion include (1) a decreased tonic production of NO and (2) an aberration in NO signaling within the endothelium or vascular smooth muscle as NO is deactivated. We tested the first hypothesis that tonic release of NO was impaired by measuring CBF before and after intravenous administration of a nonspecific NOS inhibitor. We found that during profound hypoperfusion, NOS inhibition was still able to illicit a marked decrease in CBF (Clavier *et al.,* 1994a). In subsequent experiments, we examined the hypothesis that NO signaling was impaired during hypoperfusion by examining pial vessel responses to vasodilatory agonists in animals recovering from global cerebral ischemia. Consistent with this hypothesis, pial vasodilation to topically applied acetylcholine (which depends on endothelial NO production for its action) was grossly abnormal (Clavier *et al.,* 1994b). Defects in acetylcholine-induced vasodilatory responses were less pronounced in large pial arterioles (201 $\pm$ 11 μm) than in medium (66 $\pm$ 2 μm) or small (35 $\pm$ 2 μm) arterioles, emphasizing that small vessel endothelial pathology is important. However, arteriolar dilation to nitroprusside (a direct donor of NO to vascular smooth muscle) was not

impaired (Clavier *et al.,* 1994b). These data suggest that defects in NO signaling are important in the pathology of hypoperfusion, and the defect is localized within the endothelium rather than in smooth muscle function.

Deficiency of Vascular NO and Ischemic Outcome

Loss of NO production within the vascular compartment furthers ischemic injury in brain regions that are at risk of inadequate perfusion but potentially salvageable (e.g., the penumbra in focal cerebral ischemia). Consistent with this hypothesis is the finding that nonspecific NOS inhibition with L-NAME (which inhibits all NOS isoforms, including eNOS) worsens injury in experimental stroke, whereas treatment with 7-nitroindazole (7-NI, a selective nNOS inhibitor) has no effect (Kamii *et al.,* 1996). Likewise, administration of a nonspecific NOS inhibitor as an ischemic treatment in nNOS null transgenic mice results in worsening of neurological injury (Huang *et al.,* 1994). Further, NO originating from eNOS has been clearly shown to benefit ischemic pathology because eNOS null mice show impaired cerebral hemodynamics during MCAO (Lo *et al.,* 1996). NO originating from eNOS (but not iNOS or nNOS) also appears to be required in the phenomenon of ischemic tolerance secondary to preconditioning (Gidday *et al.,* 1999).

With the knowledge that a reduction in eNOS-generated NO is detrimental in ischemia, several investigators have evaluated means of increasing NO availability to the vasculature or restoring eNOS activity during ischemia. One indirect method involves the administration of a superoxide anion scavenger, reducing available superoxide for reaction with NO. This strategy could have two benefits: first, removing a source of NO reactions and, second, reducing the toxic product of the reaction, peroxynitrite. In fact, combined treatment with a NOS inhibitor and a radical scavenger was demonstrated to provide synergistic protection from focal cerebral ischemia (Spinnewyn *et al.,* 1999). Successful scavenging of oxygen radicals following global ischemia was associated with increased accumulation of detectible NO (Shutenko *et al.,* 1999). Further, superoxide anion scavenging following transient global ischemia in piglets resulted in improved recovery of vascular reactivity to hypercapnia (Kirsch *et al.,* 1993).

The cholesterol-lowering agents 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors have been demonstrated to selectively upregulate eNOS. Prophylactic treatment with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors improved cerebral perfusion, reduced brain injury, and improved neurological function in normocholesterolemic mice exposed to focal ischemia (Endres *et al.,* 1998). These benefits were not observed in eNOS null mice treated with the inhibitor, suggesting that the mechanism of protection was linked to NO generated from eNOS. Similarly, endogenous neuroprotectants such as estrogen work in part through vascular NO to improve intraischemic perfusion and ameliorate ischemic damage in female animals (Hurn *et al.,* 1995; Palmon *et al.,* 1998; Alkayed *et al.,* 1998; Pelligrino *et al.,* 1998).

Gene therapy shows early promise as a means of overexpressing eNOS in vessels. Using replication-deficient viral vectors carrying a transgene for eNOS, vascular function can be improved in isolated rabbit carotid arteries (Ooboshi *et al.,* 1997; Kullo *et al.,* 1997). Vessels transfected with eNOS demonstrate improved vasodilation to acetylcholine and calcium ionophore (Ooboshi *et al.,* 1997), diminished contractile responses to vasoconstrictors such as phenylephrine, and increased *ex vivo* calcium dependent NOS activity (Kullo *et al.,* 1997). A similar approach has been viable in cerebral arteries. Transfection of eNOS via an adenoviral vector into canine basilar arteries increased basal cGMP production and reduced vasocontriction in response to pharmacological agonists (Chen *et al.,* 1997). These benefits were readily reversible by nonspecific NOS inhibitors.

On the other extreme, a single investigation in stroke patients has assessed possible benefits of supplying NO to the cerebral circulation compromised by ischemia (Butterworth *et al.,* 1998). In this study, sodium nitroprusside was administered as an NO donor, with the dual objective of improving CBF distribution to the ischemic penumbra and inhibiting undesirable platelet aggregation and activation of adhesion molecules. Twenty-two patients with acute ischemic stroke and 12 control patients were treated with sodium nitroprusside at a dose that minimally depressed mean arterial pressure (i.e., by 10 mmHg). Although platelet function was not altered, three of four patients evaluated for cerebral perfusion sustained improved penumbral blood flow during drug administration.

An important question is whether eNOS abnormalities are important in genetically stroke-prone animals and in ischemic vulnerability. We have tested the hypothesis that altered NOS activity could account for the high occurrence of stroke in spontaneously hypertensive stroke-prone rats (SHRSP). Although endothelium-dependent, NO-mediated cerebral vasodilation was impaired in SHRSP rats, we observed no difference in regional brain NOS activity, or in degree of calcium dependence of NOS, as compared to their hypertensive or normotensive controls (Clavier *et al.,* 1994c). In addition, although salt loading and dietary manipulations predisposed these animals to stroke, it did not change cortical NOS activity. We also tested the hypothesis that predisposition to stroke is related to impaired regulation of NOS. We found no insensitivity to inhibition of NOS or depressed CBF response to pharmacological inhibition (Izuta *et al.,* 1995). Therefore, genetic predisposition to stroke in this animal model does not appear to have a link to NOS. Likewise, others have demonstrated a poor correlation in humans between stroke frequency and eNOS gene polymorphism (exon 7) (Markus *et al.,* 1998).

Blocking the Sources of NO Overproduction

The neuronal isoform is present in a small population of neurons; however, these neurons are distributed widely in the central nervous system. A variety of experimental paradigms have demonstrated that excessive NO derived from such neuronal sources increases early ischemic brain dam-

age. Inducible NO released by microglia and infiltrating inflammatory cells contributes in the later stages of neuronal injury. Much work in primary neuronal culture and in animal models offers confirmation at the cellular and organ level that NO neurotoxicity is a major injury mechanism in stroke and experimental brain injury. Assessing the toxicity of NO and the therapeutic value of pharmacological NOS inhibition *in vivo* has been slowed and confounded by the lack of isoform-specific inhibitors with good tissue penetration and which do not compromise CBF and vascular function via eNOS. Further, cerebral ischemia is a multifactorial pathology, and isolating NO-specific mechanisms remains a complex process. Parallel injury mechanisms frequently obscure many well-predicted results *in vitro* and *in vivo*. These mechanisms include primary mitochondrial failure, acidosis and cell swelling, sodium overload and loss of transmembrane ion gradients, eicosanoid elaboration, protease activation, and many non-NO-linked radical mechanisms. Nevertheless, key pieces of evidence implicate nNOS and iNOS as sources of NO toxicity and suggest therapeutic avenues (Nishikawa *et al.,* 1994).

Numerous *in vitro* studies indicate that NO is a major mediator of oxygen–glucose deprivation (OGD), as well as glutamate excitotoxicity. In primary cortical neuronal cultures, NO mediated the loss of cell viability after NMDA exposure. Further, treatment with agents that decrease NOS catalytic activity protected against NMDA excitotoxicity, including pharmacological NO or calcineurin inhibitors and agents that bind calmodulin (Dawson *et al.,* 1991, 1993) (for a review, see Samdani *et al.,* 1997). In addition, Izumi *et al.* (1992) have shown that NOS inhibitors applied to hippocampal slices prevent glutamate- and NMDA-mediated cell death, which was reversible by L-arginine. Cultures obtained from transgenic animals deficient in nNOS were demonstrated to be resistant to OGD as compared to cultures from wild-type mice (Dawson *et al.,* 1996).

In models of focal cerebral ischemia, significant NOS inhibition reduces infarction volume when agents are employed that either are nNOS selective or are given at concentrations that do not affect vascular NO production. These results extend across species, including rats, mice, and cats. Studies have utilized the selective nNOS inhibitor 7-NI, which has been useful in providing proof-of-principle but is of limited clinical utility owing to poor aqueous solubility. 7-NI has been shown to reduce injury in the setting of both focal (Yoshida *et al.,* 1994; Escott *et al.,* 1998; Coert *et al.,* 1999) and global forebrain ischemia (Nanri *et al.,* 1998). However, its therapeutic window is limited to approximately 60 min of onset of ischemia because of a lack of a parenteral preparation (Escott *et al.,* 1998).

In contrast, AR-R 17477 is one of a series of water-soluble, heterocyclic, substituted amidines that inhibits nNOS and reduces neurological injury when administered at the onset of reperfusion. At a dose of 1 mg/kg, histology was improved when assessed at 7 days postischemia in the rat (Z. G. Zhang *et al.,* 1996). However, careful dose–response relationships must be evaluated to understand the role of NOS inhibition. For example, higher doses of AR-R 17477 (Z. G. Zhang *et al.,* 1996) were not effective and caused a reduction in CBF and increased arterial blood pressure. These hemodynamic effects suggest dose-dependent, nonselective endothelial actions that could adversely affect penumbral blood flow. We have observed previously unreported effects on blood pressure even at 3 mg/kg of this agent in unanesthetized rats (Harukuni *et al.,* 1999). Our observations are in contrast to earlier studies conducted in halothane–nitrous oxide-anesthetized animals. These anesthetic agents are known to alter vascular tone by a mechanism that involves NO (Moore *et al.,* 1994; McPherson *et al.,* 1993) and may have direct effects on neuronal NOS (Tobin *et al.,* 1994; Rengasamy *et al.,* 1997). Therefore, the use of halothane–nitrous oxide anesthesia may have masked the effects of 3 mg/kg AR-R 17477 on peripheral vascular tone, which became evident in the unanesthetized state.

Conventional pharmacological approaches to the demonstration of the toxicity of NO have been demonstrated in transgenic animal models of focal cerebral ischemia. Infarction volume after permanent MCAO was reduced in neuronal NOS null mice as compared to age-matched wild-type controls (Huang *et al.,* 1994). Similarly, genetic deficiency in nNOS also resulted in improved histological outcomes from transient, reversible MCAO and from global cerebral ischemia (Hara *et al.,* 1996; Panahian *et al.,* 1996). It must be emphasized that although transgenic models are quite useful in the study of cerebral ischemia *in vivo,* these animals have lacked the gene (and enzyme) since birth and may demonstrate a compensatory physiology that is unique to the transgenic strain. Therefore, it is gratifying to note the good agreement between data from cultured cells, nNOS deficient mice, and pharmacologically inhibited animals, all of which clearly show the importance of NO neurotoxicity during ischemia and energy failure.

The importance of nNOS in the mechanism of brain injury following transient global cerebral ischemia is less clear. nNOS appears to be the isoform responsible for NO production during forebrain ischemia in gerbils (Lei *et al.,* 1999). Further, nNOS null mice have less injury to CA1 hippocampal neurons following 5 or 10 min of global ischemia (Panahian *et al.,* 1996). However, NO appears to increase more rapidly and reproducibly following focal ischemia in rats, as compared to global ischemia in gerbils (Araki *et al.,* 1998). Inhibition of NOS is neuroprotective in some (Nakagomi *et al.,* 1997; O'Neill *et al.,* 1997), but not all, rodent models of transient forebrain ischemia (Sancerario *et al.,* 1994; Buchan *et al.,* 1994; Lei *et al.,* 1999). In an attempt to resolve these inconsistencies in rodent models, we tested the effect of a nonspecific NOS inhibitor (L-NAME) on neurological outcome in cats treated with transient global ischemia induced by aortic occlusion. Further, L-arginine administration was hypothesized to worsen neurological injury by increasing substrate availability for NO production (Kirsch *et al.,* 1997). We found that neither NOS inhibition nor L-arginine administration affected the intensity of ischemic insult (time to EEG silence during occlusion) and did not improve (or worsen) the neurological outcome. Ten minutes of global cerebral ischemia produced severe neurological injury in the

cat as measured either by standard neuropathology or by functional, neurological examination. Although reducing the ischemic duration to 5 min reduced the severity of histological injury, L-NAME treatment still remained ineffective. Therefore, it remains unclear if NO is important in global cerebral ischemic injury; however, our data in higher order animals do not support a role for NOS inhibition.

A newly identified protein with endogenous NOS inhibitory activity may also reduce NO toxicity. Regional neuronal responses to an ischemic event may be influenced by the amount of protein inhibitor of nNOS (PIN) produced concomitantly with NOS stimulation (Gillardon *et al.,* 1998). Following transient global ischemia, mRNA expression of the protein inhibitor of nNOS is more prevalent in brain regions that are resistant to ischemia-induced injury. There is also a progressive accumulation of PIN in cortical neurons that border the infarct region after MCAO. Another endogenous NO inhibitor has been detected in human plasma; circulating levels of asymmetric dimethylarginine have been correlated with carotid artery vascular pathology (Miyazaki *et al.,* 1999). These observations suggest new alternatives by which ischemic NO production could be potentially harnessed in brain. The therapeutic significance of endogenous NO inhibitors in cerebral ischemia requires further investigation.

Delayed Postischemic Injury due to Inducible NO Synthase Activity

Although iNOS is not detectable in healthy brain, NO is produced during reperfusion via iNOS in microglia and neutrophils that enter damaged brain as part of a postischemic inflammatory response (Bidmon *et al.,* 1998). These cells produce excessive NO for many hours after the onset of reperfusion (Iadecola *et al.,* 1995; Togashi *et al.,* 1998). Further, iNOS can be expressed in neurons, astrocytes, and endothelial cells under pathological conditions. iNOS immunoreactivity has been found in regions of ischemic human brain post mortem (Foster *et al.,* 1999). In rodents, functional iNOS protein can be identified within 12 hours of the ischemic insult, returning to baseline levels by approximately 7 days. Induction of iNOS results in delayed neuronal death *in vitro* and exacerbation of glutamate toxicity. Mice lacking the gene for iNOS do not express iNOS mRNA or protein after MCAO and accordingly have significantly smaller infarction volumes as compared to wild-type mice (Iadecola *et al.,* 1997).

There are important but complex interactions between iNOS and nNOS activity during experimental ischemia. One hypothesis is that the stimulus for iNOS induction and activity is nNOS-induced NO production (Higuchi *et al.,* 1998). Likewise, the finding that NMDA-induced excitotoxic brain lesions can be prevented by administration of the iNOS inhibitor aminoguanidine further suggests a relationship between nNOS-induced NO production and subsequent activation of iNOS (Lecanu *et al.,* 1998). In addition, protein kinase C has been found to be a major second messenger in the signaling pathway regulating iNOS expression in microglia, which intriguingly opens the possibility for testing protein kinase C inhibitors in treating delayed postischemic neurological injury (Chen *et al.,* 1998; Fiebich *et al.,* 1998).

NO generated via iNOS supports postischemic inflammatory responses, in part through enhanced cyclooxygenase-2 (COX-2) activity, proinflammatory prostanoids, and reactive oxygen species (Nogawa *et al.,* 1998). Delayed sources of NO production (Yoshida *et al.,* 1995) and inhibition of iNOS are therapeutically important because the window for effective treatment is wide. Inhibition of iNOS can be beneficial even when inhibitor administration is delayed, for example, 24 hours after the onset of reperfusion (F. Zhang *et al.,* 1996).

Treatments that involve iNOS include aminoguanidine, heparin, agents which increase cAMP levels within brain, and tetracyclines. Systemic administration of the specific iNOS inhibitor aminoguanidine was associated with a dose-dependent reduction in brain injury when initiated 12 or 24 hours post-permanent MCAO in rat (Nagayama *et al.,* 1998). The efficacy of aminoguanidine has been shown to be dependent on prolonged administration, for example, for a minimum of 2 days following the onset of ischemia, suggesting prolonged NO production via iNOS which cannot be substantially altered by short-term drug treatment (Zhang and Iadecola, 1998). Likewise, it has been postulated that part of the benefit of heparin in treatment of ischemic stroke may be related to attenuation of iNOS expression and NO catalytic activity (Bonmann *et al.,* 1998). In addition, pharmacological agents that increase brain levels of cyclic AMP have been proposed as potential neuroprotectants. This is based on the finding that adenosine receptor agonists [particularly adenosine A3 receptor agonists (von Lubitz *et al.,* 1999)], forskolin, and dibutyryl cyclic AMP all result in increased brain cyclic AMP and inhibition of iNOS expression and activity (Brodie *et al.,* 1998). Lastly, the tetracyclines minocycline and doxycycline have anti-inflammatory effects that are independent of their antibacterial effects. The tetracycline family may prevent microglial caspase induction, which is associated in turn with a substantial reduction in iNOS mRNA in postischemic gerbil brain (Yrjanheikki *et al.,* 1998).

Downstream Mechanisms of NO Toxicity

The precise pathway by which NO kills neurons in culture during OGD and as a consequence of *in vivo* cerebral ischemia is not known. Oxidant mechanisms have been clearly implicated, although NO per se is not a strong oxidant. A central component of the mechanism is the propensity of NO at high concentrations to react with superoxide anion and form peroxynitrite anion, which decomposes at acidic pH into hydroxyl radical and nitrogen dioxide (Beckman *et al.,* 1990; Beckman, 1990). More importantly, peroxynitrite persists in part as an activated complex with great oxidizing potential and important cellular targets such as DNA and cytoskeletal proteins (for review, see Beckman and

Koppenol, 1996). Consistent with the NO–peroxynitrite hypothesis, immunohistochemical staining for nitrotyrosine (a marker for oxidants derived from NO such as peroxynitrite) increases during transient ischemia and is reduced by nonselective NOS inhibitors (Forman *et al.,* 1998). Peroxynitrite or $ONOO^-$ is a potent oxidant, although somewhat selective due to its relatively slow rate of reaction with many biological molecules. Evidence from several laboratories suggests that many toxic effects associated with NO are, in fact, due to $ONOO^-$ toxicity. For example, one of the targets of $ONOO^-$ is the mitochondrial form of superoxide dismutase (manganese SOD or MnSOD). Therefore, $ONOO^-$ inactivation of MnSOD may result in enhanced oxidant injury within mitochondria due to impaired scavenging capacity. Reduced MnSOD activity exacerbates glutamate injury in mouse cortical cultures and is important in preventing injury after focal cerebral ischemia, potentially by blocking cytosolic redistribution of mitochondrial enzyme cytochrome *c* (Fujimura *et al.,* 1999; Li *et al.,* 1998).

There are additional mechanisms of the toxicity of NO. When arginine is of limited availability (as in ischemia), nNOS can transfer electrons from NADPH to oxygen and form superoxide. Therefore, besides synthesizing NO, nNOS can produce superoxide at low concentrations (Xia *et al.,* 1996). However, it is not clear to what extent this source of superoxide is biologically important in cerebral ischemic injury. Because NO reacts readily with iron–sulfur cluster-containing proteins, another deleterious effect may be inhibition of mitochondrial enzymes such as NADH–ubiquinone oxidoreductase and NADH:succinate oxidoreductase. NO also inhibits mitochondrial respiration by competing with oxygen at the level of cytochrome oxidase.

One pathway of NO-mediated cell damage is via activation of the enzyme poly(ADP-ribose) polymerase (PARP), also known as poly(ADP-ribose) synthetase. PARP is an abundant nuclear enzyme that helps to maintain genomic integrity in neurons and numerous other cell types. Although the sensing mechanism is not clear, PARP is activated by DNA strand nicks or tears resulting from a variety of cell stressors, including oxidant and free radical attack during reperfusion after intense ischemia. The physiological function of PARP, and of poly(ADP-ribosyl)ation, is under heavy ongoing investigation, but a likely role is to assure correct processing of DNA breaks and to prevent undesirable DNA recombination reactions. Data from our laboratory and others indicate that PARP is a powerful player in neuroinjury from experimental stroke and may be a key downstream mechanism for NO toxicity.

NO and peroxynitrite damage DNA and thus activate PARP. Once activated, PARP catalyzes the transfer of ADP-ribose units from NAD to nuclear proteins. The reaction consumes NAD in the process, and ultimately ATP as well, because NAD regeneration within the cell requires ATP. Hypothetically, intense PARP activation, such as would be likely to occur in the presence of ischemia-damaged DNA, utilizes ATP at a time when cellular energy stores are limited. Excessive PARP activation can also occur as a result of release of intracellular calcium stores (Tasker *et al.,* 1998). The finding that PARP immunostaining was rapidly increased during reperfusion from focal ischemia is consistent with this hypothesis (Endres *et al.,* 1997). Further, administration of either NOS or PARP inhibitors resulted in reduced poly(ADP-ribosyl)ation and infarction volume after transient focal cerebral ischemia (Tokime *et al.,* 1998). Likewise, mice with genetic disruption of PARP were greatly protected against glutamate toxicity and sustained a decreased infarction volume following transient MCAO, as compared to wild-type mice of the same background strain (Eliasson *et al.,* 1997). During ischemia, NAD was less severely depleted in PARP-deficient transgenics, and this finding was accompanied by a reduced overall infarction size but little change in apoptotic markers within the ischemic zone (Endres *et al.,* 1997). Pharmacological inhibition of PARP with 3-aminobenzamide produces decreased infarction volume (Endres *et al.,* 1997; Lo *et al.,* 1998). Further, other DNA repair enzymes such as apurinic/apyrimidinic endonuclease may be critical to repair of neurons after ischemia, rather than cell destruction as is the case with PARP (Edwards *et al.,* 1998; Kawase *et al.,* 1999; Fujimori *et al.,* 1999). Therefore, controlling downstream mechanisms of NO toxicity and developing specific PARP inhibitors with good brain penetration will be potential thrapeutic avenues in the future.

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References

Alkayed, N. J., Harukuni, I., Kimes, A. S., London, E. D., Traystman, R. J., and Hurn, P. D. (1998). Gender-linked brain injury in experimental stroke. *Stroke* **29,** 159–165.

Araki, T., Kato, H., Shuto, K., and Itoyama, Y. (1998). Alterations in [^{3}H]L-*N*(G)-nitroarginine binding in brain after transient global or transient focal ischemia in gerbils and rats. *Eur. J. Pharmacol.* **354,** 153–159.

Ashwal, S., Tone, B., Tian, H. R., Cole, D. J., and Pearce, W. J. (1998). Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion. *Stroke* **29,** 1037–1046.

Beasley, T. C., Bari, F., Thore, C., Thrikawala, N., Louis, T., and Busija, D. (1998). Cerebral ischemia/reperfusion increases endothelial nitric oxide synthase levels by an indomethacin-sensitive mechanism. *J. Cereb. Blood Flow Metab.* **18,** 88–96.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Beckman, J. S. (1990). Ischaemic injury mediator. *Nature* **345,** 27–28.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Bertalanffy, H., Kawase, T., and Toya, S. (1993). In vivo effect of visible light on feline cortical microcirculation. *Acta Neurochir.* **121,** 174–180.

Bhardwaj, A., Northington, F. J., Koehler, R. C., Stiefel, T., Hanley, D. F., and Traystman, R. J. (1995). Adenosine modulates *N*-methyl-D-aspartate-stimulated hippocampal nitric oxide production in vivo. *Stroke* **26,** 1627–1633.

Bhardwaj, A., Northington, F. J., Ichord, R. N., Hanley, D. F., Traystman, R. J., and Koehler, R. C. (1997a). Characterization of ionotropic glutamate receptor mediated nitric oxide production in vivo. *Stroke* **28,** 850–856.

Bhardwaj, A., Northington, F. J., Martin, L. J., Hanley, D. F., Traystman, R. J., and Koehler, R. C. (1997b). Characterization of metabotropic glutamate receptor mediated nitric oxide production in vivo. *J. Cereb. Blood Flow Metab.* **17,** 153–160.

Bidmon, H. J., Wu, J., Buchkremer-Ratzmann, I., Mayer, B., Witte, O. W., and Zilles, K. (1998). Transient changes in the presence of nitric oxide synthases and nitrotyrosine immunoreactivity after focal cortical lesions. *Neuroscience* **82,** 377–395.

Boissel, J. P., Schwarz, P. M., and Förstermann, U. (1998). Neuronal-type NO synthase: Transcript diversity and expressional regulation. *Nitric Oxide* **2,** 337–349.

Bonmann, E., Juttler, E., Krestel, H. E., and Spranger, M. (1998). Heparin inhibits induction of nitric oxide synthase by cytokines in rat brain microvascular endothelial cells. *Neurosci. Lett.* **253,** 95–98.

Boulanger, C. M., Heymes, C., Benessiano, J., Geske, R. S., Levy, B. I., and Vanhoutte, P. M. (1998). Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells. *Circ. Res.* **83,** 1271–1278.

Brian, J. E., Traystman, R. J., and McPherson, R. W. (1990). Changes in cerebral blood flow over time during isoflurane anesthesia in dogs. *J. Neurosurg. Anesth.* **2,** 122–130.

Brock, M. V., Blue, M. E., Lowenstein, C. J., Northington, F. A., Lange, M. S., Johnston, M. V., and Baumgartner, W. A. (1996). Induction of neuronal nitric oxide after hypothermic circulatory arrest. *Ann. Thorac. Surg.* **62,** 1313–1320.

Brodie, C., Blumberg, P. M., and Jacobson, K. A. (1998). Activation of the A2A adenosine receptor inhibits nitric oxide production in glial cells. *FEBS Lett.* **429,** 139–142.

Brorson, J. R., and Zhang, H. (1997). Disrupted $[Ca^{2+}]_i$ homeostasis contributes to the toxicity of nitric oxide in cultured hippocampal neurons. *J. Neurochem.* **69,** 1882–1889.

Buchan, A. M., Gertler, S. Z., Huang, Z.-G., Li, H., Chaundy, K. E., and Xue, D. (1994). Failure to prevent selective CA1 neuronal death and reduce cortical infarction following cerebral ischemia with inhibition of nitric oxide synthase. *Neuroscience* **6,** 1–11.

Butterworth, R. J., Cluckie, A., Jackson, S. H., Buxton-Thomas, M., and Bath, P. M. (1998). Pathophysiological assessment of nitric oxide (given as sodium nitroprusside) in acute ischaemic stroke. *Cerebrovasc. Dis.* **8,** 158–165.

Cai, Z., Hutchins, J. B., and Rhodes, P. G. (1998). Intrauterine hypoxia–ischemia alters nitric oxide synthase expression and activity in fetal and neonatal brains. *Brain Res. Dev. Brain Res.* **109,** 265–269.

Chen, A. F. Y., O'Brien, T. O., Tsutsui, M., Kinoshita, H., Pompili, V. J., Crotty, T. B., Spector, D. J., and Katusic, Z. S. (1997). Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery. *Circ. Res.* **80,** 327–335.

Chen, C. C., Wang, J. K., Chen, W. C., and Lin, S. B. (1998). Protein kinase C η mediates lipopolysaccharide-induced nitric-oxide synthase expression in primary astrocytes. *J. Biol. Chem.* **273,** 19424–19430.

Chen, Y., McCarron, R. M., Bembry, J., Ruetzler, C., Azzam, N., Lenz, F. A., and Spatz, M. (1999). Nitric oxide modulates endothelin 1-induced Ca^{2+} mobilization and cytoskeletal F-actin filaments in human cerebromicrovascular endothelial cells. *J. Cereb. Blood Flow Metab.* **19,** 133–138.

Clavier, N., Kirsch, J. R., Hurn, P. D., and Traystman, R. J. (1994a). Cerebral blood flow is reduced by N^{ω}-nitro-L-arginine methyl ester during delayed hypoperfusion in cats. *Am. J. Physiol.* **267,** H174–H181.

Clavier, N., Kirsch, J. R., Hurn, P. D., and Traystman, R. J. (1994b). Effect of postischemic hypoperfusion on vasodilatory mechanisms in cat. *Am. J. Physiol.* **267,** H2012–H2018.

Clavier, N., Tobin, J., Kirsch, J. R., Izuta, M., and Traystman, R. J. (1994c). Brain nitric oxide synthase activity in normal, hypertensive, and stroke prone rats. *Stroke* **25,** 1674–1678.

Cobbs, C. S., Fenoy, A., Bredt, D. S., and Noble, L. J. (1997). Expression of nitric oxide synthase in the cerebral microvasculature after traumatic brain injury in the rat. *Brain Res.* **751,** 336–338.

Coert, B. A., Anderson, R. E., and Meyer, F. B. (1999). A comparative study of the effects of two nitric oxide synthase inhibitors and two nitric oxide donors on temporary focal cerebral ischemia in the Wistar rat. *J. Neurosurg.* **90,** 332–338.

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., and Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical culture. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 6368–6371.

Dawson, V. L., Dawson, T. M., Bartley, D. A., Uhl, G. R., and Snyder, S. H. (1993). Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13,** 2651–2661.

Dawson, V. L., Kizuxhi, V. M., Huang, P. L., Snyder, S. H., and Dawson, T. M. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neurosci.* **16,** 2479–2487.

Dineman, J. L., Dawson, T. M., Schell, M. J., Snowman, A., and Snyder, S. H. (1994). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: Implications for synaptic plasticity. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4214–4218.

Edwards, M., Kent, T. A., Rea, H. C., Wei, J., Quast, M., Izumi, T., Mitra, S., and Perez-Polo, J. R. (1998). APE/Ref-1 responses to ischemia in rat brain. *Neuroreport* **9,** 4015–4018.

Eliasson, M. J., Sampei, K., Mandir, A. S., Hurn, P. D., Traystman, R. J., Bao, J., Pieper, A., Wang, Z. Q., Dawson, T. M., Snyder, S. H., and Dawson, V. L. (1997). Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med.* **3,** 1089–1095.

Endres, M., Wang, Z. Q., Namura, S., Waeber, C., and Moskowitz, M. A. (1997). Ischmic brain injury is mediated by the activation of poly(ADP-ribose) polymerase. *J. Cereb. Blood Flow Metab.* **17,** 1143–1151.

Endres, M., Laufs, U., Huang, Z., Nakamura, T., Huang, P., Moskowitz, M. A., and Liao, J. K. (1998). Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8880–8885.

Escott, K. J., Beech, J. S., Haga, K. K., Williams, S. C., Meldrum, B. S., and Bath, P. M. (1998). Cerebroprotective effect of the nitric oxide synthase inhibitors, 1-(2-trifluromethylphenyl) imidazole and 7-nitro indazole, after transient focal cerebral ischemia in rat. *J. Cereb. Blood Flow Metab.* **18,** 281–287.

Eskinder, H., Hillard, C. J., Flynn, N., Bosnjak, Z. J., and Kampine, J. P. (1992). Role of guanylate cyclase–cGMP systems in halothane-induced vasodilation in canine cerebral arteries. *Anesthesiology* **77,** 482–487.

Fabian, R. H., and Kent, T. A. (1999). Superoxide anion production during reperfusion is reduced by an antineutrophil antibody after prolonged cerebral ischemia. *Free Radicals Biol. Med.* **26,** 355–361.

Faraci, F. M., Breese, K. R., and Heistad, D. D. (1993). Nitric oxide contributes to dilatation of cerebral arterioles during seizures. *Am. J. Physiol.* **265,** H2209–H2212.

Fiebich, B. L., Butcher, R. D., and Gebicke-Haerter, P. J. (1998). Protein kinase C-mediated regulation of inducible nitric oxide synthase expression in cultured microglial cells. *J. Neuroimmunol.* **92,** 170–178.

Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999). Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat. Struct. Biol.* **6,** 233–242.

Flynn, N. M., Buljubasic, N., Bosnjak, Z. J., and Kampine, J. P. (1992). Isoflurane produces endothelium-independent relaxation in canine middle cerebral arteries. *Anesthesiology* **76,** 461–467.

Forman, L. J., Liu, P., Nagele, R. G., Yin, K., and Wong, P. Y. (1998). Augmentation of nitric oxide, superoxide, and peroxynitrite production during cerebral ischemia and reperfusion in the rat. *Neurochem. Res.* **23,** 141–148.

Foster, C., Clark, H. B., Ross, M. E., and Iadecola, C. (1999). Inducible nitric oxide synthase expression in human cerebral infarcts. *Acta Neuropathol.* **97,** 215–220.

Fujimori, M., Morita-Fujimura, Y., Kawase, M., and Chan, P. H. (1999). Early decrease of apurinic/apyrimidinic endonuclease expression after transient focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* **19,** 495–501.

Fujimura, M., Morita-Fujimura, Y., Kawase, M., Copin, J. C., Calagui, B., Epstein, C. J., and Chan, P. H. (1999). Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome *c* and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. *J. Neurosci.* **19,** 3414–3422.

Gajkowska, B., and Mossakowski, M. J. (1997). Endothelial nitric oxide synthase in vascular endothelium of rat hippocampus after ischemia: Evidence and significance. *Folia Neuropathol.* **35,** 171–180.

Gidday, J. M., Shah, A. R., Maceren, R. G., Wang, Q., Pelligrino, D. A., Holtzman, D. M., and Park, T. S. (1999). Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *J. Cereb. Blood Flow Metab.* **19,** 331–340.

Gillardon, F., Krep, H., Brinker, G., Lenz, C., Bottiger, B., and Hossmann, K. A. (1998). Induction of protein inhibitor of neuronal nitric oxide synthase/cytoplasmic dynein light chain following cerebral ischemia. *Neuroscience* **84,** 81–88.

Greenberg, R. S., Helfaer, M. A., Kirsch, J. R., Moore, L. E., and Traystman, R. J. (1994). Nitric oxide synthase inhibition with N^G-monomethyl-L-arginine reversibly decreases cerebral blood flow in piglets. *Crit. Care Med.* **22,** 384–392.

Greenberg, R. S., Helfaer, M. A., Kirsch, J. R., and Traystman, R. J. (1995). Effect of nitric oxide synthase inhibition on post-ischemic cerebral hyperemia. *Am. J. Physiol.* **239,** H341–H347.

Gunnett, C. A., Chu, Y., Heistad, D. D., Loihl, A., and Faraci, F. M. (1998). Vascular effects of LPS in mice deficient in expression of the gene for inducible nitric oxide synthase. *Am. J. Physiol.* **275,** H416–H421.

Hallenbeck, J. M., Dutka, A. J., Tanishima, T., Kochanek, P. M., Kumaroo, K. K., Thompson, C. B., Obrenovitch, T. P., and Contreras, T. J. (1986). Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. *Stroke* **17,** 246–253.

Hara, H., Huang, P. L., Panahian, N., Fishman, M. C., and Moskowitz, M. A. (1996). Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. *J. Cereb. Blood Flow Metab.* **16,** 605–611.

Harukuni, I., Traystman, R. J., and Kirsch, J. R. (1999). Effect of AR-R 17477, a potent neuronal NOS inhibitor, on infarction volume resulting from permanent focal ischemia in rats. *Crit. Care Med.* **27,** 2508–2511.

Hecker, M., Cattaruzza, M., and Wagner, A. H. (1999). Regulation of inducible nitric oxide synthase gene expression in vascular smooth muscle cells. *Gen. Pharmacol.* **32,** 9–16.

Higuchi, Y., Hattori, H., Kume, T., Tsuji, M., Adaike, A., and Furusho, K. (1998). Increase in nitric oxide in the hypoxic–ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine. *Eur. J. Pharmacol.* **342,** 47–49.

Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265,** 1883–1885.

Humphreys, S. A., and Koss, M. C. (1998). Role of nitric oxide in post-ischemic cerebral hyperemia in anesthetized rats. *Eur. J. Pharmacol.* **347,** 223–229.

Hurn, P. D., Littleton-Kearney, M. T., Kirsch, J. R., Dharmarajan, A. M., and Traystman, R. J. (1995). Postischemic cerebral blood flow recovery in the female: Effect of 17 β-estradiol. *J. Cereb. Blood Flow Metab.* **15,** 666–672.

Iadecola, C., Zhang, F., Xu, S., Casey, R., and Ross, M. E. (1995). Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J. Cereb. Blood Flow Metab.* **15,** 378–384.

Iadecola, C., Zhang, F., Casey, R., Nagayama, M., and Ross, M. E. (1997). Delayed reduction of ischemic brain injury and neurological deficits in mice lacking inducible nitric oxide synthase gene. *J. Neurosci.* **17,** 9157–9164.

Ichord, R. N., Helfaer, M. A., Kirsch, J. R., Wilson, D., and Traystman, R. J. (1994). Nitric oxide synthase inhibition attenuates hypoglycemic cerebral hyperemia in piglets. *Am. J. Physiol.* **266,** H1062–H1068.

Ignarro, L. J., Woods, K. S., and Wolin, M. S. (1982). Activation of purified soluble guanylate cyclase by protoporphyrin IX. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2870–2873.

Ignarro, L. J., Byrns, R. E., Buga, G. M., and Wood, K. S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.* **61,** 866–879.

Imaizumi, S., Woolworth, V., Fishman, R. A., and Chan, P. H. (1990). Liposome-entrapped superoxide dismutase reduces cerebral infarction in cerebral ischemia in rats. *Stroke* **21,** 1312–1317.

Izumi, Y., Clifford, D. B., and Zorumski, C. F. (1992). Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science* **257,** 1273–1276.

Izuta, M., Clavier, N., Kirsch, J. R., and Traystman, R. J. (1995). Cerebral blood flow during inhibition of brain nitric oxide synthase activity in normal, hypertensive, and stroke prone rats. *Stroke* **26,** 1079–1085.

Jensen, N. F., Todd, M. M., Kramer, D. J., Leonard, P. A., and Warner, D. S. (1992). A comparison of the vasodilating effects of halothane and isoflurane on the isolated rabbit basilar artery with and without intact endothelium. *Anesthesiology* **76,** 624–634.

Johns, R. A., Moscicki, J. C., and Difazio, C. A. (1992). Nitric oxide synthase inhibitor dose-dependently and reversibly reduces the threshold for halothane anesthesia. A role for nitric oxide in mediating consciousness? *Anesthesiology* **77,** 779–784.

Kamii, H., Mikawa, S., Murakami, K., Kinouchi, H., Yoshimoto, T., Reola, L., Carlson, E., Epstein, C. J., and Chan, P. H. (1996). Effects of nitric oxide synthase inhibition on brain infarction in SOD-1-transgenic mice following transient focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* **16,** 1153–1157.

Kawase, M., Fujimura, M., Morita-Fujimura, Y., and Chan, P. H. (1999). Reduction of apurinic/apyrimidinic endonuclease expression after transient global cerebral ischemia in rats: Implication of the failure of DNA repair in neuronal apoptosis. *Stroke* **30,** 441–448.

Kinouchi, H., Mizui, T., Carlson, E., Epstein, C. J., and Chan, P. H. (1991). Focal cerebral ischemia and the antioxidant system in transgenic mice overexpressing CuZn-superoxide dismutase. *J. Cereb. Blood Flow Metab.* **11** (Suppl. 2), S423(Abstract).

Kirsch, J. R., Helfaer, M. A., Haun, S. E., Koehler, R. C., and Traystman, R. J. (1993). Polyethylene glycol-conjugated superoxide dismutase improves recovery of postischemic hypercapnic cerebral blood flow in piglets. *Pediatr. Res.* **34,** 530–537.

Kirsch, J. R., Bhardwaj, A., Martin, L. J., Hanley, D. F., and Traystman, R. J. (1997). Neither L-arginine nor L-NAME affects neurological outcome after global ischemia in cats. *Stroke* **28,** 2259–2265.

Koenig, H. M., Pelligrino, D. A., and Albrecht, R. F. (1992). Halothane vasodilation and nitric oxide in rat pial vessels. *J. Neurosurg. Anesthesiol.* **4,** 301 (Abstract).

Kullo, I. J., Mozes, G., Schwartz, R. S., Gloviczki, P., Crotty, T. B., Barber, D. A., Katusic, Z. S., and O'Brien, T. (1997). Adventitial gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries alters vascular reactivity. *Circulation* **96,** 2254–2261.

Lecanu, L., Verrecchia, C., Margaill, I., Boulu, R. G., and Plotkine, M. (1998). iNOS contribution to the NMDA-induced excitotoxic lesion in the rat striatum. *Br. J. Pharmacol.* **125,** 584–590.

Lei, B., Adachi, N., Nagaro, T., Arai, T., and Koehler, R. C. (1999). Nitric oxide production in the CA1 field of the gerbil hippocampus after transient forebrain ischemia: Effects of 7-nitroindazole and N^G-nitro-L-arginine methyl ester. *Stroke* **30,** 669–677.

Li, Y., Copin, J. C., Reola, L. F., Calagui, B., Gobbel, G. T., Chen, S. F., Sato, S., Epstein, C. J., and Chan, P. H. (1998). Reduced mitochondrial manganese–superoxide dismutase activity exacerbates glutamate toxicity in cultured mouse cortical neurons. *Brain Res.* **814,** 164–170.

Liu, T. H., Beckman, J. S., Freeman, B. A., Hogan, E. L., and Hsu, C. Y. (1989). Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am. J. Physiol.* **256,** H589–H593.

Lo, E. H., Hara, H., Rogowska, J., Trocha, M., Pierce, A. R., Huang, P. L., Fishman, M. C., Wolf, G. L., and Moskowitz, M. A. (1996). Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene expression. *Stroke* **27,** 1381–1385.

Lo, E. H., Bosque-Hamilton, P., and Meng, W. (1998). Inhibition of poly(ADP-ribose) polymerase: Reduction of ischemic injury and attenuation of *N*-methyl-D-aspartate-induced neurotransmitter dysregulation. *Stroke* **29,** 830–836.

McPherson, R. W., Kirsch, J. R., and Traystman, R. J. (1993). N^{ω}-nitro-L-arginine methyl ester prevents cerebral hyperemia by inhaled anesthetics in dogs. *Anesth. Analg.* **77,** 891–897.

McPherson, R. W., Kirsch, J. R., Tobin, J. R., Ghaly, R. F., and Traystman, R. J. (1994). Cerebral blood flow in primates is increased by isoflurane over time and is decreased by nitric oxide synthase inhibition. *Anesthesiology* **80,** 1320–1327.

McPherson, R. W., Kirsch, J. R., Ghaly, R. F., and Traystman, R. J. (1995). Effect of nitric oxide synthase inhibition on cerebral blood flow response to hypercapnia in primates. *Stroke* **26,** 682–687.

Maggio, R., Fumagalli, F., Donati, E., Barbier, P., Racagni, G., Corsini, G. U., and Riva, M. (1995). Inhibition of nitric oxide synthase dramatically potentiates seizures induced by kainic acid and pilocarpine in rats. *Brain Res.* **679,** 184–187.

Malinski, T., Bailey, F., Zhang, Z. G., and Chopp, M. (1993). Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.* **13,** 355–358.

Markus, H. S., Ruigrok, Y., Ali, N., and Powell, J. F. (1998). Endothelial nitric oxide synthase exon 7 polymorphism, ischemic cerebrovascular disease, and carotid atheroma. *Stroke* **29,** 1908–1911.

Matsumiya, N., Koehler, R. C., Kirsch, J. R., and Traystman, R. J. (1991). Conjugated superoxide dismutase reduces extent of caudate injury after transient focal ischemia in cats. *Stroke* **22,** 1193–1200.

Meng, W., Ayata, C., Waeber, C., Huang, P. L., and Moskowitz, M. A. (1998). Neuronal NOS–cGMP-dependent Ach-induced relaxation in pial arterioles of endothelial NOS knockout mice. *Am. J. Physiol.* **274,** H411–H415.

Miyazaki, H., Matsuoka, H., Cooke, J. P., Usui, M., Ueda, S., Okuda, S., and Imaizumi, T. (1999). Endogenous nitric oxide synthase inhibitor: A novel marker of atheroschlerosis. *Circulation* **99,** 1141–1146.

Moore, L. E., Kirsch, J. R., Helfaer, M. A., Tobin, J. R., McPherson, R. W., and Traystman, R. J. (1994). Nitric oxide and prostanoids contribute to isoflurane-induced cerebral hyperemia in pigs. *Anesthesiology* **80,** 1328–1337.

Moro, M. A., Leza, J. C., Lorenzo, P., and Lizasoain, I. (1998). Peroxynitrite causes aspartate release from dissociated rat cerebellar granule neurones. *Free Radical Res.* **28,** 193–204.

Moskowitz, M. A., Sakas, D. E., Wei, E. P., Kano, M., Buzzi, M. G., Ogilvy, C., and Kontos, H. A. (1989). Postocclusive cerebral hyperemia is markedly attenuated by chronic trigeminal ganglionectomy. *Am. J. Physiol.* **257,** H1736–H1739.

Mulsch, A., Busse, R., Mordvintcev, P. I., Vanin, A. F., Nielsen, E. O., Scheel-Kruger, J., and Olesen, S. P. (1994). Nitric oxide promotes seizure activity in kainate-treated rats. *Neuroreport* **5,** 2325–2328.

Nagayama, M., Zhang, F., and Iadecola, C. (1998). Delayed treatment with aminoguanidine decreases focal cerebral ischemic damage and enhances neurologic recovery in rats. *J. Cereb. Blood Flow Metab.* **18,** 1107–1113.

Nahrwold, M. L., Lust, W. D., and Passonneau, J. V. (1977). Halothane-induced alterations of cyclic nucleotide concentrations in three regions of the mouse nervous system. *Anesthesiology* **47,** 423–427.

Nakagomi, T., Kanemitzu, H., Takagi, K., Morikawa, E., Kirino, T., and Tamura, A. (1997). Effect of L-arginine and N^{G}-nitro-L-arginine on delayed neuronal death in the gerbil hippocampus. *Neurol. Res.* **19,** 426–430.

Nakamura, K., Hatano, Y., Toda, H., Nishiwada, M., Baek, W. Y., and Mori, K. (1991). Halothane-induced relaxation of vascular smooth muscle: A possible contribution of increased cyclic GMP formation. *Jpn. J. Pharmacol.* **55,** 165–168.

Nanri, K., Montecot, C., Springhetti, V., Seylaz, J., and Pinard, E. (1998). The selective inhibitor of neuronal nitric oxide synthase, 7-nitroindazole, reduces the delayed neuronal damage due to forebrain ischemia in rats. *Stroke* **29,** 1248–1253.

Nishikawa, T., Kirsch, J. R., Koehler, R. C., Miyabe, M., and Traystman, R. J. (1994). Nitric oxide synthase inhibition reduces caudate injury following transient focal ischemia in cats. *Stroke* **25,** 877–885.

Nogawa, S., Forster, C., Zhang, F., Nagayama, M., Ross, M. E., and Iadecola, C. (1998). Interaction between inducible nitric oxide synthase and cyclooxygenase-2 after cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10966–10971.

Nozaki, K., Moskowitz, M. A., Maynard, K. I., Koketsu, N., Dawson, T. M., Bredt, D. S., and Snyder, S. H. (1993). Possible origins and distribution of immunoreactive nitric oxide synthase-containing nerve fibers in cerebral arteries. *J. Cereb. Blood Flow Metab.* **13,** 70–79.

O'Neill, M. J., Hicks, C., Ward, M., and Panetta, J. A. (1997). Neuroprotective effects of the antioxidant LY231617 and NO synthase inhibitors in global cerebral ischaemia. *Brain Res.* **760,** 170–178.

Ohta, K., Graf, R., Rosner, G., and Heiss, W. D. (1997). Profiles of cortical tissue depolarization in cat focal cerebral ischemia in relation to calcium ion homeostasis and nitric oxide production. *J. Cereb. Blood Flow Metab.* **17,** 1170–1181.

Ooboshi, H., Chu, Y., Rios, C. D., Faraci, F. M., Davison, B. L., and Heistad, D. D. (1997). Altered vascular function after adenovirus-mediated overexpression of endothelial nitric oxide synthase. *Am. J. Physiol.* **273,** H265–H270.

Palmon, S. C., Williams, M. J., Littleton-Kearney, M. T., Traystman, R. J., Kosk-Kosicka, D., and Hurn, P. D. (1998). Estrogen increases cGMP in selected brain regions and in cerebral microvessels. *J. Cereb. Blood Flow Metab.* **18,** 1248–1252.

Panahian, N., Yoshida, T., Huang, P. L., Hedleywhyte, E. T., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1996). Attenuated hippocampal damage after global cerebral ischemia in mice mutant in neuronal nitric oxide synthase. *Neuroscience* **72,** 343–354.

Papadaki, M., Tilton, R. G., Eskin, S. G., and McIntire, L. V. (1998). Nitric oxide production by cultured human aortic smooth muscle cells: Stimulation by fluid flow. *Am. J. Physiol.* **274,** H616–H626.

Pelligrino, D. A., Santizo, R., Baughman, V. L., and Wang, Q. (1998). Cerebral vasodilating capacity during forebrain ischemia: Effects of chronic estrogen depletion and repletion and the role of neuronal nitric oxide synthase. *Neuroreport* **9,** 3285–3291.

Penix, L. P., Davis, W., and Sabramaniam, S. (1994). Inhibition of NO synthase increases the severity of kainic acid-induced seizures in rodents. *Epilepsy Res.* **18,** 177–184.

Rao, A. M., Dogan, A., Hatcher, J. F., and Dempsey, R. J. (1998). Fluorometric assay of nitrite and nitrate in brain tissue after traumatic brain injury and cerebral ischemia. *Brain Res.* **793,** 265–270.

Ratych, R. E., Chuknyiska, R. S., and Bulkley, G. B. (1987). The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. *Surgery* **102,** 122–131.

Rengasamy, A., Pajewski, T. N., and Johns, R. A. (1997). Inhalational anesthetic effects on rat cerebellar nitric oxide and cyclic guanosine monophosphate production. *Anesthesiology* **86,** 689–698.

Rosenblum, W. I., Nishimura, H., and Nelson, G. H. (1990). Endothelium-dependent L-Arg- and L-NMMA-sensitive mechanisms regulate tone of brain microvessels. *Am. J Physiol.* **259,** H1396–H1401.

Samdani, A. F., Dawson, T. M., and Dawson, V. L. (1997). Nitric oxide synthase in models of focal ischemia. *Stroke* **28,** 1283–1288.

Sancerario, G., Iannone, M., Morello, M., Nistico, G., and Bernardi, G. (1994). Nitric oxide inhibition aggravates ischemic damage of hippocampus but not of NADPH neurons in gerbils. *Stroke* **25,** 436–444.

Sandor, P., Komjati, K., Reivich, M., and Nyary, I. (1994). Major role of nitric oxide in the mediation of regional CO_2 responsiveness of the cerebral and spinal cord vessels of the cat. *J Cereb. Blood Flow Metab.* **14,** 49–58.

Shimizu-Sasamata, M., Bosque-Hamilton, P., Huang, P. L., Moskowitz, M. A., and Lo, E. H. (1998). Attenuated neurotransmitter release and spreading depression-like depolarizations after focal ischemia in mutant mice with disrupted type I nitric oxide synthase gene. *J. Neurosci.* **18,** 9564–9571.

Shutenko, Z., Henry, Y., Pinard, E., Seylaz, J., Potier, P., Berthet, F., Girard, P., and Sercombe, R. (1999). Influence of the antioxidant quercetin in

vivo on the level of nitric oxide determined by electron paramagnetic resonance in rat brain during global ischemia and reperfusion. *Biochem. Pharmacol.* **57,** 199–208.

Soriano, S. G., Coxon, A., Wang, Y. F., Frosch, M. P., Lipton, S. A., Hickey, P. R., and Mayadas, T. N. (1999). Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. *Stroke* **30,** 134–139.

Spinnewyn, B., Cornet, S., Auguet, M., and Chabrier, P. E. (1999). Synergistic protective effects of antioxidant and nitric oxide synthase inhibitor in transient focal ischemia. *J. Cereb. Blood Flow Metab.* **19,** 139–143.

Tanaka, T., Okamura, T., Handa, J., and Toda, N. (1999). Neurogenic vasodilation mediated by nitric oxide in porcine cerebral arteries. *J. Cardiovasc. Pharmacol.* **33,** 56–64.

Tasker, R. C., Sahota, S. K., Cotter, F. E., and Williams, S. R. (1998). Early postischemic dantrolene-induced amelioration of poly(ADP-ribose) polymerase-related bioenergetic failure in neonatal rat brain slices. *J. Cereb. Blood Flow Metab.* **18,** 1346–1356.

Tobin, J. R., Martin, L. D., Breslow, M. J., and Traystman, R. J. (1994). Selective anesthetic inhibition of brain nitric oxide synthase. *Anesthesiology* **81,** 1264–1269.

Toda, N., and Okamura, T. (1992). Regulation of nitroxidergic nerve of arterial tone. *NIPS* **7,** 148–152.

Toda, N., Ayajiki, K., Enokibori, M., and Okamura, T. (1993). Monkey cerebral arterial relaxation caused by hypercapnic acidosis and hypertonic bicarbonate. *Am. J Physiol.* **265,** H929–H933.

Togashi, H., Mori, K., Ueno, K., Matsumoto, M., Suda, N., Saito, H., and Yoshioka, M. (1998). Consecutive evaluation of nitric oxide production after transient cerebral ischemia in the rat hippocampus using in vivo brain microdialysis. *Neurosci. Lett.* **240,** 53–57.

Tokime, T., Nozaki, K., Sugino, T., Kikuchi, H., Hashimoto, N., and Ueda, K. (1998). Enhanced poly(ADP-ribosyl)ation after focal ischemia in rat brain. *J. Cereb. Blood Flow Metab.* **18,** 991–997.

Toung, T. J., Bhardwaj, A., Dawson, V. L., Dawson, T. M., Traystman, R. J., and Hurn, P. D. (1999). Neuroprotective FK506 does not alter *in vivo* nitric oxide production during ischemia and early reperfusion in rats. *Stroke* **30,** 1279–1285.

Traystman, R. J., Moore, L. E., Helfaer, M. A., Davis, S., Banasiak, K., Williams, M., and Hurn, P. D. (1995). Nitro-L-arginine analogues: Dose- and time-related nitric oxide synthase inhibition in brain. *Stroke* **26,** 864–869.

Uggeri, M. J., Proctor, G. J., and Johns, R. A. (1992). Halothane, enflurane, and isoflurane attenuate both receptor-and non-receptor-mediated EDRF production in rat thoracic aorta. *Anesthesiology* **76,** 1012–1017.

von Lubitz, D. K., Lin, R. C., Boyd, M., Bischofberger, N., and Jacobson, K. A. (1999). Chronic administration of adenosine A3 receptor agonist and cerebral ischemia: Neuronal and glial effects. *Eur. J. Pharmacol.* **367,** 157–163.

Wang, Q., Paulson, O. B., and Lassen, N. A. (1992). Effect of nitric oxide blockade by N^G-nitro-L-arginine on cerebral blood flow response to changes in carbon dioxide tension. *J. Cereb. Blood Flow Metab.* **12,** 947–953.

Weiss, S. W., Albers, D. S., Iadarola, M. J., Dawson, T. M., Dawson, V. L., and Standaert, D. G. (1998). NMDAR1 glutamate receptor subunit isoforms in neostriatal, neocortical and hippocampal nitric oxide synthase neurons. *J. Neurosci.* **18,** 1725–1734.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6770–6774.

Yang, G., Chan, P. H., Chen, J., Carlson, E., Chen, S. F., Weinstein, P., Epstein, C. J., and Kamii, H. (1994). Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. *Stroke* **25,** 165–170.

Yenari, M. A., Kunis, D., Sun, G. H., Onley, D., Watson, L., Turner, S., Whitaker, S., and Steinberg, G. K. (1998). Hu23F2G, an antibody recognizing the leukocyte CD11/CD18 integrin, reduces injury in a rabbitt model of transient focal cerebral ischemia. *Exp. Neurol.* **153,** 223–233.

Yoshida, T., Limmroth, V., Irikura, K., and Moskowitz, M. A. (1994). The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J. Cereb. Blood Flow Metab.* **14,** 924–929.

Yoshida, T., Waeber, C., Huang, Z., and Moskowitz, M. A. (1995). Induction of nitric oxide synthase activity in rodent brain following middle cerebral artery occlusion. *Neurosci. Lett.* **21,** 214–218.

Yrjanheikki, J., Keinanen, R., Pellikka, M., Hokfelt, T., and Koistinaho, J. (1998). Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15769–15774.

Zhang, F., and Iadecola, C. (1998). Temporal characteristics of the protective effect of aminoguanidine on cerebral ischemic damage. *Brain Res.* **802,** 104–110.

Zhang, F., Casey, R. M., Ross, M. E., and Iadecola, C. (1996). Aminoguanidine ameliorates and L-arginine worsens brain damage from intraluminal middle cerebral artery occlusion. *Stroke* **27,** 317–323.

Zhang, Z. G., Chopp, M., Gautam, S., Zaloga, C., Ahang, R. L., and Schmidt, H. H. W. (1994). Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in the rat. *Brain Res.* **654,** 85–95.

Zhang, Z. G., Reif, D., Macdonald, J., Tang, W. X., Kamp, D. K., Gentile, R. J., Shakespeare, W. C., Murray, R. J., and Chopp, M. (1996). ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. *J. Cereb. Blood Flow Metab.* **16,** 599–604.

Zuo, Z., and Johns, R. A. (1997). Inhalational anesthetics up-regulate constitutive and lipopolysaccharide-induced inducible nitric oxide synthase expression and activity. *Mol. Pharmacol.* **52,** 606–612.

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CHAPTER 29

Role of Nitric Oxide in Neuronal Protection versus Apoptosis

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VARIOUS NITRIC OXIDE (NO)-RELATED REDOX SPECIES ARE CHARACTERIZED BY DISTINCT CHEMICAL REACTIVITIES THAT DIFFERENTIALLY INFLUENCE THE LIFE AND DEATH OF NEURONS IN RESPONSE TO VARIOUS INSULTS. IN THE CASE OF NO^+ EQUIVALENTS (HAVING ONE LESS ELECTRON THAN NO·), THE MECHANISM OF REACTION OFTEN INVOLVES S-NITROSYLATION OR TRANSFER OF THE NO GROUP TO CYSTEINE THIOL (OR MORE PROPERLY THIOLATE ANION) TO FORM AN RS–NO; FURTHER OXIDATION OF CRITICAL THIOLS CAN POSSIBLY THEN FORM DISULFIDE BONDS FROM NEIGHBORING CYSTEINE RESIDUES. INCREASING EVIDENCE, BOTH PHYSIOLOGICAL AND CHEMICAL, SUGGESTS THAT THE ACTIVITIES OF THE *N*-METHYL-D-ASPARTATE (NMDA) SUBTYPE OF GLUTAMATE RECEPTOR AND CASPASE ENZYMES CAN BE DECREASED BY S-NITROSYLATION, AS CAN OTHER SIGNALING MOLECULES INVOLVED IN NEURONAL APOPTOTIC PATHWAYS, TO AFFORD NEUROPROTECTION. LEFT UNCURBED, OVERSTIMULATION OF THE NMDA RECEPTOR (NMDAR) LEADS TO EXCESSIVE Ca^{2+} INFLUX AND ACTIVATION OF A VARIETY OF NEURODESTRUCTIVE PATHWAYS INCLUDING NO· GENERATION, WHICH LEADS TO APOPTOSIS, IF THE INITIAL INSULT IS MILD, OR NECROSIS, IF MORE SEVERE.

OVER THE PAST DECADE, BEGINNING WITH AN INITIAL REPORT ON THE NMDAR, EVIDENCE HAS ACCUMULATED THAT S-NITROSYLATION CAN REGULATE THE BIOLOGICAL ACTIVITY OF A GREAT VARIETY OF PROTEINS. IN SOME WAYS THIS REACTION IS AKIN TO PHOSPHORYLATION, MYRISTOYLATION, PALMITOYLATION, ETC., BUT IN OTHER ASPECTS S-NITROSYLATION HAS BROADER IMPLICATIONS FOR AUTOCRINE AND PARACRINE SIGNALING BECAUSE THE REACTION CAN OCCUR EITHER INTRACELLULARLY (AS ON CASPASES) OR EXTRACELLULARLY (AS IN THE CASE OF THE NMDAR). THUS, THIS CHEMICAL REACTION IS GAINING ACCEPTANCE AS A NEWLY RECOGNIZED MOLECULAR SWITCH TO CONTROL PROTEIN FUNCTION VIA REACTIVE THIOL GROUPS, SUCH AS THOSE ENCOUNTERED ON THE NMDAR AND IN THE ACTIVE SITE OF CASPASES.

ONE METHOD OF PRODUCING S-NITROSYLATION OF THE NMDAR AND CASPASES IS THE ADMINISTRATION OF NITROGLYCERIN. NITROGLYCERIN CAN BE NEUROPROTECTIVE IN ACUTE FOCAL ISCHEMIA–REPERFUSION MODELS VIA MECHANISMS OTHER THAN INCREASING CEREBRAL BLOOD FLOW. IN CONTRAST, NO· ITSELF DOES NOT APPEAR TO REACT WITH THIOL UNDER PHYSIOLOGICAL CONDITIONS. IN FACT, THE FAVORED REACTION OF NO· WITH $O_2^{\cdot-}$ (SUPEROXIDE ANION) TO FORM HIGH CONCENTRATIONS OF $ONOO^-$ (PEROXYNITRITE) LEADS TO NEUROTOXICITY. A THIRD NO-RELATED SPECIES WITH ONE ADDED ELECTRON COMPARED TO NO· IS NITROXYL ANION (NO^-, WHICH MAY EXIST IN BOTH SINGLET AND TRIPLET STATES). NO^-—UNLIKE NO· BUT REMINISCENT OF NO^+ TRANSFER—CAN ALSO REACT EITHER DIRECTLY (IN THE SINGLET STATE) OR INDIRECTLY (IN THE TRIPLET STATE, VIA FORMATION OF VERY LOW CONCENTRATIONS OF $ONOO^-$) WITH CRITICAL THIOL GROUPS OF THE NMDAR AND MAY THUS ACT TO CURTAIL EXCESSIVE Ca^{2+} INFLUX AND PROVIDE NEUROPROTECTION FROM EXCITOTOXIC INSULTS.

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Introduction: S-Nitrosylation to Regulate Protein Function

Redox modulation by covalent modification of sulfhydryl (thiol) groups on protein cysteine residues can regulate protein function. If they possess a sufficient redox potential, oxidizing agents can react to form adducts on single sulfhydryl groups, or, if two free sulfhydryl groups are vicinal (in close proximity), disulfide bonds may possibly be formed. Reducing agents can regenerate free sulfhydryl (-SH) groups by donating an electron(s). One example of a protein possessing the potential for physiological regulation by redox agents is the NMDAR; another is the family of caspase enzymes, which have a critical cysteine residue in their active site. The redox modulatory sites of the NMDAR consist of critical cysteine residues which, when chemically reduced, increase the magnitude of NMDA-evoked responses. In contrast, after oxidation, NMDA-evoked responses are decreased in size.

Considering endogenous redox agents, in addition to the usual suspects including glutathione, lipoic acid, and reactive oxygen species, nitric oxide-related species have more recently come to the fore. This has occurred largely because of the rediscovery and application to biological systems of work from the early part of the twentieth century showing the organic synthesis of nitrosothiols (RS–NO) (Stamler *et al.,* 1992a). NO-related species include nitric oxide ($NO\cdot$) and nitrosonium ion equivalents (NO^+), with one less electron than $NO\cdot$, as well as nitroxyl anion (NO^-) with one additional electron compared to $NO\cdot$ (Stamler *et al.,* 1992b). Recent evidence suggests these redox-related forms, or their functional equivalents, are important pharmacologically and physiologically, participating in distinctive chemical reactions.

NO^+ can be transferred (not as free NO^+ but as an intermediate that functionally donates NO^+) from either endogenous or exogenous donors to thiol. This reaction is termed S-nitrosylation and forms nitrosothiols; for example, we believe they are formed on the NMDAR (NMDAR-SNO). The reaction of the NO group with NMDAR thiol decreases NMDA-evoked responses similar to an oxidizing agent. A consensus motif of amino acids composed of nucleophilic residues surrounding a critical cysteine, which increases the susceptibility of the cysteine sulfhydryl to S-nitrosylation by NO^+ donors, has been proposed (see below). NMDAR subunits contain multiple typical consensus motifs for S-nitrosylation (Stamler *et al.,* 1997), and site-directed mutagenesis work on recombinant NMDAR subunits indicate that these cysteine residues indeed react with NO^+ donors, but only if the cysteine residues are in the reduced (free sulfhydryl) state. NO^- in its singlet or high-energy state can also react with thiol, for example, on the NMDAR (Lipton and Stamler, 1994). However, $NO\cdot$ in general will not react with thiol; instead, it can lead to a neurodestructive pathway via reaction with O_2^- to form $ONOO^-$ (peroxynitrite) (Beckman *et al.,* 1990; Dawson *et al.,* 1993; Lipton *et al.,* 1993). This review summarizes these processes and the evidence for and against their existence in the nervous system.

Reactive Nitrogen Intermediates as Physiological Messengers or Pathological Mediators of Apoptosis and Necrosis

Reactive nitrogen intermediates (RNI) are generated by the progressive oxidation of the terminal guanidine residue of L-arginine by nitric oxide synthase (NOS) to produce the NO group, although the exact NO-related species generated is still contentious. Further oxidation leads ultimately to nitrate, with RNI including NO_2, N_2O_3, N_2O_4, and physiological NO adducts such as *S*-nitrosothiols and peroxynitrite. Three different forms of NOS have been identified, two of which are constitutive and occur in neurons, namely, nNOS (or NOS1) and eNOS (or NOS3; eNOS was originally cloned from endothelial cells). Inducible NOS (iNOS or NOS2) occurs in the nervous system in astrocytes and microglia. The constitutive forms of NOS are activated by Ca^{2+} via calmodulin.

The NO group has a dual role as physiological messenger and as a contributor to lethal processes. Classically, ''NO'' mediates endothelium-dependent relaxation, takes part in neurotransmission, and is a key player in the cellular immune response (Dawson *et al.,* 1992). Multiple reactions occur between oxygen, superoxide, and transition metals with the following products: N_2O_3 [equivalent to $(NO_2^-)(NO^+)$], peroxynitrite ($OONO^-$), and metal–NO adducts, respectively. These reactions determine the biological activity of the NO group in its various redox-related forms. Other reactions involving the transfer of NO^+ equivalents (with one less electron than NO) result in nitrosative reactions at nucleophilic centers with critical cysteine sulfhydryls, producing *S*-nitrosothiol formation. This reaction, termed S-nitrosylation, occurs preferentially at specific consensus motifs of amino acid residues centered around a critical cysteine sulfhydryl that reacts with the NO group, and it serves to regulate protein function akin to phosphorylation of critical serine, threonine, or tyrosine residues (Stamler *et al.,* 1992b, 1997; Lipton *et al.,* 1993). Accordingly, thiol- and transition metal-containing proteins serve as major target sites for NO-related species (Stamler, 1994). NO–target interaction achieves both cGMP-dependent and cGMP-independent transducing mechanisms. Cyclic GMP-independent NO-induced responses account for the antimicrobial, the cytostatic, and in many cases the cytotoxic capacity of NO-related species. Excess production of $NO\cdot$ has been shown to underlie, at least in part, glutamate-induced neuronal toxicity in cultures of cortical and striatal neurons (Dawson *et al.,* 1991). Finally, radicals generated by the interaction of $NO\cdot$ with oxygen species can induce DNA damage (Noronha-Dutra *et al.,* 1993). This may activate poly(ADP-ribose) polymerase (PARP), which inturn consumes ATP, thus depleting energy stores and resulting in necrosis from a fail-

ure of homeostatic mechanisms (Zhang *et al.,* 1994). Our own studies have focused on the cytotoxicity of nitric oxide in conjunction with reactive oxygen species in cerebrocortical neurons.

NO-Induced Neuronal Cell Death: Contribution to Apoptosis

Excessive activation of excitatory amino acid receptors and the subsequent generation of free radical species have been implicated as a mechanism for neurotoxicity in both acute and chronic neurological diseases, ranging from stroke, epilepsy, and head trauma to Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, HIV-associated dementia, and glaucoma (Rothman and Olney, 1987; Choi, 1988; Meldrum and Garthwaite, 1990; Coyle and Puttfarken, 1993; Lipton and Rosenberg, 1994; Dreyer and Lipton, 1999). This form of neuronal cell death has been termed ''excitotoxicity'' by John Olney. The underlying process responsible for neuronal cell death after overactivation of glutamate receptor subtypes—of which the NMDAR plays a prominent role because of its high permeability to Ca^{2+}—have only recently begun to be clarified. In a wide variety of neurologic disorders, excitotoxicity may be related to excessive glutamate release and/or lack of clearance, which results in excessive stimulation of NMDARs (Lipton and Rosenberg, 1994). This can result in either an acute or chronic process, possibly dependent on the level of NMDAR stimulation and NO·/peroxynitrite generation (Bonfoco *et al.,* 1995). Other excitatory amino acid receptor subtypes also contribute to these processes, but in many cases the NMDAR has a prominent role.

The interaction of glutamate with excitatory amino acid receptors initiates a cascade of events involving excessive Ca^{2+} entry and activation of several enzymes, including phospholipases, proteases, and NOS (Rothman and Olney, 1987; Choi, 1988; Meldrum and Garthwaite, 1990; Coyle and Puttfarken, 1993; Lipton and Rosenberg, 1994). Phospholipase A_2 activation leads to the generation of arachidonic acid and other metabolites as well as to the formation of oxygen free radicals. This can lead to a combination of oxidative and nitrosative stress, culminating in peroxynitrite formation and neuronal cell death (Beckman *et al.,* 1990; Lipton *et al.,* 1993). The cell death pathway can be either necrotic or apoptotic, depending on the intensity of the insult (Bonfoco *et al.,* 1995). To show this, we investigated whether apoptosis or necrosis can be induced in cerebrocortical neurons in culture by overstimulation of glutamate receptors, with consequent influx of excessive Ca^{2+} and downstream production of NO· and O_2^{-}. We used high and low concentrations of glutamate agonists (such as NMDA), NO· donors [such as 3-morpholinosydnonimide (SIN-1) and *S*-nitrosocysteine (SNOC)], or peroxynitrite. We found that exposure of cortical cultures to relatively short durations or low concentrations of NMDA, SNOC, SIN-1, or peroxynitrite induced delayed neuronal cell death characterized by apoptotic features. In contrast, intense exposure to high concentrations of NMDA or peroxynitrite induced relatively rapid necrotic cell death in neurons (Bonfoco *et al.,* 1995). Superoxide dismutase (SOD) and catalase attenuated neuronal cell death, most likely by reducing the formation of peroxynitrite, as they were only effective if peroxynitrite had not yet formed. These findings suggest that the intensity of the original insult may determine the ensuing pathway to either necrotic or apoptotic neuronal cell death. The nature of the original insult as wll as the decision to enter the necrotic versus the apoptotic pathway might have therapeutic implications in terms of the possible effectiveness of SOD/catalase or NMDAR antagonists, as well as the necessary timing of such interventions.

High concentrations of NO (2 μM or more) have also been reported to disrupt mitochondrial respiration as well as glycolysis, leading to severe ATP depletion. Such profound alteration in ATP would likely result in necrosis (Brorson *et al.,* 1999). However, NO can also lead to altered Ca^{2+} homeostasis and mitochondrial depolarization, events that have been linked to apoptosis (Ankarcrona *et al.,* 1995; Bonfoco *et al.,* 1995).

NO and Inhibition of Cell Death

In addition to the contribution of NO to neuronal cell death described earlier, it is important to outline the mechanisms whereby NO-related species can also be neuroprotective. Recent work has suggested that S-nitrosylation of critical cysteine thiol groups of a modulatory site(s) of the NMDAR, of p21ras during MAP kinase signaling, and of the active site of caspase enzymes can decrease the activity of these proteins, thereby contributing to neuroprotection. For example, curtailing excessive activity of the NMDAR by S-nitrosylation (transfer of NO^+ to protein thiol), or by reaction of thiol with nitroxyl anion (NO^-), can be neuroprotective (Lipton *et al.,* 1993; Lipton and Stamler, 1994; Stamler *et al.,* 1997; Kim *et al.,* 1999). Similarly, downstream from NMDAR activation, S-nitrosylation of a critical cysteine residue in p21ras, or of the cysteine residue in the active site of all known caspase enzymes, decreases their activity and also affords protection to neurons from NMDAR-mediated apoptotic events (Melino *et al.,* 1997; Tenneti *et al.,* 1997; Yun *et al.,* 1998). The activity of other proteins involved in apoptosis may also be affected by S-nitrosylation, such as that of transglutaminase (Melino *et al.,* 1997). Thus, depending on its redox state, the NO group can contribute to excitotoxicity (via formation of peroxynitrite in conjunction with superoxide anion) or can provide neuroprotection in at least some types of neurons (by downregulating the activity of both the NMDAR and its downstream activation of p21ras and caspases). Caspases in a variety of other cell types have also been shown to undergo S-nitrosylation (Dimmeler *et al.,* 1997; Kim *et al.,* 1997; Mannick *et al.,* 1999).

Besides directly inhibiting p21ras and caspases, NO may interfere with the execution of apoptosis at different steps, without necessarily affecting the rate of cell death, for example, by changing the mode of demise from apoptosis to

necrosis (Melino *et al.,* 1997). NO can inhibit caspase activation by mechanisms in addition to S-nitrosylation, for example, by NO-dependent formation of cGMP, which in some cell systems can interfere with cell death signaling upstream from caspase activation (Mannick *et al.,* 1994; Kim *et al.,* 1997; Hebesteit *et al.,* 1998). Moreover, since a well-documented action of NO is inhibition of the mitochondrial respiratory chain, it seems conceivable that the resulting ATP depletion might be relevant to the effects of NO on cell death. In fact, recent results have suggested that NO might prevent caspase activation by inhibiting mitochondrial respiration, and thereby lowering intracellular ATP levels (Leist *et al.,* 1999). The prevention of cell death in this system is only ephemeral. Cell demise is delayed, and doomed cells die eventually by necrosis. When nonmitochondrial, glycolytic ATP generation was supported via glucose supplementation to the culture medium, death reverted to its apoptotic form.

In vivo, halting the apoptotic program may have two possible implications: (i) neurons protected by NO via stopping the apoptotic execution cascade would have time to recover from a transient or mild insult, and thus survive; or (ii) neurons exposed to a lethal, normally apoptotic insult would eventually lyse without being removed by phagocytosis. Thus, depending on the situation, endogenous mediators, such as NO-related species, either may prevent cell demise entirely or convert an apoptotic insult into a necrotic one. In the latter case, the release of factors from dead cells and the ensuing inflammation could further aggravate tissue damage.

Cysteine Thiols React with NO-Related Species

As alluded to earlier, many of the neuroprotective actions of NO are mediated by S-nitrosylation of proteins critical for neuronal survival. A key question in determining the life or death outcome for cells concerns the mechanism and conditions that favor these nitrosylation reactions. This section outlines some of those conditions. Free endogenous nitrosonium ion (NO^+) exists only at low pH. However, functional equivalents of NO^+ can be transferred to thiol, or, more properly perhaps, thiolate anion (RS^-), at physiological pH. For example, transfer of NO^+ equivalents occurs from one nitrosothiol to another, a reaction termed transnitrosylation, that is, R–SH + R'–SNO → R–SNO + R'–SH. Since transfer of NO^+ involves thiolate anion (RS^-), it is pH dependent (Arnelle and Stamler, 1995). Endogenous nitrosothiols, such as *S*-nitrosoglutathione, have been demonstrated to react in this manner and to exist in brain and in lung at concentrations approaching tens of micromolar (Arnelle and Stamler, 1995; Hogg *et al.,* 1996; Kluge *et al.,* 1997). Additionally, evidence has shown that nNOS can, in conjunction with glutathione, produce *S*-nitrosoglutathione (Mayer *et al.,* 1998), an NO^+ equivalent and endogenous donor. The enzymatic machinery underlying the formation and breakdown of nitrosothiols is just beginning to be characterized. For example, thioredoxin reductase was shown to catalyze the homolytic cleavage of nitrosothiol (R–SNO) to nitric oxide ($NO\cdot + RS\cdot$) (Nikitovic and Holmgren, 1996).

Classically, there was no precedent for direct reaction of $NO\cdot$ with thiols under anaerobic conditions (Pryor and Lightsey, 1981; Pryor *et al.,* 1982). However, Ischiropoulos and co-workers reported that under particular conditions, for example, in the presence of an electron acceptor such as O_2, $NO\cdot$ could react with thiol to form a nitrosothiol (Gow *et al.,* 1997). However, this reaction may have been artificially facilitated by nominal amounts of copper that contaminated the solutions (J. Beckman, personal communication, 1998). Thus, most authorities agree that the reaction of $NO\cdot$ with thiol does not proceed directly. In fact, the reaction of $NO\cdot$ and O_2^{-} to form peroxynitrite is kinetically favored if both of these reactants are present (Beckman, 1994).

The next important concept in considering the chemical biology of reactions of the NO group involves our image of the local diffusion and ephemeral nature of $NO\cdot$. Bredt and colleagues demonstrated that nNOS is located in close proximity to the NMDAR by virtue of the PDZ domain of nNOS (Brenman *et al.,* 1996). nNOS interacts via its PDZ domain with the carboxyl-terminal tail of NR1, the subunit of the NMDAR that is essential for functional activity. Therefore, NO group production occurs very near one of its potential targets, the NMDAR. With this localization, restricted diffusional constraints and the need for high local concentrations to facilitate NO reactions should not present a problem.

With some of the chemical reactions of these NO-related species in hand, we now turn our attention to the mechanism of S-nitrosylation or transfer of the NO moiety to cysteine sulfhydryl groups on the NMDAR. In the 7 years since we first proposed that S-nitrosylation can modulate protein function using the NMDAR as the archetypal protein (Lipton *et al.,* 1993), S-nitrosylation has also been shown to regulate the activity of various other ion channels, G proteins, growth factors, enzymes, and transcription factors (Stamler *et al.,* 1997). These reactions of NO-related species do not involve the well-known activation of guanylate cyclase by reaction with the heme group to increase cGMP formation. Rather, they involve reactions with cysteine sulfhydryls on an increasing number of protein targets to provide modulation of function, analogous to phosphorylation of critical serine, threonine, or tyrosine residues. S-Nitrosylation may be more versatile than phosphorylation in regulating protein activity since S-nitrosylation can occur on either extracellular or intracellular cysteine sulfhydryl groups, whereas phosphorylation and similar posttranslational events are exclusively intracellular. For example, the critical cysteine sulfhydryls on the NMDAR that react with the NO group are all located extracellularly (Choi *et al.,* 2000; Kim *et al.,* 1999). Moreover, the chemical reactivity of NO-related species is related to the local redox milieu and peptide environment, pH, temperature, and the presence of catalytic amounts of transition metals.

Previously, we had suggested that reaction of the NO group with regulatory sulfhydryl(s) of the redox modulatory site(s) of NMDAR results in downregulation of receptor ac-

tivity (Lipton *et al.,* 1993). In addition to our group, other groups had also shown that NO donors could decrease NMDAR function (Lei *et al.,* 1992; Manzoni *et al.,* 1992; Manzoni and Bockaert, 1993), but the exact mechanism of the reaction has remained contentious (Fagni *et al.,* 1995). The redox basis for this reaction will be presented below for both endogenous and recombinant NMDARs.

S-Nitrosylation, NMDAR Activity, and Neuroprotection

We and others reported that the NO group can decrease NMDAR activity (Hoyt *et al.,* 1992; Lei *et al.,* 1992; Manzoni *et al.,* 1992). We have shown that this reaction of NO occurs at a redox modulatory site(s) of the receptor, consisting of critical cysteine sulfhydryls or thiol groups (Lei *et al.,* 1992; Lipton *et al.,* 1993; Kohr *et al.,* 1994; Sullivan *et al.,* 1994). Interestingly, in recombinant NMDARs, not all redox sites (defined as cysteine residues that react with redox agents) of the NMDAR react with the NO group, just a subset of such cysteines (Choi *et al.,* 2000; Kim *et al.,* 1999).

In native neurons (e.g., cerebrocortical and retinal ganglion cells), we measured the amplitude of NMDA-evoked responses, monitored by whole-cell and single-channel recording with a patch electrode or by digital calcium imaging with the Ca^{2+}-sensitive dye fura-2 (Lei *et al.,* 1992; Lipton *et al.,* 1993). We found that sulfhydryl-reducing agents such as dithiothreitol (DTT), which promote the formation of free thiol groups, increased NMDA responses, predominantly by increasing the opening frequency of NMDAR-operated channels. In contrast, oxidizing agents such 5,5'-dithio (2-bisnitrobenzoic acid) (DTNB) decreased NMDA responses, by forming thiobenzoate protein conjugates at single sulfhydryl groups or perhaps by facilitating disulfide bond formation. In addition to the DTT and DTNB results, we knew that thiols on the NMDAR were involved because under our conditions *N*-ethylmaleimide (NEM), a relatively specific agent for alkylating thiols, irreversibly blocked the effects of these redox reagents while itself slightly decreasing responses to NMDA (Lei *et al.,* 1992; Lipton *et al.,* 1993). Importantly, under our specific conditions, NEM also prevented the subsequent effects of NO donors, indicating that reactions of thiol and NO groups were involved. Both our group and that of Joël Bockaert (Manzoni and Bockaert, 1993) have also demonstrated that endogenous production of NO can decrease NMDAR activity, indicating the potential physiological importance of this effect. In these experiments implicating the involvement of endogenous NO, inhibition of NOS was found to enhance subsequent NMDAR responses. Heretofore, however, there has not been universal consensus on the mechanism of action of NO in this system.

As an example of an NO^+ chemical reaction at the NMDAR, we found that *S*-nitrosocysteine decreases NMDAR activity as demonstrated by whole-cell recording or by digital calcium imaging (Lipton *et al.,* 1993). During single-channel recording, *S*-nitrosocysteine (SNOC) decreased the opening frequency of NMDAR-operated channels in outside-out patches from cerebrocortical neurons (Lipton *et al.,* 1998). In the presence of copper, zinc-superoxide dismutase (Cu,Zn-SOD), SNOC attenuated NMDA-evoked Ca^{2+} influx, a prerequisite for NMDAR-mediated neurotoxicity. Not surprisingly, therefore, under the same conditions, application of SNOC ameliorated NMDAR-mediated neurotoxicity. These findings can be explained best by SNOC donating NO^+ equivalents. Thus, S-nitrosylation or facile transfer of an NO^+ equivalent to thiol groups of the NMDAR results in a nitrosothiol derivative of the NMDAR, which decreases receptor activity. Under these conditions, any NO· produced by alternative homolytic cleavage of SNOC is prevented from entering a neurotoxic pathway of $ONOO^-$ formation via reaction with $O_2^{\cdot-}$ because of the presence of excess SOD (Lipton *et al.,* 1993; Lipton and Stamler, 1994). Rather, NO group transfer leads to downregulation of NMDAR activity, possibly through facilitation of disulfide formation. The fact that EDTA can prevent the effects of the NO group on NMDAR activity (Fagni *et al.,* 1995) supports rather than refutes this chemistry. In particular, metals can facilitate nitrosative reactions involving NO· and O_2 (Stamler *et al.,* 1992b; Lipton *et al.,* 1996). In general, nitrosation of redox sites is facilitated by oxygen, transition metals, and perhaps $O_2^{\cdot-}$ (Stamler, 1994; Gow *et al.,* 1997). The common event is the transfer of an NO^+ equivalent or another intermediate with NO^+-like character to form an RS–NO, in this case on the NMDAR.

S-Nitrosylation of Recombinant NMDARs

In order to better understand redox mechanisms postulated to exist based on the previously mentioned experiments on primary neurons with native NMDARs, we have turned to recombinant systems. Our work on S-nitrosylation and other redox reactions of recombinant NMDARs in the *Xenopus* oocyte expression system is instructive but also must be interpreted with a degree of caution (Sullivan *et al.,* 1994; Sucher *et al.,* 1996). We do not yet fully understand how to form recombinant NMDARs that exactly mimic native receptors, and therefore conclusions based on site-directed mutagenesis studies of cysteine residues must be viewed with tempered enthusiasm. In fact, in the course of performing polymerase chain reaction (PCR) studies based on primers containing the cysteines known to be unique to NMDAR subunits, our group discovered a new NMDAR subunit (originally termed NMDAR-L or χ-1, but more recently named NR3A) (Ciabarra *et al.,* 1995; Sucher *et al.,* 1995; Das *et al.,* 1998). Additional unidentified NMDAR subunits probably remain to be identified. Thus, it is not yet possible to understand definitively the native NMDAR responses on the basis of recombinant subunits.

This statement notwithstanding, our data show that the cysteines at position 744 and 798 of the NR1 subunit are not only important to redox reactions in general, but also have

some (albeit minor) influence on the effect of NO and Zn^{2+} on the NMDAR; however, this can only be seen after chemical reduction of the receptor with DTT because of disulfide bond formation at this site. Structural evidence from crystallography experiments for disulfide formation between these cysteines has come from two cysteine residues on GluR2, which are homologous to Cys-744 and Cys-798 of NR1 and which were shown to form a disulfide bond (Armstrong *et al.,* 1998). Aizenman *et al.,* (1998) did not chemically reduce the NMDAR and thus failed to see the effect of S-nitrosylation at cysteine residues 744 and 798 of NR1. In an extensive series of experiments using site-directed mutagenesis of all cysteines on the known NMDAR subunits, we found that additional cysteine residues on the NR2A subunit contribute in a more important manner to the NO effect than Cys-744 and Cys-798 of NR1, indicating that the NMDAR is polynitrosylated to modulate its physiological function in a manner resembling the ryanodine receptor (Xu *et al.,* 1998). In brief, our results show two things. First, specific NMDAR subunit combinations manifest larger NO-induced decreases in activity than other receptor subunit combinations (e.g., a greater effect of NO is observed for NR1/NR2A than NR1/NR2B receptors) (Omerovic *et al.,* 1995; Sucher *et al.,* 1996). Second, seven cysteine residues on NR1 and NR2A mediate the effects of NO, Zn^{2+}, or redox agents on the NMDAR; six of these cysteine residues work in pairs, that is, apparently forming three disulfide bonds after exposure to oxidizing agents or, in the case of four of these cysteine residues, after reaction with NO^+ donors. One additional cysteine residue appears to be S-nitrosylated only and apparently does not form a disulfide. The effects of NO group transfer would be expected to be either relatively long lasting (in the case of disulfide formation) or more transient (in the case of reaction with a single thiol), and indeed both such effects have been observed on primary neurons. Although it appears that the NO effects are predominantly due to reaction with the single cysteine sulfhydryl on the NR2A subunit, the four other cysteine residues also contribute somewhat by reacting with NO^+, but only if they are in the reduced state (Choi *et al.,* 2000). In contrast, the voltage-independent effects of Zn^{2+} on NR1/NR2A receptors are influenced by all of the aforementioned six cysteine residues that act in pairs and whose redox status can be modulated by DTT and DTNB.

By site-directed mutagenesis we found that a critical cysteine residue (Cys-399) on the NR2A subunit reacts under physiological (nonreducing) conditions by S-nitrosylation (transfer of NO^+) to underlie the major component of NO modulation of NMDAR activity. In cell systems expressing NMDARs with mutant NR2A subunits in which this single cysteine has been replaced by an alanine, the effect of endogenous NO is lost. Thus endogenous S-nitrosylation can serve as a regulatory mechanism for ion channel activity.

Inspection of NR1 and NR2 subunits revealed that an additional four cysteine residues resembled the consensus motif for S-nitrosylation (see below). In addition to NR2A Cys-399, only Cys-744 and Cys-798 in the M3–M4 extracellular linker of NR1 (as mentioned above), and Cys-87 and Cys-320 of the extracellular N terminus of NR2A fit the consensus motif. Therefore, we also mutated these cysteines to alanines, yielding the heteromeric receptor NR1(C744A, C798A)/NR2A(C87A,C320A,C399A). The crystal structure of the GluR2 ligand-binding domain reveals a disulfide bond between cysteine residues homologous to NR1 C744 and C798, and this may also be true for NR2A C87 and C320 (Armstrong *et al.,* 1998). In the form of disulfide, these additional cysteine residues would not possess free thiol groups, and would therefore not be expected to react with NO. Indeed, under physiological conditions, we could not detect a statistically significant difference in SNOC-induced inhibition of NMDA-evoked currents between NR1/NR2A (C399A) and NR1(C744A,C798A)/NR2A(C87A,C320A, C399A) receptors. However, if we pretreated NR1/NR2A (C399A) with DTT to reduce the disulfide bonds to free thiol groups, we then observed an additional small but statistically significant inhibition by NO donors ($11 \pm 4\%$, $n = 10$; $p < 0.05$ by ANOVA). Reminiscent of polynitrosylation of the ryanodine receptor, these results suggest that multiple cysteine residues may react with NO, depending on the redox state of the NMDAR, and this may account for prior divergent findings in different laboratories (Lipton *et al.,* 1998; Aizenman and Potthoff, 1999). Our results indicate, however, that even though NR1(C744,C798) and NR2A(C87, C320) can possibly interact with NO, the effect is quite small and occurs under nonphysiological, chemical reducing conditions. Hence, the main effect of both exogenous and endogenous NO on NMDAR responses occurs at NR2A(C399) under physiological conditions, that is, in the absence of prior expsure to reducing or oxidizing agents (Choi *et al.,* 1999).

Other effects of NO on the NMDAR are of course not ruled out by these findings. It is also true that the effects of Zn^{2+} on the NMDAR and that of redox agents can be confused because some reducing agents (such as DTT) bind Zn^{2+}, because EDTA chelates Zn^{2+}, and because Zn^{2+} may also be coordinated, at least in part, by cysteine residues. It had been proposed by Bockaert and co-workers that NO can react with a Zn^{2+} site of the NMDAR (Fagni *et al.,* 1995). However, we can now explain all of these previous findings with our more recent work showing that the effect of Zn^{2+} can be influenced by the redox state of the six cysteine residues on the NMDAR that are discussed above, four of which can also react with NO^+. Hence, it appears that at least some sites of NO^+ and Zn^{2+} action may share common cysteine residues.

Nitroglycerin Decreases NMDAR Activity and Attenuates Neurotoxicity

On the basis of the above findings, the ideal NO group donor drug would be one that reacts readily with critical thiol groups of the NMDAR to inhibit excessive Ca^{2+} influx but does not produce NO· to react with $O_2^{\cdot-}$, so it will not lead to the formation of peroxynitrite ($ONOO^-$). We therefore studied nitroglycerin (NTG) as an exemplary compound.

Specifically, alkyl nitrates such as NTG do not directly liberate nitric oxide (NO·) to any significant extent; rather, they react readily with thiol groups to form derivative thionitrites (RS–NO) or thionitrates ($RS–NO_2$) (together, these are represented as $RS–NO_x$, with $x = 1$ or 2; Fig. 1) (Lei *et al.,* 1992; Lipton *et al.,* 1993). Using whole-cell recording with patch clamp electrodes and digital calcium imaging with fura-2 on primary cerebrocortical neurons, we found that NTG inhibited NMDA-evoked currents and Ca^{2+} influx (Lei *et al.,* 1992; Lipton *et al.,* 1993). Strong evidence that this effect of NTG is mediated by its reactions with thiol in the previously illustrated manner came from a series of chemical experiments. These studies showed that under our conditions specific alkylation of thiol groups with NEM completely abrogated the inhibitory effect of NTG on subsequent NMDA-evoked responses (Lei *et al.,* 1992). Moreover, more recent work on recombinant NMDAR subunits has revealed that specific thiol-reactive agents, such as 2-aminoethylmethanethiosulfonate (MTSEA) and 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET), can completely block the effect of NO on NMDAR activity, again consistent with the fact that NO/thiol group reactions account for this effect (Choi *et al.,* 2000; Kim *et al.,* 1999).

The finding that NTG could inhibit NMDA-evoked responses was consistent with the demonstration that similar concentrations of NTG could also significantly ameliorate NMDA-induced neuronal killing in cerebrocortical cultures (Lei *et al.,* 1992; Lipton *et al.,* 1993). Additional *in vivo* data show that high doses of nitroglycerin are neuroprotective in rat models of focal ischemia under conditions of constant systemic blood pressure and modestly increased cerebral blood flow in the penumbra (Lipton and Wang, 1996). These parameters are held stable either by inducing tolerance to the systemic effects of NTG through chronic transdermal application (Sathi *et al.,* 1993), or by intravenous infusion of a pressor agent concurrently with nitroglycerin (Lipton and Wang, 1996). Although difficult to prove *in vivo,* it appears likely that the decrease in stroke size observed after treatment with NTG is at least in part due to its effect on decreasing NMDAR activity, although several other beneficial actions also occur (see below) (Lipton and Wang, 1996).

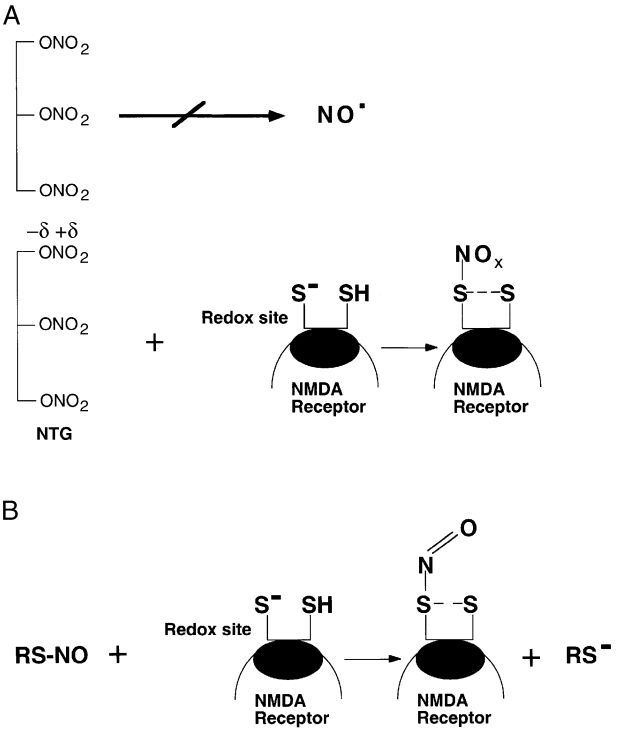


Figure 1 Schematic model of S-nitrosylation of the NMDA receptor. Chemical mechanism of action of nitroglycerin (NTG) with thiol groups (A) and of *S*-nitrosocysteine (an RS–NO) with thiol groups (B) on the NMDA receptor. NTG itself does not directly generate nitric oxide (NO·). Instead, NTG transfers NO_x^+ (where $x = 1$ or 2) to critical thiol groups of NMDA receptor cysteine residues (in some cases this may facilitate disulfide bond formation, indicated by a dashed line). In the case of RS–NO, NO^+ is transferred to thiol. This reaction results in decreased activity of NMDA receptor-operated channels. [Reproduced with permission from Lipton, S. A. (1999). Neuronal protection and destruction by NO. *Cell Death Differ.* **6,** 943–951.]

S-Nitrosylation of Caspases

As introduced earlier, another example of beneficial NTG and S-nitrosylation reactions that can prevent neuronal cell death involves caspases. Caspases are members of the interleukin-1β-converting enzyme (ICE)–CED-3 protease family of enzymes that play a crucial role in mammalian apoptosis either during development or due to growth factor deprivation. Additionally, caspases have been implicated in the pathway to neuronal apoptosis from mild excitotoxic insults (Tenneti *et al.,* 1997, 1998). More intense excitotoxic injuries evoke rapid and irreversible energy compromise, leading to the failure of ionic homeostasis with consequent swelling and lysis (Ankarcrona *et al.,* 1995; Bonfoco *et al.,* 1995); this form of cell death represents necrosis and is not dependent on caspases (Tenneti *et al.,* 1998). One new regulatory pathway of caspase activity involves S-nitrosylation. In a recent development in the apoptosis field, in primary cerebrocortical neurons (Tenneti *et al.,* 1997), and in other cell types (Dimmeler *et al.,* 1997; Melino *et al.,* 1997; Ogura *et al.,* 1997), caspase activity has been found to be decreased by S-nitrosylation or transfer of an NO^+-like group to a critical cysteine sulfhydryl that is located in the active site in all caspase enzymes. This finding has important implications for the regulation of apoptosis by the NO group: under specific redox conditions that favor nitrosylation of caspases, apoptosis can be attenuated by decreasing caspase activity, whereas conditions favoring the generation of NO· will lead, via reaction with O_2^{-}, to the production of peroxynitrite and consequent cell death (either apoptotic or necrotic depending on the intensity of the insult).

A Variety of Targets for S-Nitrosylation

After NMDAR activity was shown to be regulated by NO-related species, similar data were presented for the

Ca^{2+}-activated K^+ channel of cardiac muscle (Bolotina *et al.,* 1994). In this case, donors of NO^+ equivalents were shown to activate the channel, and, similar to findings at the NMDAR in our laboratory, NEM blocked the effect by irreversibly alkylating thiol groups. Along similar lines, several other ion channels, enzymes, G proteins, transcription factors, and other proteins are either upregulated or downregulated by similar mechanisms of S-nitrosylation or donation of NO^+ equivalents to regulatory sulfhydryl centers (Stamler *et al.,* 1997). The list will undoubtedly grow, just as phosphorylation, myristoylation, and palmitoylation have become recognized as important biochemical processes for regulatory function. Interestingly, palmitoylation may be aimed at similar critical thiol group targets, resulting in thioester bond formation. In fact, on some proteins such as SNAP-25 it is possible that S-nitrosylation and palmitoylation may compete for the same sulfhydryl group, possibly with different physiological outcomes (Hess *et al.,* 1993).

In contrast to better known intracellular regulatory processes such as phosphorylation, S-nitrosylation of critical cysteine residues can occur extracellularly, intracellularly, or possibly even within the putative membrane-spanning region of a protein. From this point of view, S-nitrosylation may offer additional versatility in the location of control that can be exerted compared to phosphorylation and other better known posttranslational forms of modification (Choi *et al.,* 2000).

Proposed Consensus Motif for S-Nitrosylation

The primary amino acid sequence of functionally important sites for posttranslational modification of proteins are distinguished by the occurrence of certain patterns or motifs. In many cases such motifs constitute only a very minor part of the entire protein primary sequence. Thus, small patterns often are not detected by overall alignment of protein sequences that are only distantly or not at all related. Such motifs, however, can be identified by the occurrence of a particular cluster of residue types in the primary sequence. A collection of such sequence fingerprints has been developed for PROSITE, a data base of biologically significant sites and patterns that can be used to identify families of functionally related proteins or sites for posttranslational modification. Examples of such motifs are the consensus sequence patterns required for glycosylation or phosphorylation.

In an attempt to define a possible consensus motif that might be required or at least be facilitatory for S-nitrosylation, in collaboration with the laboratories of Nikolaus Sucher and Jonathan Stamler, we initially examined the putative target sites for redox modulation of NMDARs. Most importantly, cysteine residues in motifs similar to that described later for the NMDAR have been shown by various chemical criteria to be nitrosylated on proteins such as hemoglobin, $p21^{ras}$, cyclooxygenase, and others (Stamler *et al.,* 1997). As discussed earlier, two cysteines (abbreviated C in the single letter amino acid code) in the NR1 subunit have been found by site-directed mutagenesis to be necessary for the persistent component of redox modulation of that receptor (Sullivan *et al.,* 1994), and in the reduced state they can be S-nitrosylated (Choi *et al.,* 2000). Unexpectedly, however, these cysteines, C744 and C798, appear to be conserved in all ionotropic glutamate receptors when the sequences are aligned by overall similarity (Moriyoshi *et al.,* 1991; Sucher *et al.,* 1995). Nonetheless, among the ionotropic glutamate receptors, only NMDARs are exquisitely sensitive to redox modulation and NO effects (Aizenman *et al.,* 1989; Lei *et al.,* 1992). Inspection of the immediate amino acid neighbors of these cysteines revealed that the NR1 cysteines are distinguished from the cysteines conserved in the other ionotropic glutamate receptors in that they are preceded at position -2 by a polar amino acid (G, S, T, C, Y, N, Q), an acidic (D, E) or basic (K, R, H) amino acid at position -1, and an acidic amino acid at position $+1$. On the basis of this observation, we constructed the degenerate amino acid pattern designated (G,S,T,C,Y,N,Q)(K,R,H,D,E)C(D,E) in standard single letter amino acid code and used it in a search of the Protein Identification Resource (PIR) and Swiss Protein (SW) databases with the program Find patterns of the GCG software pckage (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711). In the PIR database (Release 44.0; March 1995), 3878 sequences out of 77,573 contained this pattern at least once, and in the SW database (Release 31.0; March 1995), 2383 out of 43,470 sequences contained this pattern. Viral, bacterial, plant, and animal sequences contained this motif.

Among candidates for regulation by S-nitrosylation that were identified by the database search were ion channels (NMDAR, voltage-sensitive Na^+ channel, cyclic nucleotide-gated channel), transporters (Ca^{2+}-ATPase, K-transporter), receptors (inositol trisphosphate receptor, nerve growth factor receptor), enzymes (oxidoreductases, dehydrogenases, adenylate and guanylate cyclases, proteases, DNA topoisomerases, DNA and RNA polymerases, kinases, phosphatases), transcription factors (helix–loop–helix proteins, NF-κB, zinc finger proteins), small GTP-binding proteins (rab, ras, sas, ypt), cell adhesion molecules (integrins, neural cell adhesion molecule), cell adhesion substrates (laminin, collagen), cyclins, and coagulation factors (IXa, Xa, XIII).

In fact, 20 of 27 proteins that had been listed in a review (Stamler, 1994) as bioregulatory targets of nitrosylation contain the full motif. The presence of the putative nitrosylation motif in guanylate cyclase suggests that the NO group may regulate the functional activity of guanylate cyclase by S-nitrosylation in addition to the interaction with the heme group of this enzyme. It is possible, however, that S-nitrosylation might occur at sites other than the proposed motif, or that the motif may only be evident in the tertiary rather than the primary structure of some proteins (this appears to be true for the caspases, for example). Moreover, it appears that a certain subset of the motif (basic or acidic amino acid residue–cysteine residue–acidic residue) may bear the highest statistical correlation to the propensity for nitrosylation (Stamler *et al.,* 1997). Although the proposed motif was de-

fined post hoc on the basis of our results with redox modulation of the NMDAR, it should allow us to identify additional possible target proteins for S-nitrosylation. Most importantly, this motif predicts a target sequence that can be subjected to site-directed mutagenesis in order to experimentally verify its importance for S-nitrosylation. Finally, for the NMDAR, site-directed mutagenesis has shown that the major determinant of S-nitrosylation occurs at the cysteine residue at position 399 of the NR2A subunit, which fits the proposed consensus motif for nitrosylation. Reversed-phase HPLC evidence has demonstrated, on a peptide containing the N-terminal region of this NMDAR subunit, that *S*-nitrosothiol formation indeed occurs by chemical criteria (Choi *et al.,* 2000).

Proposed Nitroxyl Reactions with Cysteine Thiol

It has been proposed that NO^- can be generated in two forms, NOH or HNO, both of which may exist in the triplet or singlet states (Bonner and Stedman, 1996; Wink and Feelisch, 1996). For example, in the singlet state, two antibonding electrons occupy a single outer π^* (pi antibonding molecular orbital), with spins opposed. In the triplet state the two π^* antibonding orbitals each contain an electron with spins aligned (with a z component of electron spin of $+1$, 0, or -1; hence, the designation ''triplet state''). In the singlet state, we have found evidence that NO^-/HNO (pK^a 4.7) can react most likely directly with critical thiols of the NMDAR to yield either an R–SNH–OH derivative or possibly a disulfide, in which case hydroxylamine (NH_2OH) is also formed (Fig. 2A) (Doyle *et al.,* 1988; Arnelle and Stamler, 1995). Triplet NO^- does not react directly with thiol groups but can react with O_2, forming peroxynitrite (Stamler *et al.,* 1992b; Arnelle and Stamler, 1995; Butler *et al.,* 1995), which does react with thiol (Radi *et al.,* 1991). At low concentrations peroxynitrite decreases NMDA receptor activity by oxidizing critical thiol groups (Kim *et al.,* 1996), whereas it is neurodestructive at higher concentrations (Beckman *et al.,* 1990; Dawson *et al.,* 1993; Lipton *et al.,* 1993). We therefore speculate that triplet NO^- is acting here via formation of low concentrations of $ONOO^-$ to downregulate NMDA receptor activity (Fig. 2B). These reactions decrease NMDAR activity (Lipton *et al.,* 1993; Lipton and Stamler, 1994). The chemistry of S-nitrosylation of the NMDAR (Choi *et al.,* 2000) and nitroxyl reactions with the NMDAR has been detailed (Kim *et al.,* 1999).

Conclusions

Possible chemical reactions of the NO group are dictated by its redox state. In the case of NO^+ equivalents, this mechanism appears to involve S-nitrosylation and possibly further oxidation of critical thiols to disulfide bonds on the NMDAR to decrease channel activity; a cysteine residue, known to be present in the active site of all known caspase enzymes, can also be S-nitrosylated, resulting in inhibition of enzyme ac-

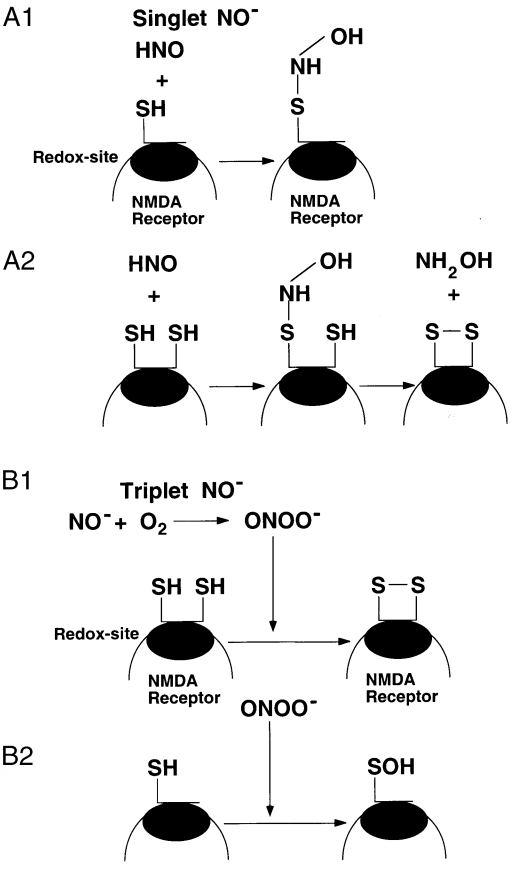


Figure 2 Proposed mechanism for the action of NO^- (nitroxyl anion). It has been proposed that NO^- can be generated in two forms, NOH or HNO, both of which may exist in the triplet or singlet states (Gallup, 1975). For example, in the singlet state, two antibonding electrons occupy a single outer π^* (pi antibonding molecular orbital), with spins opposed. In the triplet state the two π^* antibonding orbitals each contain an electron with spins aligned (with a z component of electron spin of $+1$, 0, or -1). (A) In the singlet state, NO^-/HNO (pK_a 4.7) can react with critical thiols of the NMDA receptor to yield an R-SNH-OH derivative (A1) or disulfide (A2), in which case hydroxylamine (NH_2OH) is also formed (Arnelle and Stamler, 1995). These reactions would downregulate NMDA receptor activity (Doyle *et al.,* 1988; Lipton *et al.,* 1993; Sullivan *et al.,* 1994). To confirm this type of reaction scheme, we have shown that hydroxylamine forms when an NO^- generating drug reacts with vicinal thiols. Thus, reactions of singlet NO^- are likely to be neuroprotective. (B) Triplet NO^-, rather than reacting directly with thiol, tends to react with molecular oxygen to form peroxynitrite ($ONOO^-$) (Hughes and Nicklin, 1971; Bonner and Stedman, 1996), which can also oxidize thiols to disulfide (B1) (Radi *et al.,* 1991); this reaction can be reversed with DTT. Alternatively, $ONOO^-$ can oxidize thiol to produce an $-$OH modification of sulfur (a sulfenic acid or R-SOH, as shown in B2), which is also reversible with DTT (Becker *et al.,* 1998; Stamler and Hausladen, 1998). Indeed, we have demonstrated that at low concentrations peroxynitrite downregulates NMDA receptor activity, apparently reacting at a redox modulatory site(s) on the receptor, as DTT can reverse this effect (Kim *et al.,* 1996). Such effects of triplet NO^-/O_2, although supportive of the molecular mechanism entertained here and theoretically neuroprotective, are likely to be more tenuous *in vivo,* as peroxynitrite is a powerful oxidant that has been shown to account for NO-related neurotoxicity (Beckman *et al.,* 1990; Dawson *et al.,* 1993; Lipton *et al.,* 1993). [Adapted with permission from Kim *et al.,* (1999). *Neuron* **24,** 461–469.]

tivity. These effects of S-nitrosylation, and undoubtedly others, can lead to neuroprotection by ''NO donors'' such as NTG, which favor S-nitrosylation or related reactions. In contrast, NO· (with one more electron than NO^+) kinetically prefers to react with $O_2^{\cdot-}$ to yield peroxynitrite ($ONOO^-$), which is neurodestructive. Finally, nitroxyl anion (NO^-) has one additional electron compared to NO· and can exist in at least two different chemical states (singlet and triplet) with distinct reactivities that can directly or indirectly support reactions with thiol groups on the NMDAR, among other proteins.

It is becoming increasingly evident that in addition to those of NMDARs and caspase enzymes, the biological activities of many other proteins containing critical cysteine residues can be regulated by S-nitrosylation and other redox reactions, in a sense similar to the type of control exerted by phosphorylation (Lipton *et al.,* 1993). This type of chemical reaction may represent a new and ubiquitous pathway for the molecular control of protein function by potentially reactive sulfhydryl centers.

Acknowledgments

This review is based on work performed in a close collaboration between the laboratories of Stuart Lipton, then at Harvard Medical School, Jonathan Stamler at Duke Medical Center, Pierluigi Nicotera at the University of Konstanz (Germany), and Nikolaus Sucher at the Hong Kong University of Science and Technology. We are grateful to the many members of our laboratories who contributed. The work was presented at several recent meetings, and this is reflected in the proceedings of these meetings and other reviews. However, the present chapter has been updated to reflect more current work.

References

Aizenman, E., and Potthoff, W. K. (1999). Lack of interaction between nitric oxide and the redox modulatory site of the NMDA receptor. *Br. J. Pharmacol.* **126,** 296–300.

Aizenman, E., Lipton, S. A., and Loring, R. H. (1989). Selective modulation of NMDA responses by reduction and oxidation. *Neuron* **2,** 1257–2163.

Aizenman, E., Brimecombe, J. C., Potthoff, W. K., and Rosenberg, P. A. (1998). Why is the role of nitric oxide in NMDA receptor function and dysfunction so controversial? *Prog. Brain Res.* **118,** 53–71.

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. (1995). Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15,** 961–973.

Armstrong, N., Sun, Y., Chen, G.-Q., and Gouaux, E. (1998). Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature* **395,** 913–917.

Arnelle, D. R., and Stamler, J. S. (1995). NO^+, NO·, and NO^- donation by *S*-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch. Biochem. Biophys.* **318,** 279–285.

Becker, K., Savvides, S. N., Keese, M., Schirmer, F. H., and Karplus, P. A. (1998). Enzyme inactivation through sulfhydryl oxidation by physiologic NO-carriers. *Nat. Struct. Biol.* **5,** 267–271.

Beckman, J. S. (1994). The physiological and pathological chemistry of nitric oxide. *In* ''The Biological Chemistry of Nitric Oxide'' (J. Lancaster, ed.), Academic Press, San Diego.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagaon, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368,** 850–853.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., and Lipton, S. A. (1995). Apoptosis and necrosis: Two distinct events induced respectively by mild and intense insults with NMDA or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7162–7166.

Bonner, F. T., and Stedman, G. (1996). The chemistry of nitric oxide and redox-related species. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. S. Stamler, eds.), pp. 3–27, Wiley, Chichester, England.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santilliano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. *Cell* **84,** 757–767.

Brorson, J. R., Schumacker, P. T., and Zhang, H. (1999). Nitric oxide acutely inhibits neuronal energy production. *J. Neurosci.* **19,** 147–158.

Butler, A. R., Flitney, F. W., and Williams, D. L. (1995). NO, nitrosonium ions, nitrosothiols and iron-nitrosyls in biology: A chemist's perspective. *Trends Pharmacol. Sci.* **16,** 18–22.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1,** 623–634.

Choi, Y.-B., Chen, H.-S. V., and Lipton, S. A. (2000). Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. *Nat. Neurosci.* **3,** 15–21.

Ciabarra, A. M., Sullivan, J. M., Gahn, L. G., Pecht, G., Heinemann, S., and Sevarino, K. A. (1995). Cloning and characterization of χ-1: A developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *J. Neurosci.* **15,** 6498–6508.

Coyle, J. T., and Puttfarken, P. (1993). Oxidative stress, glutamate and neurodegenerative disorders. *Science* **262,** 689–695.

Das, S., Sasaki, Y. F., Rothe, T., Premkumar, L. S., Takasu, M., Crandall, J. E., Dikkes, P., Connor, D. A., Rayudu, P., Cheung, W., Chen, H.-S. V., Lipton, S. A., and Nakanishi, N. (1998). Increased NMDA current and spine density in mice lacking the NMDAR subunit, NR3A. *Nature* 377–381.

Dawson, T. M., Dawson, V. L., and Snyder, S. H. (1992). A novel neuronal messenger molecule in brain: The free radical, nitric oxide. *Ann. Neurol.* **32,** 297–311.

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., and Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 6368–6371.

Dawson, V. L., Dawson, T. M., Bartley, D. A., Uhl, G. R., and Snyder, S. H. (1993). Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13,** 2651–2661.

Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A. M. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1β-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.* **185,** 601–607.

Doyle, M. P., Surendra, M. N., Broene, R. D., and Guy, J. K. (1988). Oxidation and reduction of hemoproteins by trioxodinitrite(II). The role of nitrosyl hydride and nitrite. *J. Am. Chem. Soc.* **110,** 593–599.

Dreyer, E. B., and Lipton, S. A. (1999). New perspectives on glaucoma. *JAMA* **281,** 306–308.

Fagni, L., Olivier, M., Lafon-Cazal, M., and Bockaert, J. (1995). Involvement of divalent ions in the nitric oxide-induced blockade of *N*-methyl-D-aspartate receptors in cerebellar granule cells. *Mol. Pharmacol.* **47,** 1239–1247.

Gallup, G. A. (1975). *Inorg. Chem.* **14,** 563–565.

Gow, A. J., Buerk, D. G., and Ischiropoulos, H. (1997). A novel reaction mechanism for the formation of *S*-nitrosothiol *in vivo*. *J. Biol. Chem.* **272,** 2841–2845.

Hebesteit, H., Dibbert, B., Balatti, I., Braun, D., Schapowal, A., Blaser, K., and Simon, H.-U. (1998). Disruption of fas receptor signaling by nitric oxide in eosinophils. *J. Exp. Med.* **187,** 415–425.

Hess, D. T., Patterson, S. I., Smith, D. S., and Skene, J. H. P. (1993). Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* **366,** 562–565.

Hogg, N., Singh, R. J., and Kalyanaraman, B. (1996). The role of glutathione in the transport and catabolism of nitric oxide. *FEBS Lett.* **382,** 223–228.

Hoyt, K. R., Tang, L.-H., Aizenman, E., and Reynolds, I. J. (1992). Nitric oxide modulates NMDA-induced increases in intracellular Ca^{2+} in cultured rat forebrain neurons. *Brain Res.* **592,** 310–316.

Hughes, M. N., and Nicklin, H. G. (1971). *J. Chem. Soc. A,* 164.

Kim, W.-K., Rayudu, P. V., Mullins, M. E., Stamler, J. S., and Lipton, S. A. (1996). Down regulation of NMDA receptor activity in cortical neurons by peroxynitrite. *In* ''The Biology of Nitric Oxide'' (S. Moncada, J. S. Stamler, S. Gross, and E. A. Higgs, eds.), Vol. 5, p. 126. Portland Press, London.

Kim, W.-K., Choi, Y.-B., Rayudu, P. V., Das, P., Asaad, W., Arnelle, D. R., Stamler, J. S., and Lipton, S. A. (1999). Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO^-. *Neuron* **24,** 461–469.

Kim, Y.-M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Kluge, I., Gutteck-Amsler, U., Zollinger, M., and Do, K. Q. (1997). *S*-nitrosoglutathione in rat cerebellum: Identification and quantification by liquid chromatography-mass spectrometry. *J. Neurochem.* **69,** 2599–2607.

Kohr, G., Eckardt, S., Lüddens, H., Monyer, H., and Seeburg, P. H. (1994). NMDA receptor channels: Subunit-specific potentiation by reducing agents. *Neuron* **12,** 1031–1040.

Lei, S. Z., Pan, Z.-H., Aggarwal, S. K., Chen, H.-S. V., Hartman, J., Sucher, N. J., and Lipton, S. A. (1992). Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. *Neuron* **8,** 1087–1099.

Leist, M., Single, B., Naumann, H., Fava, D., Simon, B., Kuhnle, S., and Nicotera, P. (1999). Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. *Exp. Cell Res.* **249,** 396–403.

Lipton, S. A., and Rosenberg, P. A. (1994). Mechanisms of disease: Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* **330,** 613–622.

Lipton, S. A., and Stamler, J. S. (1994). Actions of redox-related congeners of nitric oxide at the NMDA receptor. *Neuropharmacology* **33,** 1229–1233.

Lipton, S. A., and Wang, Y. F. (1996). NO-related species can protect from focal cerebral ischemia/reperfusion. *In* ''Pharmacology of Cerebral Ischemia, 1996'' (J. Krieglstein, ed.), pp. 183–191. Medpharm Scientific Publishers, Stuttgart.

Lipton, S. A., Choi, Y.-B., Pan, Z.-H., Lei, S. Z., Chen, H.-S. V., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Lipton, S. A., Choi, Y.-B., Sucher, N. J., Pan, Z.-H., and Stamler, J. S. (1996). Redox state, NMDA receptors, and NO-related species. *Trends Pharmacol. Sci.* **17,** 186–187.

Lipton, S. A., Rayudu, P. V., Choi, Y.-B., Sucher, N. J., and Chen, H.-S. V. (1998). Redox modulation of the NMDA receptor by NO-related species. *Prog. Brain Res.* **118,** 73–82.

Mannick, J. B., Asano, K., Izumi, K., Kleff, E., and Stamler, J. S. (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* **79,** 1137–1146.

Mannick, J. B., Hausladen, A., Liu, L., Hess, D. T., Zeng, M., Miao, Q. X., Kane, L. S., Gow, A. J., and Stamler, J. S. (1999). Fas-induced caspase denitrosylation. *Science* **284,** 651–654.

Manzoni, O., and Bockaert, J. (1993). Nitric oxide synthase activity endogenously modulates NMDA receptors. *J. Neurochem.* **61,** 368–370.

Manzoni, O., Prezeau, L., Marin, P., Deshager, S., Bockaert, J., and Fagni, L. (1992). Nitric oxide-induced blockade of NMDA receptors. *Neuron* **8,** 653–662.

Mayer, B., Pfeiffer, S., Schrammel, A., Koesling, D., Schmidt, K., and Brunner, F. (1998). A new pathway of nitric oxide/cyclic GMP signaling involving *S*-nitrosoglutathione. *J. Biol. Chem.* **273,** 3264–3270.

Meldrum, B., and Garthwaite, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11,** 379–387.

Melino, G., Bernassola, F., Knight, R. A., Corasaniti, M. T., Nisticò, G., and Finazzi-Agrò, A. (1997). S-nitrosylation regulates apoptosis. *Nature* **388,** 432–433.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1991). Molecular cloning and characterization of the rat NMDA receptor [see comments]. *Nature* **354,** 31–37.

Nikitovic, D., and Holmgren, A. (1996). *S*-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J. Biol. Chem.* **271,** 19180–19185.

Noronha-Dutra, A. A., Epperlein, M. M., and Woolf, N. (1993). Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing. *FEBS Lett.* **321,** 59–62.

Ogura, T., Tatemichi, M., and Esumi, H. (1997). Nitric oxide inhibits CPP32-like activity under redox regulation. *Biochem. Biophys. Res. Commun.* **236,** 365–369.

Omerovic, A., Chen, S.-J., Leonard, J. P., and Kelso, S. R. (1995). Subunit-specific redox modulation of NMDA receptors expressed in *Xenopus* oocytes. *J. Recept. Sign. Transduc. Res.* **15,** 811–827.

Pryor, W. A., and Lightsey, J. W. (1981). Mechanisms of nitrogen dioxide reactions: Initiation of lipid peroxidation and the production of nitrous acid. *Science* **214,** 435–437.

Pryor, W. A., Church, D. F., Govinden, C. K., and Crank, G. (1982). Oxidation of thiols by nitric oxide and nitrogen dioxide: Synthetic utility and toxicological implications. *J. Org. Chem.* **47,** 156–159.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266,** 4244–4250.

Rothman, S. M., and Olney, J. W. (1987). Excitotoxicity and the NMDA receptor. *Trends Neurosci.* **10,** 299–302.

Sathi, S., Edgecomb, P., Warach, S., Manchester, K., Donaghey, T., Stieg, P. E., Jensen, F. E., and Lipton, S. A. (1993). Chronic transdermal nitroglycerin (NTG) is neuroprotective in experimental rodent stroke models. *Soc. Neurosci. Abstr.* **19,** 849.

Stamler, J. S. (1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78,** 931–936.

Stamler, J. S., and Hausladen, A. (1998). Oxidative modifications in nitrosative stress. *Nat. Struct. Biol.* **5,** 247–249.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992a). S-nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992b). Biochemistry of nitric oxide and its redox activated forms. *Science* **258,** 1898–1902.

Stamler, J. S., Toone, E. J., Lipton, S. A., and Sucher, N. J. (1997). (S)NO signals: Translocation, regulation, and a consensus motif. *Neuron* **18,** 691–696.

Sucher, N. J., Akabarian, S., Chi, C. L., Leclerc, C. L., Awobuluyi, M., Deitcher, D. L., Wu, M. K., Yuan, J. P., Jones, E. G., and Lipton, S. A. (1995). Developmental and regional expression pattern of a novel NMDA receptor-like subunit (NMDAR-L) in the rodent brain. *J. Neurosci.* **15,** 6509–6520.

Sucher, N. J., Awobuluyi, M., Choi, Y.-B., and Lipton, S. A. (1996). NMDA receptors: From genes to channels. *Trends Pharmacol. Sci.* **17,** 348–355.

Sullivan, J. M., Traynelis, S. F., Chen, H.-S. V., Escobar, W., Heinemann, S. F., and Lipton, S. A. (1994). Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. *Neuron* **13,** 929–936.

Tenneti, L., D'Emilia, D. M., and Lipton, S. A. (1997). Suppression of neuronal apoptosis by S-nitrosylation of caspases. *Neurosci. Lett.* **236,** 139–142.

Tenneti, L., D'Emilia, D. M., Troy, C. M., and Lipton, S. A. (1998). Role of caspases in *N*-methyl-D-aspartate-induced apoptosis of cerebrocortical neurons. *J. Neurochem* **71,** 946–959.

Wink, D. A., and Feelisch, M. (1996). Formation and detection of nitroxyl and nitrous oxide. *In* ''Methods in Nitric Oxide Research'' (M. Feelisch and J. S. Stamler, eds.), pp. 403–412. Wiley, Chichester, England.

Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998). Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* **279,** 234–237.

Yun, H.-Y., Gonzalez-Zulueta, M., Dawson, V. L., and Dawson, T. M. (1998). Nitric oxide mediates *N*-methyl-D-aspartate receptor-induced activation of $p21^{ras}$. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5773–5778.

Zhang, J., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1994). Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science* **263,** 687–689.

CHAPTER 30

Nitric Oxide and Autoimmune Disease in the Nervous System

Pathobiology of Inflammation and Demyelination

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THIS CHAPTER DISCUSSES THE PRACTICAL ASPECTS OF MEASURING NITRIC OXIDE (NO) AND NITRIC OXIDE SYNTHASE (NOS) IN BIOLOGICAL SPECIMENS FROM ANIMALS AND HUMANS. IT DEALS WITH THE ISSUES OF EXPERIMENTAL PARADIGMS FOR INDUCING NO AND THE REASONS FOR THE DIFFERENCES IN THE ABILITY TO INDUCE NO IN RODENT AND HUMAN CELLS, WITH AN EMPHASIS ON THE IMPLICATIONS OF USING ANIMAL MODELS FOR DERIVING USEFUL INFORMATION ABOUT TREATING HUMAN DISEASE. A SECOND ASPECT OF THE CHAPTER REVIEWS THE ROLE OF NO IN AN ANIMAL MODEL FOR MULTIPLE SCLEROSIS (MS), EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE), AS WELL AS EVIDENCE FOR NO PRODUCTION IN MS. GENERAL CONCEPTS REGARDING THE REGULATION OF NO OF AND BY THE IMMUNE RESPONSE ARE DISCUSSED WITH SPECULATIONS REGARDING THE INTERACTIONS OF THESE MOLECULES IN ALTERING THE DISEASE COURSE IN MS PATIENTS. THE CONCLUSION OF THIS CHAPTER IS THAT NO REGULATES GENE TRANSCRIPTION AS WELL AS THE INTEGRITY, STRUCTURE, AND FUNCTION OF PROTEINS AND MOLECULES AND, IN THE CONTEXT OF AUTOIMMUNE DISEASE, MAY ACT AS A PRO- OR ANTI-INFLAMMATORY MOLECULE.

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) thought to be of immune origin. Activated T cells and immunoglobulin-producing B cells converge in the white matter where an inflammatory milieu is created, initiating the influx of macrophages from the blood and the activation of the endogenous macrophages called microglia. Although the major amount of supporting data that free radicals contribute to the reactive gliosis, oligodendrocyte cell death, and demyelination is derived from the animal model for MS, experimental autoimmune encephalomyelitis (EAE) (see below), there is some evidence from patient material as well. Free radicals or their metabolites as well as lipid peroxidation products are elevated in plasma and cerebrospinal fluid (CSF) of MS patients (Glabinski *et al.,* 1993; Hunter *et al.,* 1985). Spectroscopic changes have demonstrated lipid and protein oxidation in brain samples containing MS lesions (Levine and Warren, 1998). Antioxidants, thought to provide protection against oxidative stress, are elevated in MS sera (Gutowski *et al.,* 1998). Myeloperoxidase is present in MS brain macrophages/microglia (Nagra *et al.,* 1997), thereby offering an additional mechanism for free radical generation in the CNS. With regard to the etiology of MS, it has been postulated both that free radicals are the consequence of immune activation of inflammatory

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cells as well as that infections or trauma initiate free radical formation which then activates T cells and sets off an autoimmune cascade (Cooper, 1997).

This chapter will deal with the issues of production and regulation of nitric oxide (NO) *in vivo* and *in vitro* in both MS tissues and cells. This discussion and the analysis of the role of NO in the EAE model will be presented in the format of general principles, allowing a better understanding of how nitric oxide may function in the presence or absence of free radicals of oxygen. Furthermore, the concept that NO may function in both inflammation and repair, thereby acting in both a proinflammatory and anti-inflammatory capacity, will be considered.

Methods Used for Detection of iNOS/NO in CNS Tissues

Inducible nitric oxide synthase (iNOS or type II NOS) has been identified as the isoform of interest in the inflammatory lesions in MS and EAE. Its presence and function have been assessed in tissues such as brain and spinal cord, sera, and cerebrospinal fluid, as well as in *ex vivo* leukocytes derived from patients with MS or animals with EAE, using biochemical, immunohistochemical, and molecular techniques. Identification of NO-triggered events or ''footprints'' is also useful to substantiate the presence of functional iNOS enzyme and NO production. iNOS generates higher levels of NO than type I or III NOS isoforms: *ex vivo* or *in vitro* 10^6 cells can produce nanomoles to micromoles of NO in a period of hours to days, thereby allowing measurement with the colorimetric assay for nitrite (NO_2^-) and nitrate (NO_3^-). Nevertheless, there are some major differences in the stimuli required and kinetics of NO production when comparing human and rodent cells, which need to be carefully considered when interpreting negative results (reviewed in Parkinson *et al.,* 1997; Mitrovic *et al.,* 1996). These will be discussed in the context of comparisons of MS and EAE.

Determination of NO_2^- Plus NO_3^- and Citrulline Levels

The fluorometric assay for nitrite (NO_2^-) plus nitrate (NO_3^-) and the radiochemical assay for L-citrulline provide greater sensitivity at lower arginine concentrations than other NOS enzyme assays and are suitable end-point assays for studying enzyme kinetics. Such assays have been used in assessing NO_2^-/NO_3^- levels in CSF and sera and in assessing NO produced by MS leukocytes *ex vivo* with or without stimulation and by human and rodent glia stimulated by cytokines in *in vitro* cultures. The radiochemical assay for citrulline can be performed using either [^{3}H]- or [^{14}C]arginine, with separation of arginine from citrulline on ion-exchange resins at low pH. This assay can be used for stoichiometric and kinetic analyses of NOS activity when NOS activity is low in samples; it is superior in sensitivity to the fluorimetric analysis and can be adapted to high-throughput assays using spectrophotmetric analysis on plate readers. In aqueous or biological solutions, such as culture supernatants, sera, and CSFs, NO oxidizes rapidly to equilibrium mixtures to NO_2^- plus NO_3^-. Since the ratio between these two species varies considerably in biological samples, it is advisable to measure the sum of both metabolites to avoid underestimation of NO. This is especially true in human samples, where NO_2^- is rapidly oxidized to NO_3^-, in comparison to what has been found in rodent samples (Ikeda *et al.,* 1995). Colorimetric determination, based on diazotization of sulfanilic acid and (1-naphthyl)ethylenediamine (Griess reagent) has been widely used. For ease in assessment, enzymatic conversion of NO_3^- to NO_2^- by nitrate reductase, followed by fluorimetric or colorimetric NO_2^- assay, is preferred by many investigators. Neverteless, a quantitative conversion of NO_3^- to NO_2^- to NO can be achieved using boiling vanadium(III) under acidic conditions, thereby yielding measurement of total NO (NO_x) which is, in some samples, more or less equivalent to NO_2^- levels determined by the Griess reagent plus nitrate reductase (Ding *et al.,* 1997; Ding and Merrill, 1997).

In Situ Detection of NOS Protein, mRNA, and Activity

Several histochemical and molecular techniques have been used to identify the presence, anatomical location, cellular distribution, and function of iNOS in MS brain and spinal cord (Parkinson *et al.,* 1997; Mitrovic *et al.,* 1996). Isoform-specific anti-NOS antibodies have been developed and are now commercially available from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotech (Santa Cruz, CA), among others; use of such polyclonal and monoclonal antibodies to human iNOS has demonstrated the enzyme in human tissues including brain, lung, *ex vivo* leukocytes, and *in vitro* cultured human glia. iNOS protein can be induced in human glia within a few hours of stimulation with cytokines *in vitro* (Ding *et al.,* 1997; Lee *et al.,* 1993). Important considerations when attempting to stain human tissues or cultured cells with these reagents are (1) the specificity to a NOS domain is distinct from the C-terminal flavoprotein reductase domain, which has considerable homology with other flavoprotein reductases, leading to nonspecific staining; (2) species cross-reactivity for iNOS reagents is lower than that for the two constitutive isoforms; (3) some reagents identifying the isoform-specific N-terminal region of human iNOS may work on cultured cells or on Western blots but not on tissue sections because of inaccessible epitopes (rendered so by fixation), natural endogenous epitope masking, or low antibody affinity (Parkinson *et al.,* 1997).

Northern blot analysis or reverse transcriptase–polymerase chain reaction (RT-PCR) for iNOS mRNA in extracted samples, and *in situ* hybridization or *in situ* RT-PCR (RT-IS-PCR) for iNOS mRNA coupled to immunohistochemical identification of specific cells or iNOS protein-containing

cells, have been used in MS tissues (Lee *et al.,* 1993; Bagasra *et al.,* 1995; Ding and Merrill, 1997; DeGroot *et al.,* 1997). mRNA for iNOS is not seen in uninduced human glia *in vitro* but can be seen within 2 to 8 hours after cytokine induction (Lee *et al.,* 1993; Ding *et al.,* 1997). Since enzyme activity occurs and NO is only formed when NOS is conformationally in a head to head dimer, the presence of neither mRNA nor protein means that functional iNOS enzyme exists in cells or tissues (Ding *et al.,* 1997; Ding and Merrill, 1997). As in the case of other oxidoreductases, NOS has intrinsic diaphorase activity and can transfer electrons from NADPH to alternative electron acceptors such as ferricyanide, cytochrome *c,* and tetrazolium salts. The reaction of NOS with nitroblue tetrazolium in the presence of NADPH is the basis of the NADPH diaphorase (NADPHd) staining in tissues for identification of the presence of NOS. There is some discrepancy as to which cell type produces iNOS in MS tissues when comparing the studies using NADPHd staining compared to iNOS staining (Lee *et al.,* 1993; Brosnan *et al.,* 1994; Bö *et al.,* 1995; Bagasra *et al.,* 1995; DeGroot *et al.,* 1997; Ding and Merrill, 1997). Because not all NADPHds are iNOS and not all NOS functions as NADPHd, and since fixation may greatly influence the NADPHd detection, the use of specific antibodies with double staining for cell subtypes is a more reliable technique for identifying iNOS-positive cells in tissues (Mitrovic *et al.,* 1996; Parkinson *et al.,* 1997).

Indirect Detection of NO Formation by Assessment of ''Footprints''

When assays for detection of functional enzyme or NO and its metabolites are not easy or possible, as in brain and spinal cord tissue, an indirect assessment must be made through examining the chemical reactants induced or modified by NO. Additionally, the disparity in the kinetics of the presence of iNOS enzyme, which occurs days before the appearance of NO in culture supernatants or the extractability of L-citrulline from cell lysates of human cells (glia or macrophages), suggests that confirmatory assays for the production of NO are desirable (Ding and Merrill, 1997). NO or donors of NO can stimulate the activity of soluble guanylate cyclase, which then increases intracellular cGMP; thus, a cGMP radioimmunoassay is an indirect method of determining NO production. However, it is crucial to remember that this is not quantitative, nor is it specific for NO. Nevertheless, this assay can be used to show the presence of NO in biological systems, and it has been used to confirm studies demonstrating the signaling functions of NO in neurons. NO as a neurotransmitter can be mimicked by addition of soluble guanylate cyclase activators and upregulated by cGMP phosphodiesterase inhibitors (Ding *et al.,* 1997; Ding and Merrill, 1997).

NO reacts with superoxide anion (O_2^-) to form the powerful oxidizing agent peroxynitrite ($ONOO^-$). $ONOO^-$ can nitrate phenolic rings, forming 3-nitrotyrosine (NT) (Beckman *et al.,* 1994). Antibodies to NT have been developed and successfully used in many inflammatory tissues including MS and EAE brain and spinal cord to identify nitrated tyrosines on proteins (Bagasra *et al.,* 1995; DeGroot *et al.,* 1997; van der Veen *et al.,* 1997; Hooper *et al.,* 1997; Merrill *et al.,* 1998). This is of significance given that NT staining (a) is not seen in normal tissues, (b) colocalizes with areas of active lesion formation in MS and EAE where iNOS staining is also seen, and (c) will remain as evidence of previous foci of inflammation long after iNOS is downregulated as in burnt out lesions in MS brain tissue (Merrill *et al.,* 1998). Furthermore, a delayed kinetics of cGMP production and nitrotyrosine staining in cultured human glial cells has been shown to parallel the NO and L-citrulline detection, thereby validating the use of NO ''footprints'' as indicators of functional enzyme in a given biological system (Ding *et al.,* 1997; Ding and Merrill, 1997).

Evidence for iNOS/NO Production in MS

Brain and Spinal Cord Tissue

Two separate reports published from the same laboratory have demonstrated in MS tissue the colocalization of the purported macrophage marker *Ricinus communis* agglutinin (RCA-1) lectin and iNOS mRNA, as detected by reverse transcriptase–*in situ* PCR (Bagasra *et al.,* 1995; Hooper *et al.,* 1997). There have been no published studies using double immunostaining with a cell-specific marker and an iNOS-specific antibody in MS or EAE tissue. Indeed, no studies have colocalized NT to specifically labeled cells in MS lesions (Brosnan *et al.,* 1994; Bagasra *et al.,* 1995; Bö *et al.,* 1995; DeGroot *et al.,* 1997). There is some disagreement in the published reports on MS tissues as to whether astrocytes, microglia, or indeed both cell types make iNOS. Two studies using NADPHd as an indicator of iNOS found that it colocalized with astrocytes only (Brosnan *et al.,* 1994; Bö *et al.,* 1995). Three other studies found no iNOS staining in astrocytes, but did find NADPHd staining in CD68-positive cells (DeGroot *et al.,* 1997) or iNOS mRNA in RCA-1-positive cells (Bagasra *et al.,* 1995; Hooper *et al.,* 1997). Since NADPHd is not iNOS specific and does not directly correlate with the NOS activity of any of the three isoforms *in vivo* in tissues (Tracey *et al.,* 1993; Gonzalez-Hernandez *et al.,* 1996), we produced a specific antibody to a unique sequence in the amino terminus of human iNOS to assess the presence of this isoform in cells *in vitro* (Ding and Merrill, 1997) and *in vivo.* It is clear that several cell types might be able to produce iNOS and NO, and because the production of NO is elevated in MS patients (Boullerne *et al.,* 1995; Johnson *et al.,* 1995), it is important to quantitate both iNOS and NT staining in specific cell types in tissues from MS patients containing lesions at different stages of development. Data generated in this laboratory have shown that NO is produced by up to five distinct cell types in MS lesions: blood-borne macrophages, perivascular microglia, activated parenchymal microglia, gliotic astrocytes, and cells associ-

ated with endothelial cells at the blood–brain barrier (BBB) (possibly pericytes or Mato cells). The amount of iNOS and NT staining appears to correlate with the stage of the lesion (Merrill *et al.,* 1998).

Sera and Cerebrospinal Fluid

Analyses of CSF and sera from MS patients in several studies have demonstrated increased NO_2^-/NO_3^- levels compared to noninflammatory neurological controls (Johnson *et al.,* 1995; Yamashita *et al.,* 1997; Giovanni *et al.,* 1997, 1998; Giovanni, 1998), although one study found no differences (Ikeda *et al.,* 1995), the reasons for which are not identifiable since all studies used the Griess reaction method. In the positive studies, the NO_2^-/NO_3^- levels in the CSF were 10–20 μM and 40% above controls, whereas serum levels were 5–10 times the amount determined in CSF and 20% above controls. In the Ikeda study, where MS NO production was not elevated, NO_2^-/NO_3^- levels were under 10 μM, suggesting the possibility of problems with sample preservation, assay inhibitors, or background (Ikeda *et al.,* 1995). One published study demonstrated a relationship of NO to relapses, whereas a second study found no relationship to disability (Yamashita *et al.,* 1997; Giovanni *et al.,* 1997). Other indicators that NO may be chronically upregulated in MS CNS have been reported; these include increased CSF neopterin, a precursor of iNOS cofactor tetrahydrobiopterin (Johnson *et al.,* 1995) and circulating antibodies to an immunogenic nitroso amino acid, *S*-nitrosocysteine (Boullerne *et al.,* 1995).

Blood Macrophages *ex Vivo*

Although it has been somewhat difficult *in vitro* to demonstrate that human blood-borne MΦ make iNOS/NO, after cross-linking of CD69, CD23, and the receptor for HIV-1/gp120, human MΦ can be activated to produce NO (DeMaria *et al.,* 1994; Bukrinsky *et al.,* 1995; Vouldoukis *et al.,* 1995). Additionally, human alveolar MΦ in lung tissue of individuals with a wide spectrum of acute and chronic inflammatory conditions have been shown to be iNOS positive *in vivo* (Kobzik *et al.,* 1993; Nicholson *et al.,* 1996), and/or to produce iNOS *ex vivo* (Nicholson *et al.,* 1996). López-Moratalla *et al.,* (1997) have shown that peripheral blood MΦ, freshly isolated from MS patients, but not controls, constitutively express iNOS and produce NO. In a second study, NO production by blood cells was associated with increased production of proinflammatory cytokines and, therefore, could be considered as a marker of mononuclear cell activation, and thus an indirect marker of disease activity in the peripheral blood of MS patients. Sarchielli *et al.* (1997) demonstrated that both basal NO and stimulated NO production by *ex vivo* peripheral blood mononuclear cells (PBMC) were significantly elevated in patients with active lesions as detected by MRI than those without active lesions. Patients without active disease produced NO levels that were no different from controls. During relapse, NO production by PBMCs was elevated over that in stable stages. NO levels fell during recovery phase from relapses. NO production by PBMCs was positively correlated with their production of interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and γ-interferon (IFN-γ) and negatively correlated with IL-10, and transforming growth factor β (TGF-β).

Other Neurodegenerative Diseases

Type II nitric oxide synthase has been identified in other neurodegenerative disease tissues. iNOS and NT staining have been seen in astrocytes in progressive supranuclear palsy, a nonhereditary neurodegenerative disorder involving neurofibrillary tangles and neuronal loss in the brain stem, basal ganglia, and cerebellum (Komori *et al.,* 1998). Oxidative stress and lipid peroxidation have been implicated in Alzheimer's disease (AD). Vodovotz *et al.* (1996) were the first to identify, by immunohistochemical techniques, iNOS in tangle-bearing neurons of AD brain tissue. Several studies have found iNOS in AIDS brains by PCR, although by the highly sensitive RT-IS-PCR, Bagasra *et al.* (1997) were not able to see the iNOS message. As in the MS cases, NO_2^-/NO_3^- levels were high in sera and CSF of HIV-1-positive patients, and an albumin quotient indicated NO was being produced intrathecally (Giovanni *et al.,* 1998). NT staining in neurofibrillary tangles (NFT) as well as in neurons lacking NFTs has been seen in AD. Interestingly, no reaction was seen in extracellular NFTs, amyloid deposits, or dystrophic neurites of senile plaques. NT was undetectable in cerebral cortex of age-matched control brains (Good *et al.,* 1996; Smith *et al.,* 1997). These findings provide evidence that $ONOO^-$ may be involved in the inflammatory sequelae and oxidative damage of AD pathology. They also contribute to our understanding that NO and its activity can be detected in pathological states in the CNS.

Production of NO by Human Glia and Macrophages

Human Glial Cells

Because the production of iNOS and NO in the CNS has such potentially harmful effects on both neurons and oligodendrocytes and may play a role in such diseases as CNS AIDS, and MS, the cells of the nervous system may have evolved a protective mechanism to prevent spurious levels of NO formation even in the presence of iNOS gene induction (Xie *et al.,* 1992). We and others have shown that human fetal astrocytes and microglia respond to IFN-γ and IL-1β by producing iNOS and mRNA within hours and iNOS protein within a day *in vitro.* However, in our experience, these cells do not make NO for a period of 24 to 48 hours after the appearance of the enzyme, at which time the production is between 10 and 20 μM; this is so only if nitrate is reduced to nitrite before analysis using the Griess reagent (Lee *et al.,* 1993; Ding *et al.,* 1997; Ding and Merrill, 1997). Examining production of just nitrite within the first 24 to 48 hours usually reveals production of less than 10 μM (Lee *et al.,* 1993;

Hua *et al.,* 1998). Such a delayed NO production is in contrast to the appearance of iNOS and NO production by rodent glia *in vitro* within 8 to 24 hours of stimulation (Simmons and Murphy, 1992). Many human cells such as heptacytes, chondrocytes, endothelial cells, and some glioblastoma cells have been shown to express iNOS and release large amounts of NO after stimulation with bacterial products and/or cytokines, showing similar kinetic patterns to that in rodent glial cells (Chakravarthy *et al.,* 1995; Blanco *et al.,* 1995; Grabowski *et al.,* 1996). Although human glial cells exhibit kinetics of iNOS transcription and translation in a time frame that is similar to other cell types, their kinetics of NO production is different compared to these other human cell types (Ding *et al.,* 1997; Ding and Merrill, 1997). Work from the laboratory of Dennis Stuehr has identified the fact that iNOS is not a functional enzyme until it is in a conformationally active state created by its dimerization (Baek *et al.,* 1993). The binding of heme and tetrahydrobiopterin (BH_4) to the enzyme play a significant role in forming and stabilizing active dimeric iNOS (Tzeng *et al.,* 1995). To understand if this temporal lag between iNOS enzyme synthesis and NO production was due to insufficient intracellular levels of the iNOS cofactor BH_4, we added BH_4 to the cultures. We were able to show a significant increase in NO production in a dose- and time-dependent manner, suggesting that the rate-limiting step in NO production in these human cells is cofactor induction and activation of functional iNOS enzyme (Ding *et al.,* 1997; Ding and Merrill, 1997).

As in rodent cultures, anti-inflammatory agents are able to inhibit NO production by human glia. In our laboratory, TGF-β, IL-4, IL-13, and IFN-β all inhibit NO and TNF-α production in human fetal glial cell cultures (St. Pierre *et al.,* 1996). IFN-β inhibited at lower concentrations than were required for inhibition by IL-13 and IL-4; furthermore, all the aforementioned cytokines inhibited TNF-α production better (e.g., at lower concentrations and to a greater degree) than they inhibited NO in these cultures. These findings support the observations that TNF-α does not directly induce NO and that the regulation of TNF-α and NO are dependent on different pathways. Although a separate study failed to find that IL-10 or TGF-β inhibited NO production, this same study and one other did confirm that nanamolar concentrations of IFN-β could inhibit NO production by human astrocytes (Guthikonda *et al.,* 1998; Hua *et al.,* 1998), and that the inhibition was at the transcriptional level (Hua *et al.,* 1998). Stewart *et al.* (1998), in a rodent culture system, demonstrated that NO-mediated damage by astrocytes to mitochondrial activities of complexes II/III, and IV in neighboring neurons is inhibitable by IFN-α/β. Compounds that raise cAMP will also inhibit NO production by human glia. Using the phosphodiesterase inhibitors pentoxifylline (PTX), rolipram, and isobutyl methyl xanthine (IBMX), we were able to demonstrate inhibition of both TNF-α and NO in human glial cell cultures; iloprost, a prostacyclin analog, was more potent than PTX or IBMX but less potent than rolipram in inhibition. As with the anti-inflammatory cytokines, TNF-α was more easily inhibited than NO (St. Pierre *et al.,* 1996). It has been observed in other cell systems in rodents that elevation of cAMP results in a downregulation of TNF-α and NO; the mechanism proposed has been through the inhibition of NF-κB transcription or elevation of IL-10 (St. Pierre *etal.,* 1996). Rolipram and PTX have been successfully used *in vivo* in the EAE model to reduce disease, and IFN-β is used to treat MS patients (Mitrovic *et al.,* 1996; Parkinson *et al.,* 1997). It is therefore likely that these *in vivo* effects may well be due to the inhibition of the purported contributors to pathology, TNF-α and NO.

Human Macrophages

Whereas NO production in murine macrophages accumulates in a linear fashion following a lag of 6 hours after stimulation of cytokines (Evans *et al.,* 1994), human mononuclear phagocytes express iNOS mRNA and protein but produce no or little NO *in vitro* after such stimulation (Murray and Teitelbaum, 1992; Padgett and Pruett, 1992; Schneemann *et al.,* 1993; Albina, 1995). Although posttranslational regulation has been speculated to be the reason for this, some investigators have excluded the possibility of the regulation by BH_4, as increasing BH_4 levels do not enable the cells to produce high levels of NO (Weinberg *et al.,* 1995). This suggests that NO production in human glia and macrophages may be regulated differently. Clearly, glial cells and macrophages could respond differently to cytokine stimulation, thereby initiating different mechanisms for regulating iNOS catalytic activity. Studies in which tissues from humans with a variety of diseases have been examined have clearly shown that human macrophages do make iNOS and NO *in vivo* (see earlier) (Bagasra *et al.,* 1995; Nicholson *et al.,* 1996; Vodovotz *et al.,* 1996; Weinberg *et al.,* 1995). Again, whereas cross-linking of lectin-like receptors may be important in mimicking an endogenous signaling event in macrophages, and although cross-linking of cytokine receptors alone is not adequate for NO induction (at least *in vitro*), there are intriguing links between stress-induced neuroendocrine pathways and NO in these cells. β-Endorphin, a peptide released by the pituitary in response to stress, activates iNOS in human monocytes. The iNOS induction was dependent on a β-endorphin-triggered increase in cAMP (Aymerich *et al.,* 1998). This might indicate that stress-induced conditions are critical for macrophage activation and that more than one signal is required for NO induction in macrophages, thereby keeping a safety lock on the unnecessary triggering of a potentially harmful event *in vivo.*

NO/$ONOO^-$ Role in MS Pathology

NO Production by Oligodendrocytes

Rodent oligodendrocytes respond *in vitro* to the cytokines IL-1β and IFN-γ by iNOS/NO production; both of these cytokines have been implicated in MS (Murphy *et al.,* 1995; Merrill and Murphy, 1996). The production of NO by oli-

godendrocyte cultures is of interest, given that within the whole primary oligodendrocyte population, which is composed of bipotential precursors, committed oligodendrocytes, and mature myelin basic protein (MBP)-producing oligodendrocytes, there is a subset of NO-sensitive cells (Mitrovic *et al.,* 1995, 1996; Merrill *et al.,* 1993). Primary rat oligodendrocytes produce NO as a consequence of the induction of the iNOS gene. Inhibition of transcription or translation results in the absence of iNOS protein in oligodendrocytes and the lack of detectable NO in the culture supernatants. The protein and mRNA are identical in size to those induced in other human and rodent glial cells (Merrill *et al.,* 1997). The levels of iNOS and mRNA and the function of iNOS enzyme *in vitro* (as determined by L-citrulline production) indicate that microglia are better producers than oligodendrocytes and astrocytes. Alveolar macrophages appear to be better producers than brain microglia under the conditions tested. The fact that oligodendrocytes can produce NO may not be surprising, given that Gold *et al.* (1996) have demonstrated iNOS activation in the myelinating cell in the peripheral nervous system, the Schwann cell. Some NO-producing cells seem to be refractory to NO-mediated toxicity, as in the case of microglia and NOS-positive neurons (Dawson and Dawson, 1994; Mitrovic *et al.,* 1994). Cells such as astrocytes, macrophages, and hepatocytes, while insensitive to self-injury from endogenously produced NO, may be more sensitive to exogenous NO from a neighboring cell. Nevertheless, these cells seem to be able to repair the NO-mediated damage, possibly by converting to glycolysis during the repair phase (Drapier and Hibs, 1988; Stadler *et al.,* 1991; Mitrovic *et al.,* 1994). In the case of oligodendrocytes, it will be important to determine whether the NO-producing oligodendrocytes are also insensitive to their own chronically produced endogenous NO.

Free Radical-Mediated Oligodendrocytes and Myelin Damage

The brain is considered a target for oxidative stress and formation of lipid peroxides for several reasons: (a) large O_2 stores are required for maintenance of electrical activity, (b) phospholipids containing fatty acids with multiple bonds are more ubiquitous than in peripheral tissue, (c) free divalent iron is elevated in the brain, (d) reduced glutathione (GSH) is low in the nervous system, (e) there are elevated levels of enzymes generating active oxygen and nitrogen species in the CNS, and (f) there are cells in the CNS that are exquisitely sensitive to free radical injury such as neurons and oligodendrocytes (Thorburne and Juurlink, 1996; Glozman and Yavin, 1997).

In models of trauma and disease *in vivo* and *in vitro,* oligodendrocytes are easily damaged. Rodent microglia or macrophages will produce free radicals and kill oligodendrocytes when activated by virus or following cross-linking of cytokine receptors, FcR, or cell surface adhesion molecules such as ICAM-1 (intracellular adhesion molecule-1) (Griot *et al.,* 1990; Zajicek *et al.,* 1992; Ulvestad *et al.,* 1994; Merrill and Benveniste, 1996). This killing can be inhibited by antibodies to TNF-α or adhesion molecules, by anti-inflammatory cytokines such as TGF-α, or by free radical scavengers (Griot *et al.,* 1990; Merrill and Benveniste, 1996). Microglial cytotoxicity and NO production appear to be mediated partially and indirectly by TNF-α. That is, TNF-α does not induce NO by itself, nor can soluble TNF-α directly kill oligodendrocytes; TNF-α associated with the cell surface of microglia may play a role in the cytotoxicity (Zajicek *et al.,* 1992; Merrill *et al.,* 1993; Merrill and Benveniste, 1996). When exposed to reactive oxygen species (ROS) or NO or inducers of free radicals *in vitro* or *in vivo,* the oligodendrocyte is more vulnerable to injury than are microglia or astrocytes (Griot *et al.,* 1990; Ulvestad *et al.,* 1994; Juurlink and Husain, 1994; Husain and Juurlink, 1995; Mitrovic *et al.,* 1994, 1995, 1996; Thorburne and Juurlink, 1996). CSF from MS patients induces oligodendrocyte damage *in vitro* via glial cell-produced NO (Xiao *et al.,* 1996). Hypoxic–ischemic conditions cause white matter damage and oligodendrocyte death (Rice *et al.,* 1980; Qi and Dawson, 1993). Oligodendrocytes are exquisitely sensitive to lipid peroxidation caused by free radicals produced under anoxia (Husain and Juurlink, 1995). It has also been shown that electrical conduction in demyelinated and newly remyelinated axons is particularly sensitive to direct inhibtion by NO (Redford *et al.,* 1997). Nitric oxide can impair mitochondrial function in oligodendrocytes and astrocytes (Mitrovic *et al.,* 1994; Bolaños *et al.,* 1994), and it induces a necrotic-like cell death in oligodendrocytes *in vitro* (Merrill *et al.,* 1993; Mitrovic *et al.,* 1995). Nevertheless, not all oligodendrocytes from primary brain cultures are killed by NO. Indeed, we have discovered NO-resistant oligodendrocyte cell lines, suggesting that there are differential sensitivities among maturational subsets of oligodendrocytes to the toxic effects of this molecule (Mackenzie-Graham *et al.,* 1994; Mitrovic *et al.,* 1994; Juurlink *et al.,* 1998). As noted above, a cell that makes NO may be able to protect itself from such toxic effects on intracellular proteins. This seems to be the case for microglial cells (Simmons and Murphy, 1992; Mitrovic *et al.,* 1994) and astrocytes (Bolaños *et al.,* 1994).

Reduced glutathione (GSH), as an electron donor, scavenges hydrogen peroxide (H_2O_2) and organic peroxides in a biochemistry catalyzed by glutathione peroxidase (GPx). If lipid peroxides are not scavenged, Fe^{2+} will drive H_2O_2 to · OH (hydroxyl radical) and convert lipid hydroperoxides to alkoxyl radicals; Fe^{3+} will lead to peroxyl radicals. Juurlink *et al.* (1998) report that oligodendrocytes have 23-fold more iron than astrocytes, while having only half the amount of GSH and less than 15% of the levels of GPx as astrocytes (Thorburne and Juurlink, 1996). Exposure of oligodendrocytes to glutamate induces cystine depletion through the action of a glutamate–cystine exchange, thereby depleting glutathione and causing free radical-mediated cell death (Yonezawa *et al.,* 1996; Oka *et al.,* 1998). In preliminary work from the Merrill laboratory, oligodendrocytes appear to be producing lower levels of manganese superoxide dis-

mutase (SOD) than microglia or astrocytes in the presence of inflammatory stimulators. Requirements for iron during myelination by immature oligodendrocytes may lead to an undesirable mismanagement of iron in oligodendrocytes as the result of iron acquisition (via transferrin receptors) and storage (via ferritin) (Connor and Menzies, 1995, 1996). In other words, stress during situations where myelin turnover or repair is required might in fact lead to risk for the oligodendrocyte. This hypothesis might shed light on the previously mentioned *in vitro* observation that mature oligodendrocytes produce NO (Merrill *et al.,* 1997) and are less sensitive to the effects of NO than more immature oligodendrocytes (Mackenzie-Graham *et al.,* 1994). Free radical damage to cells must then be considered in the context of not only the state of maturation of the cell, but also the concomitant production of endogenous free radicals in the presence of defense mechanisms to protect againt the consequences of this biological activity.

Regulation of Molecules at the Blood–Brain Barrier

The Th1 response, which has been shown to be critical in unmasking latent EAE in resistant mouse strains, has been shown to be driven by IL-12 (Segal and Shevach, 1996). The transcription of the p40 chain of the IL-12 heterodimer is regulated by NO (Rothe *et al.,* 1996). Blocking ROS or NO production with mitochondrial inhibitors or antioxidants abrogates transcription of and subsequent production of these proinflammatory cytokines (Schulze-Osthoff *et al.,* 1993; Ziegler-Heitbrock *et al.,* 1993; St. Pierre *et al.,* 1996), supporting the hypothesis that free radicals can regulate gene transcription and subsequent events at the BBB. Activation and contact between blood-borne inflammatory cells and cerebrovascular endothelial cells (EC) involves ICAM-1 on EC and its integrin receptor on leukocytes, the lymphocyte function-associated antigen-1 (LFA-1). Cross-linking of ICAM-1 activates T cells and macrophages, resulting in the production of proinflammatory molecules IFN-γ, IL1-β, and NO (Merrill *et al.,* 1993; Merrill and Benveniste, 1996; Koyama *et al.,* 1996). When cytokines are induced and NO produced, there is an alteration in the permeability of the BBB, an increase ICAM-1 and LFA-1, and induction of another adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), on EC and glia. VCAM-1 is a ligand for the integrin very late antigen-4 (VLA-4). It is the expression of VLA-4 and its interaction with VCAM-1 that are crucial for the preferential entry of $CD4^+$ T cells into the CNS (Schenk *et al.,* 1996; Baron *et al.,* 1993). Additionally, HLA-DR, a class II major histocompatability complex molecule noted for its role in antigen presentation, is also likely to be critical for T cell adhesion to EC (Goodall *et al.,* 1992; Masuyama *et al.,* 1986; Schenk *et al.,* 1996). IL1-β, TNF-α, IL-6, and NO are all elevated in EAE and MS (Murphy *et al.,* 1995; Merrill and Murphy, 1996). NF-κB influences the inflammatory response and affects those ctokines and chemokines influencing BBB permeability and cell trafficking into the CNS: IL1-β, IL-6, TNF-α, IFN-γ, the colony stimulating factors (CSFs), IL-8, MCP-1, HLA-DR, VCAM-1, and ICAM-1 (Dröge *et al.,* 1994; Raes *et al.,* 1995). NO and reactive oxygen intermediates (ROI) increase TNF-α, IL-6, and IL-1β gene expression *in vitro* and *in vivo* (Magrinat *et al.,* 1992; Eigler *et al.,* 1993).

In mice, IFN-γ or lipopolysaccharide (LPS)-induced MHCII, is inhibited by NO donor agents or stimulants that induce NO (Sicher *et al.,* 1994, 1995). In vascular smooth muscle cells and EC, NO inhibits ICAM-1 and VCAM-1 (De Caterina *et al.,* 1995; Shin *et al.,* 1996). Inhibition of Na^+/K^+-ATPase by ouabain activates NF-κB, leading to the expression of VCAM-1 and iNOS (Bereta *et al.,* 1995). Activator protein 1 (AP-1)-dependent transcriptional activation in some cells can be induced both by pro- and antioxidant conditions. Pyrolidone dithiocarbamate (PDTC) induces ICAM-1 via the stimulation of c-*fos* and c-*jun* binding to the AP-1 consensus site (Meyer *et al.,* 1993; Muñoz *et al.,* 1996), suggesting that ICAM-1 and VCAM-1 are upregulated by prooxidants through NF-κB. This finding might fit with the observation that these adhesion molecules are usually inhibited by scavengers such as NO and PDTC (Marui *et al.,* 1993; De Caterina *et al.,* 1995), though ICAM-1 can be upregulated by antioxidants through the AP-1 site. ROS can also regulate the amplification of cells and their migration across the BBB. Oxygen intermediates induce, while NO inhibits, macrophage colony-stimulating factor (M-CSF) (Satriano *et al.,* 1993; Peng *et al.,* 1995). Because the thiol compound diethyldithiocarbamate (DDTC) increases M-CSFs in other cells, there is the suggestion that NO may be acting as an oxygen radical scavenger, preventing M-CSF gene transcriptional activation (East *et al.,* 1992). Thus, NO influences the nature of the cytokines and the subsequent production or inhibition of molecules on trafficking leukocytes and EC, which will affect cell migration into the CNS parenchyma.

Once cells have crossed the BBB, they are directed to migrate toward gradients created by chemoattractant cytokines. These small chemokines can be cell specific in determining the immigration patterns of lymphocytes and macrophages to certain inflammatory regions, and they are also capable of enhancing activation by integrins through redistribution of adhesion molecules, thereby modulating leukocyte–EC interactions (Del Pozo *et al.,* 1995; Ransohoff *et al.,* 1996). NO inhibits expression of the chemokines IL-8 and MCP-1 in EC (De Caterina *et al.,* 1995; Zeiher *et al.,* 1995). However, NO upregulates IL-8 transcription in leukocytes and melanoma cells (Remick and Villarete, 1996). ROS (H_2O_2 ·OH) and also paraquat upregulate IL-8 in stimulated cells, whereas antioxidants such as DMSO, mannitol, ethanol, and thiourea inhibit this induction (DeForge *et al.,* 1992; Bianchi *et al.,* 1993; Remick and Villarete, 1996; Tanaka *et al.,* 1997). Thus, NO can affect events subsequent to migration of cells across the BBB. Clearly, $NO/ONOO^-$ may influence the activation and migration of T cells across the BBB; by regulating gene expression of MHCII and adhesion molecules, as well as chemoattractants, NO can be either pro- or anti-inflammatory during the generation of CNS lesions. Nevertheless, once NO is induced in the context of

vulnerable cells whose function is altered in the presence of free radicals as in the brain parenchyma, it may contribute to pathology.

CNS Inflammation in EAE

Immunization of animals with one of the myelin proteins (MBP), proteolipid protein (PLP), or myelin-oligodendrocyte protein (MOG) induces EAE, the most widely used model for MS. In this model, helper T (Th) lymphocytes specific for myelin proteins are essential for EAE development (Mokhtarian *et al.,* 1984; Zamvil *et al.,* 1985; Lemire and Weigle, 1986; van der Veen *et al.,* 1990). The pathogenesis of EAE involves the infiltration of activated myelin-specific T cells into the CNS, where they initiate inflammation, setting off a chain of events leading to, among others, the phagocytosis of myelin by macrophages.

Th1 versus Th2 Paradigm

Th cells differentiate into at least two functionally distinct subsets (Cher and Mosmann, 1987). Inflammatory Th1 cells characteristically produce IL-2, IFN-γ, and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (Cher and Mosmann, 1987; Mosmann and Coffman, 1989; Gajewski *et al.,* 1989). Th1 and Th2 cells are reciprocally inhibitory (Fiorentino *et al.,* 1991): the Th2 products IL-4 and IL-10 inhibit Th1 cell differentiation or activation. IL-4 induces Th2 cell differentiation (Mosmann, 1991; Gautam *et al.,* 1992; Powrie *et al.,* 1993), whereas Th1 cell differentiation is induced by IL-12 and IFN-γ (Hsieh *et al.,* 1993). Similar but not identical differences are observed among human Th cells (Maggi *et al.,* 1992; Taga and Tosato, 1992). Myelin-specific Th subsets have also been demonstrated in MS (Voskuhl *et al.,* 1993; Correale *et al.,* 1995).

Traditionally, the CNS and some other tissues are thought to be relatively immune-privileged sites, indicative of the absence of routine immune surveillance. Initially, this was thought to be a passive process solely due to the blood–brain barrier; more recently, however, the active nature of immune privilege has been suggested (Streilen, 1993), and the concept of the brain as an immunoincompetent site is being scrutinized. A direct link between the brain and cerebral lymph nodes has been suggested (Cserr and Knopf, 1992). Intracerebrally introduced antigens are at least as immunogenic as in extracerebral sites (Gordon *et al.,* 1992), although the mechanism by which antigens in the brain are processed and presented to T cells remains unclear.

The concept of cross-regulatory Th subsets could be of particular significance to CNS inflammation. Immune privilege may be synonymous with noninflammatory immunity, or with suppression of inflammation. In the case of the brain, suppression of inflammation is likely to be crucial (Cserr and Knopf, 1992; Harling-Berg *et al.,* 1991), because of the particular vulnerability of the brain for inflammatory processes. Studies suggest that brain cells produce anti-inflammatory cytokines during the first days following a viral infection in the CNS, whereas the appearance of proinflammatory cytokines coincides with the onset of CNS inflammation (Wesselingh *et al.,* 1992). Th1 cell products stimulate TNF-α production in macrophages and microglia, which is considered important in demyelination (Renno *et al.,* 1995) and is downregulated by IL-10 (Fiorentino *et al.,* 1991). Involvement of IL-10 (Kennedy *et al.,* 1992; Rott *et al.,* 1994) or IL-4 (Racke *et al.,* 1994) in EAE remission or inhibition has been suggested. These arguments strongly implicate Th2-type cytokines in the regulation of CNS inflammation by Th1 cells. However, it remains to be proved which anti-inflammatory brain-derived products are important during remissions, and what their source is. A central role for Th2 cells in EAE remissions has become less likely (Khoruts *et al.,* 1995; Lafaille *et al.,* 1997).

Nitric Oxide in EAE

Free radicals, particularly the reactive oxygen intermediates, are thought to be involved in inflammatory processes (Winrow *et al.,* 1993) by exacerbating inflammation and causing tissue damage. The role of free radicals in the induction of inflammation involves the expression of inflammatory mediators, such as cytokines, adhesion molecules, and eicosanoids (Halliwell and Gutteridge, 1995). CNS tissue is particularly vulnerable to oxidative damage (Konat and Wiggins, 1985; Griot *et al.,* 1990; Fisher *et al.,* 1993; Dawson and Dawson, 1994; Bongarzone *et al.,* 1995; Connor and Menzies, 1995, 1996; Thorburne and Juurlink, 1996; Yonezawa *et al.,* 1996; Glozman and Yavin, 1997; Smith *et al.,* 1997), suggesting that any form of oxidation could be an important factor in the pathogenesis of CNS inflammation in MS and EAE. Several reports indirectly implicate free radical involvement in autoimmune CNS inflammation. For instance, antioxidant treatment ameliorates EAE (Ruuls *et al.,* 1995; Hansen *et al.,* 1995).

The free radical species that has been most convincingly linked to MS or EAE activity is nitric oxide (NO), because the inhibition of NOS inhibits disease progression in animal models *in vivo* and oligodendrocyte cell death *in vitro* (Merrill *et al.,* 1993; Mitrovic *et al.,* 1994; Cross *et al.,* 1994; Bagasra *et al.,* 1995; Zielasek *et al.,* 1995; Zhao *et al.,* 1996; Ding *et al.,* 1998). Type II NOS can be induced in endogenous glia in the CNS (microglia and astrocytes) and in inflammatory cells invading from the blood; NO production in EAE and MS tissues can be validated by electron paramagnetic resonance spectroscopy and spin trap technologies as well as footprinting the production of NO via the presence of nitrotyrosine and elevated cGMP levels (Mitrovic *et al.,* 1996). Constitutive isoforms of NOS are present in endothelial cells and neurons (Knowles and Moncada, 1994). During inflammation, the NO concentration in the CNS increases substantially (Hooper *et al.,* 1995; Okyda *et al.,* 1995). Furthermore, in EAE, encephalitogenic T cells stimulate NO production by macrophages (Misko *et al.,* 1995). Independent studies from at least four laboratories, using both the rat

and mouse models of EAE, have demonstrated that NO is involved in EAE development and progression (reviewed in Parkinson *et al.,* 1997). However, two additional studies have demonstrated variable consequences (Zielasek *et al.,* 1995) or aggravated EAE (Ruuls *et al.,* 1996) following inhibition of NOS. Furthermore, iNOS-deficient mice develop more severe EAE than normal mice (Fenyk-Melody *et al.,* 1998; Sahrbacher *et al.,* 1998).

There are several explanations for the seemingly irreconcilable differences in the effects of antagonizing NOS in the various models, which have been dealt with fully elsewhere (Parkinson *et al.,* 1997). In brief, however, the doses used in the acute rat model, where inhibition of NOS failed to protect, were lower than optimal, and the route of administration was different from that used in the mouse models. Additionally, neither of the negative studies demonstrated that the administration of a NOS inhibitor actually inhibited iNOS *in situ* nor that NO production in the affected tissue was blocked. Such data are important in the evaluation of the results. Furthermore, more information on the bioavailability of aminoguanidine and other standard NOS analog inhibitors in the CNS of rats and mice is necessary to help rationalize these conflicting data and instruct us in the future use of such compounds for treating inflammation in the spinal cord and brain.

Although the scientific documentation for a role for NO in cell death or damage is strong, several lines of evidence indicate that NO may in fact be protective under certain circumstances, perhaps best illustrated by the NO-deficient mouse models (see earlier). For instance, the NO molecule is a weak oxidant, but it reacts more rapidly as an antioxidant (Hogg *et al.,* 1993). Many nontoxic functions have been ascribed to NO, namely, smooth muscle relaxant and neurotransmitter. Interestingly, NO may even protect against lipid peroxidation (Jessup *et al.,* 1992; Hogg *et al.,* 1993; van der Veen and Roberts, 2000), and it may be involved in protection against cytokine-induced damage (Matthys *et al.,* 1995) as well as in inhibition of leukocyte adhesion and migration through the endothelial cell layer (Kubes *et al.,* 1994). In addition, NO has been implicated as a major macrophage-derived immunosuppressive factor for T-cell immunity (Mills, 1991; Tomioka and Saito, 1991) and as an inhibitor of NF-κB activation in the CNS (Togashi *et al.,* 1997). Clearly, depending on the chronicity of production, concentrations reached, and the temporal and spatial production of NO, this molecule may function as either a pro- or anti-inflammatory mediator.

Some reports have raised the possibility that NO is involved in the differential regulation of Th subsets. NO reportedly inhibits Th1 but not Th2 cell stimulation *in vitro* (Taylor-Robinson *et al.,* 1994). Th1 cells (but not Th2 cells) produce NO as well, suggesting an autoregulatory pathway in Th1 cells. Furthermore, iNOS-deficient mice, besides clearing pathogens less well, develop stronger Th1-type immune responses (Wei *et al.,* 1995), suggesting that NO has a potential role in regulating the balance between Th subsets *in vivo* as well. However, this concept has been challenged (Nukaya *et al.,* 1995). This laboratory has demonstrated that NO derived from an NO donor inhibits both Th1 and Th2 clones to a similar degree (R. C. van der Veen *et al.,* unpublished observation). This indicates that NO inhibits the activation of differentiated T cells from both Th subsets, although it does not exclude the possibility for a role of NO in the differentiation of uncommitted Th cells during the early stages of an immune response. Interestingly, the inhibition by NO was very specific for T-cell proliferation. Other functions, most notably the production of cytokines, were not critically affected. Similar results were obtained with NO produced by macrophages.

PEROXYNITRITE IN EAE

NO can be transformed by the free radical superoxide (O_2^-) to form the powerful oxidant peroxynitrite ($ONOO^-$). Peroxynitrite induces lipid peroxidation (Beckman, 1994) and may thus be involved in myelin degradation. NO-induced neuronal damage is mediated by peroxynitrite (Ohkuma *et al.,* 1995; Bolaños *et al.,* 1995). These findings suggest that peroxynitrite mediates damage in the CNS. Peroxynitrite-like activity has been demonstrated in the inflamed CNS (Hooper *et al.,* 1997; van der Veen *et al.,* 1997; Cross *et al.,* 1998). Indeed, Hooper *et al.,* (1997) reported the inhibition of EAE with the presumed peroxynitrite scavenger uric acid. Interestingly, NO levels in the CNS of these protected mice were still elevated, suggesting that NO may not be the major detrimental factor in this disease.

Peroxynitrite may represent at least part of the NO-associated toxicity, especially for its strong induction of lipid peroxidation. Lipid peroxidation causes arachidonic acid to be transformed into prostaglandin-like, but cyclooxygenase-independent F2-isoprostanes (Morrow *et al.,* 1992; Takahashi *et al.,* 1992). Likewise, docosahexaenoic acid (C22:6) is transformed into neuroprostanes, novel products that are formed in the CNS and testes only. Besides serving as markers of lipid peroxidation, isoprostanes may exert strong biological effects (Hooper *et al.,* 1997). We demonstrated the formation of F2-isoprostanes and neuroprostanes in peroxynitrite-treated myelin *in vitro,* as well as in EAE. Interestingly, NO inhibited peroxynitrite-induced lipid peroxidation in myelin (van der Veen and Roberts, 1999).

Lipid peroxidation by peroxynitrite results in the phagocytosis of the oxidized low density lipoprotein (LDL) by macrophages, which has been studied extensively in relation to atherosclerosis. Phagocytosis of oxidized LDL was demonstrated to involve specific receptors on macrophages separate from the scavenger receptors (Ottnad *et al.,* 1995). Receptors for oxidized LDL may recognize oxidized lipids or lipid–protein complexes in general, since they are also involved in the uptake of oxidized erythrocytes (Ottnad *et al.,* 1995). Phagocytosis of oxidized LDL has been shown to occur in MS plaques (Newcombe *et al.,* 1994), indicating that a similar process may occur with oxidized myelin as the target. Although peroxynitrite may be important in EAE, it may not be essential for this condition, since iNOS-deficient mice develop severe EAE (Fenyk-Melody *et al.,* 1998;

Sahrbacher *et al.,* 1998). The formation of peroxynitrite in these mice is unknown, but in the absence of strong NO fluxes from iNOS, peroxynitrite supposedly will not be formed in high quantities, even locally. In our experience, peroxynitrite-like activity, as indicated by nitrotyrosine staining, was restricted to phagocytes during EAE (van der Veen *et al.,* 1997), indicating that constitutive forms of NOS may not produce enough NO for peroxynitrite formation. However, the formation of low but undetectable levels of peroxynitrite cannot be ruled out.

Superoxide Anion Regulation of NO Activity in EAE

Most recently, our laboratory has studied the T-cell inhibitory role of NO (see earlier) in relation to EAE, based on the strong expression of iNOS in cells in the CNS during EAE, of which only a subfraction coexpressed peroxynitrite-like activity (van der Veen *et al.,* 1997). Since O_2^- has a strong affinity for NO, the possibility was studied that superoxide regulates the effect of NO on T cells. Peritoneal macrophages completely inhibited the proliferation of prestimulated, myelin-specific T cells, which was reversed in the presence of high levels of a NOS inhibitor (L-NMA). In the presence of low levels of L-NMA, which induced only a slight increase in T-cell proliferation, a substantial increase was induced when phorbol ester (PMA) was added simultaneously (Fig. 1A). The further addition of SOD reversed the effect of PMA, indicating that O_2^- production is responsible for the inactivation of NO. Neither PMA itself nor O_2^- or H_2O_2 had a substantial direct effect on T-cell proliferation. The presence of low levels of L-NMA was important to observe this effect, because in its absence, PMA only slightly inactivated NO. Apparently in this situation the NO production overwhelms or simply inhibits the O_2^- production (Ruuls *et al.,* 1995; Hansen *et al.,* 1995).

Role of NADPH Oxidase in the Regulation of NO Activity

Since extracellular SOD inhibited the effect of O_2^- on NO activity, and since O_2^- cannot penetrate the cell membrane, the O_2^- appears to be produced extracellularly. An important source for extramembraneous O_2^- production is NADPH oxidase. Therefore, the T-cell regulation by macrophages from NADPH oxidase-deficient [$p47^{phox}$(KO) knockout] mice became a key model in which to address this issue.

The degree to which macrophages derived from $p47^{phox}$ KO mice were able to inhibit the effect of NO on T cells can be examined by determining if NADPH oxidase forms a major source of NO-inactivating O_2^-. Peritoneal exudate cells (PEC) from normal wild-type (WT) and $p47^{phox}$ KO mice inhibited T-cell proliferation equally well, which was reversed with high levels of L-NMA, as expected. In the presence of low levels of L-NMA, stimulation with PMA reversed the inhibitory effect of NO in WT mice (Fig. 1B). However, in KO mice the addition of PMA had no effect, demonstrating that the lack of functional NADPH oxidase results in the preservation of NO activity. This suggests that

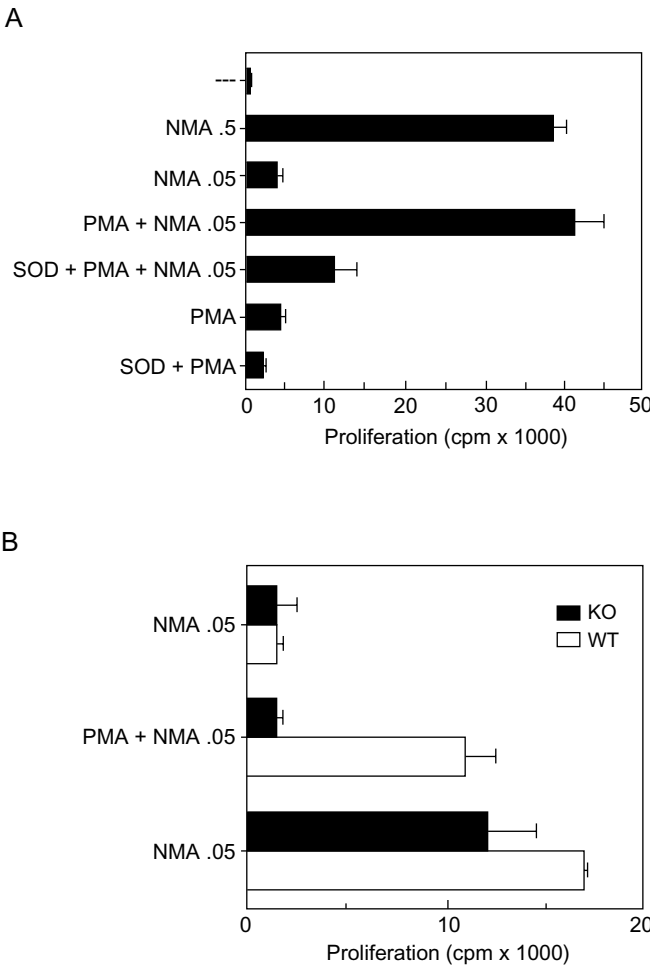


Figure 1 (A) Superoxide reverses the inhibitory effect of NO. Normal PEC were stimulated with IFN-γ and LPS, while cloned Th cells were stimulated with anti-CD3 monoclonal antibody separately. After 16 hours, the stimulated T cells were transferred to the PEC cultures in the presence of L-NMA (NMA), PMA, or SOD as indicated, and simultaneously pulsed with [^{3}H] TdR. (B) Cells from NADPH oxidase-deficient mice do not inhibit NO activity. Similar cocultures were set up with PEC from normal wild-type (WT) or $p47^{phox}$ knockout (KO) mice.

O_2^- production by NADPH oxidase inhibits the activity of NO in normal PEC.

Apparently, other oxidases besides NADPH oxidase do not contribute substantially to the NO-neutralizing O_2^- production, at least not in this system, which is centered around the induction of oxidases by PMA. Possibly, the localization (and timing) of O_2^- production as well as its quantity are crucial. A schematic representation of the possible sequence of events leading to either NO or $ONOO^-$ production is shown in Fig. 2. Intracellularly produced NO diffuses freely through the cell membrane, and it inhibits T-cell proliferation if NO remains unaffected by O_2^- (owing to the presence of SOD or a mutation in NADPH oxidase). However, when NADPH oxidase is activated and assembled in the cell membrane simultaneously, it produces O_2^- extracellularly, which binds NO, while it is just on its way out of the cell.

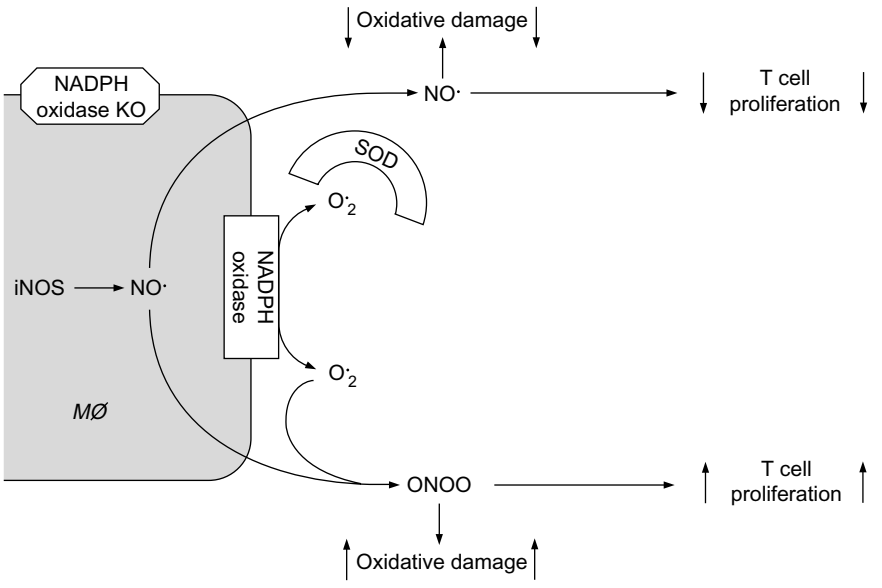


Figure 2 Schematic representation of the regulation of NO activity by O_2^-. See text for explanation.

This leads to the formation of $ONOO^-$ outside of the cell, simultaneous with the inactivation of NO and subsequently the proliferation of T cells, among others.

There may be many biological consequences of the processes described here. If NO is left intact, it can regulate immune activity in inflamed tissue, or it can serve as an antioxidant during oxidative processes such as lipid peroxidation. If, on the other hand, peroxynitrite is formed, it induces oxidative damage such as lipid peroxidation. This may damage the tissue locally and result in enhanced myelin degradation. At the same time, the immune-regulatory activity of NO is lost, resulting in the enhancement of inflammatory processes, further aggravating tissue damage. Thus, NO presents a double-edged sword: either it is antiinflammatory (as NO) or proinflammatory (as $ONOO^-$), which is regulated by O_2^- produced by NADPH oxidase. This dualism may explain the controversy over the role of NO in CNS inflammation.

ROLE OF NADPH OXIDASE IN EAE

Based on the data presented here, our prediction was that NADPH oxidase-deficient mice develop EAE to a lesser degree than normal mice. Indeed, the disease expression in these KO mice was not just less severe than in normal mice, it was virtually absent, both clinically and histologically. This indicates that NADPH oxidase forms an essential component in the development of EAE. Besides a direct role for reactive oxygen species in EAE development, this result could also indicate that NO suppresses EAE in the absence of O_2^- from NADPH oxidase, whereas in its presence NO is inhibited enough for EAE to develop. The generation of immunity was apparently normal in the KO mice, since lymph node cells from immunized KO mice proliferated just as well in response to the immunogen *in vitro* as lymph node cells from wild-type mice. On the other hand, spleen cells from NADPH oxidase KO mice showed only a weak or, more frequently, no proliferative response to the immunogen at all. In contrast, spleen cells from wild-type mice responded well. Despite the absence of proliferation in the KO mice, their spleen cells responded to the encephalitogen with cytokine production to a similar degree as wild-type mice. Since only the proliferation of spleen cells, not the cytokine production, was inhibited in the KO mice, the resemblance with the effect of NO on T-cell stimulation (see earlier) became apparent.

Therefore, the proliferation of spleen cells was tested in the presence of L-NMA, which indeed uncovered a good proliferative response to the immunogen in KO spleen, whereas only slightly increasing proliferation in most normal spleen cells (Fig. 3). This indicates that in the absence of

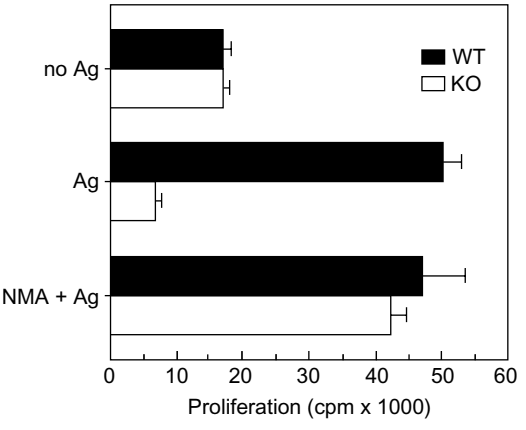


Figure 3 NADPH oxidase-deficient spleen cells display stronger NO activity. Spleen cells from KO and WT mice, previously immunized with myelin oligodendrocyte glycoprotein 35-55 in complete Freund's adjuvant, were incubated with MOG 35-55, some in the presence of L-NMA (1 m*M*).

NADPH oxidase, a spleen cell subpopulation produces enough NO during an immune response *in vitro* to inhibit the proliferation of T cells completely, although NO is less active in spleens from normal mice. These NO-producing spleen cells may be the same as the more recently described splenic Sca1/Mac1$^+$ macrophages, which suppress T-cell responses with their NO production (Johnson *et al.,* 1998). Apparently, O_2^- production by NADPH oxidase inactivates NO in the spleen environment in normal mice.

In conclusion, O_2^- from NADPH oxidase was shown to regulate the inhibitory activity of NO toward T-cell proliferation. This was shown both in isolated adherent PEC as well as in total spleen cell cultures. In addition, NADPH oxidase-deficient mice are resistant to EAE induction, indicating that NO may be highly active in these mice. In other words, the balance between O_2^- and NO may regulate the extent of an immune response. This may also hold true in target tissues, such as the CNS, where NO could downregulate inflammatory responses if left untouched by O_2^-. If indeed a central role for NO in immune responses *in vivo* can be confirmed, this would be of extreme significance to many disorders, both inflammatory or infectious, in which the activity of NO (not simply its production) could be either too low or too high, respectively.

Conclusions

Overall, it seems fair to think that the evolution of the disease pathology in both EAE and MS involves NO. Macrophages and microglia appear to be the major producers of NO and $ONOO^-$. Early in the disease induction, NO may be anti-inflammatory in that NO reduces T-cell clonal expansion and inhibits induction of adhesion molecules and MHC class II molecules, thereby inhibiting trafficking of and antigen presentation by blood-borne cells into the CNS. Nevertheless, as a consequence of such cellular movement into the CNS and recruitment of endogenous glia, the continued production of NO in the parenchyma of the brain certainly affects myelin and the myelin-producing cell the oligodendrocyte in a negative way. Future challenges to a fuller understanding of the mechanisms of action of NO in the chronic progression of disease include the development of more precise techniques for measurement of endogenous NO levels in CSF and brain and spinal cord tissue. Our ability to detect NO metabolites and track the ''footprints'' of peroxynitrite formation will be of value in designing clinical protocols and assessing outcomes of therapeutic agents that inhibit iNOS and NO.

References

Albina, J. E. (1995). On the expression of nitric oxide synthase by human macrophages. Why no NO? *J. Leukocyte Biol.* **58,** 643–649.

Aymerich, M. S., Bengoechea-Alonso, M. T., Lopez-Zabalza, M. J., Santiago, E., and Lopez-Moratalla, N. (1998). Inducible nitric oxide synthase (iNOS) expression in human monocytes triggered by endorphin through an increase in cAMP. *Biochem. Biophys. Res. Commun.* **245,** 717–721.

Baek, K. J., Thiel, B. A., Lucas, S., and Stuehr, D. J. (1993). Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J. Biol. Chem.* **268,** 21120–21129.

Bagasra, O., Michaels, F. H., Zheng, Y. M., Bobroski, L. E., Spitsin, S. V., Fu, Z. F., Tawadros, R., and Koprowski, H. (1995). Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 12041–12045.

Bagasra, O., Bobroski, L., Sarker, A., Bagasra, A., Saikumari, P., and Pomerantz, R. J. (1997). Absence of the inducible form of nitric oxide synthase in the brains of patients with the acquired immunodeficiency syndrome. *J. Neurovirol.* **3,** 153–167.

Baron, J. L., Madri, J. A., Ruddle, N. H., Hashim, G., and Janeway, C. A. (1993). Surface expression of β4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* **177,** 57–68.

Beckman, J. S. (1994). Peroxynitrite versus hydroxyl radical: The role of nitric oxide in superoxide-dependent cerebral injury. *Ann. N.Y. Acad. Sci.* **738,** 69.

Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol. Chem. Hoppe-Seyler* **375,** 81–88.

Bereta, J., Cohen, M. C., and Bereta, M. (1995). Stimulatory effects of oubain on VCAM-1 and iNOS expression in murine endothelial cells: Involvement of NF-κB. *FEBS Letts.* **377,** 21–25.

Bianchi, M., Fantuzzi, G., Bertini, R., Perin, L., Salmona, M., and Ghezzi, P. (1993). The pneumotoxicant paraquat induces IL-8 mRNA in human mononuclear cells and pulmonary epithelial cells. *Cytokine* **5,** 525–530.

Blanco, F. J., Geng, Y., and Lotz, M. (1995). Differentiation-dependent effects of IL-1β and TGFβ on human articular chondrocyte proliferation are related to inducible nitric oxide synthase expression. *J. Immunol.* **154,** 4018–4026.

Bö, L., Dawson, T. M., Wesselingh, S., Mork, S., Choi, S., Kong, P. A., Hanley, D., and Trapp, B. D. (1995). Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. *Ann. Neurol.* **36,** 778–786.

Bolaños, J. P., Peuchen, S., Heales, S. J. R., Land, J. M., and Clark, J. B. (1994). Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J. Neurochem.* **63,** 910–916.

Bolaños, J. P., Heales, S. J., Land, J. M., and Clark, J. B. (1995). Effect of peroxynitrite on the mitochondrial respiratory chain: Differential susceptibility of neurones and astrocytes in primary culture. *J. Neurochem.* **64,** 1965–1972.

Bongarzone, E. R., Pasquini, J. M., and Soto, E. F. (1995). Oxidative damage to proteins and lipids of CNS myelin produced by in vitro generated reactive oxygen species. *J. Neurosci. Res.* **41,** 213–221.

Boullerne, A. I., Petry, K. G., Meynard, M., and Geffard, M. (1995). Indirect evidence for nitric oxide involvement in multiple sclerosis by characterization of circulating antibodies directed against conjugated *S*-nitrocysteine. *J. Neuroimmunol.* **60,** 117–124.

Brosnan, C. F., Battistini, L., Raine, C. S., Dickson, D. W., Casadevall, A., and Lee, S. C. (1994). Reactive nitrogen intermediates in human neuropathology: An overview. *Dev. Neurosci.* **16,** 152–161.

Bukrinsky, M. I., Nottet, H. S. L. M., Schmidtmayerova, H., Dubrovsky, L., Flanagan, C. R., Mullins, M. E., Lipton, S. A., and Gendelman, H. E. (1995). Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HIV-1)-infected monocytes: Implications for HIV-associated neurological disease. *J. Exp. Med.* **181,** 735–745.

Chakravarthy, U., Stitt, A. W., McNally, J., Bailie, J. R., Hoey, E. M., and Duprex, P. (1995). Nitric oxide synthase activity and expression in retinal capillary endothelial cells and pericytes. *Curr. Eye Res.* **14,** 285–294.

Cher, D. J., and Mosmann, T. R. (1987). Two types of murine helper T cell clone II. Delayed-type hypersensitivity is mediated by Th1 clones. *J. Immunol.* **138,** 3688–3694.

Connor, J. R., and Menzies, S. L. (1995). Cellular management of iron in the brain. *J. Neurol. Sci.* **134,** 33–44.

Connor, J. R., and Menzies, S. L. (1996). Relationship of iron to oligodendrocytes and myelination. *GLIA* **17,** 83–93.

Cooper, R. L. T. (1997). Multiple sclerosis: An immune legacy? *Med. Hypo.* **49,** 307–311.

Correale, J., Gilmore, W., McMillan, M., Li, S., McCarthy, K., Le, T., and Weiner, L. P. (1995). Patterns of cytokine secretion by autoreactive proteolipid protein-specific T cell clones during the course of multiple sclerosis. *J. Immunol.* **154,** 2959–2968.

Cross, A. H., Misko, T. P., Lin, R. F., Hickey, W. F., Trotter, J. L., and Tilton, R. G. (1994). Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J. Clin. Invest.* **93,** 2684.

Cross, A. H., Manning, P. T., Keeling, R. M., Schmidt, R. E., and Misko, T. P. (1998). Peroxynitrite formation within the central nervous system in active multiple sclerosis. *J. Neuroimmunol.* **88,** 45.

Cserr, H. F., and Knopf, P. M. (1992). Cervical lymphatics, the blood–brain barrier and the immunoreactivity of the brain: A new view. *Immunol. Today* **13,** 507–512.

Dawson, T. M., and Dawson, V. L. (1994). Nitric oxide: Actions and pathological roles. *Neuroscientist* **1,** 9–20.

De Caterina, R., Libby, P., Peng, H.-B., Thannickal, V. J., Rajavshisth, T. B., Gimbrone, M. A., Jr., Shin, W. S., and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. *J. Clin. Invest.* **96,** 60–68.

DeForge, L. E., Fantone, J. C., Kenney, J., and Remick, D. G. (1992). Oxygen radical scavengers selectively inhibit interleukin 8 production in human whole blood. *J. Clin. Invest.* **90,** 2123–2129.

DeGroot, C. J. A., Ruuls, S. R., Theeuwes, J. W. M., Dijkstra, C. D., and Van der Walk, P. (1997). Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* **56,** 10–20.

Del Pozo, M., Sanchez-Mateos, P., Nieto, M., and Sanchez-Madrid, F. (1995). Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix: Involvement of cAMP signalling pathway. *J. Cell. Biol.* **131,** 495–506.

De Maria, R., Cifone, M. G., Trotta, R., Rippo, M. R., Festuccia, C., Santoni, A., and Testi, R. (1994). Triggering of human monocyte activation through CD69, a member of the natural killer cell gene complex family of signal transducing receptors. *J. Exp. Med.* **180,** 1999–2004.

Ding, M., and Merrill, J. E. (1997). The kinetics and regulation of the induction of Type I nitric oxide synthase and nitric oxide in human fetal glial cell cultures. *Mol. Psychiatry* **2,** 117–119.

Ding, M., St. Pierre, B. A., Parkinson, J. F., Medberry, P., Wong, J. L., Rogers, N. E., Ignarro, I. J., and Merrill, J. E. (1997). Inducible nitric oxide synthase and nitric oxide production in human fetal astrocytes and microglia: A kinetic analysis. *J. Biol. Chem.* **272,** 11327–11335.

Ding, M., Zhang, M., Wong, J. L., Rogers, N. E., Ignarro, L. J., and Voskuhl, R. R. (1998). Cutting edge: Antisense knockdown of inducible nitric oxide synthase inhibits induction of experimental autoimmune encephalomyelitis. *J. Immunol.* **160,** 2560.

Drapier, J. C., and Hibbs, J. B. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine dependent inhibition of mitochondrial iron–sulfur enzymes in the macrophage effector cells. *J. Immunol.* **140,** 2829–2838.

Dröge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, H.-P., Roth, S., and Gmünder, H. (1994). Functions of glutathione and glutathione disulfide in immunology and immunopathology. *FASEB J.* **8,** 1131–1138.

East, C. J., Abboud, C. N., and Borch, R. F. (1992). Diethyldithiocarbamate induction of cytokine release in human long-term bone marrow cultures. *Blood* **80,** 1172–1177.

Eigler, A., Sinha, B., and Endres, S. (1993). Nitric oxide-releasing agents enhance cytokine-induced tumor necrosis factor synthesis in human mononuclear cells. *Biochem. Biophys. Res. Commun.* **196,** 494–501.

Evans, T., Carpenter, A., and Cohen, J. (1994). Inducible nitric-oxide-synthase mRNA is transiently expressed and destroyed by a cyclohexamide-sensitive process. *Eur. J. Biochem.* **219,** 563–569.

Fenyk-Melody, J. E., Garrison, A. E., Brunnert, S. R., Weidner, J. R., Shen, F., Shelton, B. A., and Mudgett, J. S. (1998). Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J. Immunol.* **160,** 2940.

Fiorentino, D. F., Mosmann, T. R., Howard, M., and O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147,** 3815–3822.

Fisher, M., Plante, G. M., and Doyle, E. M. (1993). Inhibition of inflammatory cell-mediated oxidation and interleukin-1β generation by a 21-aminosteroid, U74500A. *J. Neurol. Sci.* **119,** 189.

Gajewski, T. F., Schell, S. R., Nau, G., and Fitch, F. W. (1989). Regulation of T-cell activation: Differences among T-cell subsets. *Immunol. Rev.* **111,** 80–110.

Gautam, S. C., Chikkala, N. F., and Hamilton, T. A. (1992). Anti-inflammatory action of IL-4. Negative regulation of contact sensitivity to trinitrochlorobenzene. *J. Immunol.* **148,** 1411–1415.

Giovanni, G. (1998). Cerebrospinal fluid and serum nitric oxide metabolites in patients with multiple sclerosis. *Multiple Sclerosis* **4,** 27–30.

Giovanni, G., Heales, S. J. R., Silver, N. C., O'Riordan, J., Miller, R. F., Land, J. M., Clark, J. B., and Thompson, E. J. (1997). Raised serum nitrate and nitrite levels in patients with multiple sclerosis. *J. Neurol. Sci.* **145,** 77–81.

Giovanni, G., Miler, R. F., Heales, S. J. R., Land, J. M., Harrison, M. J. G., and Thompson, E. J. (1998). Elevated cerebrospinal fluid and serum nitrate and nitrite levels in patients with central nervous system complications of HIV-1 infection: A correlation with blood–brain-barrier dysfunction. *J. Neurol. Sci.* **156,** 53–58.

Glabinski, A., Tawsek, N. S., and Bartosz, G. (1993). Increased generation of superoxide radicals in the blood of MS patients. *Acta Neuropathol.* **36,** 778–786.

Glozman, S., and Yavin, E. (1997). Lipid peroxides are generated by the fetal rat brain after episodes of global ischemia in utero. *Neurochem. Res.* **22,** 201–208.

Gold, R., Zielasek, J., Kiefer, B., Toyka, K. V., and Hartung, H.-P. (1996). Secretion of nitrite by schwann cells and its effect on T-cell activation in vitro. *Cell. Immunol.* **168,** 69–77.

Gonzalez-Hernandez, T., Perez de la Cruz, M. A., and Mantolan-Sarmiento, B. (1996). Histochemical and immunohistochemical detection of neurons that produce nitric oxide: Effect of different fixative parameters and immunoreactivity against non-neuronal NOS antisera. *J. Histochem. Cytochem.* **44,** 1399–1413.

Good, P. F., Werner, P., Hsu, A., Olanow, C. W., and Perl, D. P. (1996). Evidence for neuronal oxidative damage in Alzheimer's disease. *Am. J. Pathol.* **149,** 21–28.

Goodall, C. A., Curtis, A. S. G., and Lang, S. C. (1992). Modulation of adhesion of lymphocytes to murine brain endothelial cells in vitro: Relation to Class II major histocompatability complex expression. *J. Neuroimmunol.* **37,** 9–22.

Gordon, L. B., Knopf, P. M., and Cserr, H. F. (1992). Ovalbumin is more immunogenic when introduced into brain or cerebrospinal fluid than into extracerebral sites. *J. Neuroimmunol.* **40,** 81–88.

Grabowski, P. S., Macpherson, H., and Ralston, S. H. (1996). Nitric oxide production in cells derived from the human joint. *Br. J. Rheumatol.* **35,** 207–222.

Griot, C., Vandevelde, M., Richard, A., Peterhans, E., and Stocker, R. (1990). Selective degeneration of oligodendrocytes mediated by reactive oxygen species. *Free Radical Res. Commun.* **11,** 181–193.

Guthikonda, P., Baker, J., and Mattson, D. H. (1998). Interferon-beta-1-b (IFN-β) decreases induced nitric oxide (NO) production by a human astrocytoma cell line. *J. Neuroimmunol.* **82,** 133–139.

Gutowski, N. J., Pinkham, J. M., Akanmu, D., Chirico, S., and Murphy, R. P. (1998). Free radicals in inflammatory neurological disease: Increased

lipid peroxidation and haptoglobin levels in Guillain Barre syndrome. *Irish J. Med. Sci.* **167,** 43–46.

Halliwell, B., and Gutteridge, J. M. C. (1995). ''Free Radicals in Biology and Medicine.'' Oxford Univ. Press (Clarendon), Oxford.

Hansen, L. A., Willenborg, D. O., and Cowden, W. B. (1995). Suppression of hyperacute and passively transferred experimental autoimmune encephalomyelitis by the antioxidant, butylated hydroxyanisole. *J. Neuroimmunol.* **62,** 69.

Harling-Berg, C. J., Knopf, P. M., and Cserr, H. F. (1991). Myelin basic protein infused into cerebrospinal fluid suppresses experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **35,** 45–51.

Hogg, N., Kalyanaraman, J. J., Struck, A., and Parthasarathy, S. (1993). Inhibition of low-density lipoprotein oxidation by nitric oxide. *Eur. Biochem. Soc.* **334,** 170.

Hogg, N., Kalyanaraman, B., and Darley-Usmar, V. (1995). Oxidant and antioxidant effects of nitric oxide and superoxide in the vasculature. *In* ''The Oxygen Paradox'' (K. J. A. Davies and F. Ursini, eds.), pp. 317–324. Cleup Univ. Press, Padora, Italy.

Hooper, D. C., Ohnishi, S. T., Kean, R., Numagami, Y., Dietzschold, B., and Koprowski, H. (1995). Local nitric oxide production in viral and autoimmune diseases of the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 5312–5315.

Hooper, D. C., Bagasra, O., Marini, J. C., Zborek, A., Ohnishi, S. T., Kean, R., Champion, J. M., Sarker, A. B., Bobroski, L., Farber, J. L., Akaike, T., Maeda, H., and Koprowski, H. (1997). Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: Implications for the treatment of multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2528–2533.

Hsieh, C.-S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., and Murphy, K. M. (1993). Development of Th1 CD4+ T cells through IL-12 produced by listeria-induced macrophages. *Science* **260,** 547–549.

Hua, L. L., Liu, J. S. H., Brosnan, C. F., and Lee, S. C. (1998). Selective inhibition of human glial inducible nitric oxide synthase by interferon-γ: Implications for multiple sclerosis. *Ann. Neurol.* **43,** 384–387.

Hunter, M. I. S., Nlemadim, B. C., and Davidson, D. L. W. (1985). Lipid peroxidation products and antioxidant proteins in plasma and cerebrospinal fluid from multiple sclerosis patients. *Neurochem. Res.* **10,** 1645–1652.

Husain, J., and Juurlink, B. H. J. (1995). Oligodendroglial precursor cell susceptibility to hypoxia is related to poor ability to cope with reactive oxygen species. *Brain Res.* **698,** 86–94.

Ikeda, M., Sato, I., Matsunga, T., Takahashi, M., Yuasa, T., and Murota, S.-I. (1995). Cyclic guanosine monophosphate (cGMP), nitrite and nitrate in the cerebrospinal fluid in meningitis, multiple sclerosis and Guillain Barre syndrome. *Int. Med.* **34,** 734–737.

Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T., and Stocker, R. (1992). The participation of nitric oxide in cell-free adhesion and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochim. Biophys. Acta* **1180,** 73–82.

Johnson, A. W., Land, J. M., Thompson, E. J., Bolanos, J. P., Clark, J. B., and Heales, S. J. (1995). Evidence for increased nitric oxide production in multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **58,** 107–116.

Johnson, B. D., Hanke, C. A., Becker, E. F., and Truitt, R. L. (1998). Sca1+/Mac1+ nitric oxide-producing cells in the spleens of recipients early following bone marrow transplant suppress T cell responses in vitro. *Cell. Immunol.* **189,** 149.

Juurlink, B. H. J., and Husain, J. (1994). Hyperthermic injury of oligodendrocyte precursor cells: Implications for dysmyelination disorders. *Brain Res.* **641,** 353–356.

Juurlink, B. H. J., Thorburne, S. K., and Hertz, L. (1998). Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress. *GLIA* **22,** 371–378.

Kennedy, M. K., Torrance, D. S., Picha, K. S., and Mohler, K. M. (1992). Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol.* **149,** 2496–2505.

Khoruts, A., Miller, S. D., and Jenkins, M. K. (1995). Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J. Immunol.* **155,** 5011–5017.

Knowles, R. G., and Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.* **298,** 249–258.

Kobzik, L., Bredt, D. S., Lowenstein, C. J., Drazen, J., Gaston, B., Sugarbaker, D., and Stamler, J. S. (1993). Nitric oxide synthase in human and rat lung: Immunocytochemical and histochemical localization. *Am. J. Respir. Cell Mol. Biol.* **9,** 371–377.

Komori, T., Shibata, N., Kobayashi, M., Sasaki, S., and Iwata, M. (1998). Inducible nitric oxide synthase (iNOS)-like immunoreactivity in argyrophilic, tau-positive astrocytes in progressive supranuclear palsy. *Acta Neuropathol.* **95,** 338–344.

Konat, G. W., and Wiggins, R. C. (1985). Effect of reactive species on myelin membrane proteins. *J. Neurochem.* **45,** 11–13.

Koyama, Y., Tanaka, Y., Saito, K., Abe, M., Nakatsuka, K., Morimotto, I., Aurion, P. E., and Eto, S. (1996). Cross-linking of intercellular adhesion molecule-1 (CD54) induces AP-1 activation and IL-1β transcription. *J. Immunol.* **157,** 5097–5103.

Kubes, P., Kurose, I., and Granger, D. N. (1994). NO donors prevent integrin-induced leukocyte adhesion but not P-selectin-dependent rolling in postischemic venules. *Am. J. Physiol.* **267,** H931–937.

Lafaille, J. J., Keere, F. V., Hsu, A. L., Baron, J. L., Haas, W., Raine, C. S., and Tonegawa, S. (1997). Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med.* **186,** 307–312.

Lee, S. C., Dickson, D. W., Liu, W., and Brosnan, C. F. (1993). Induction of nitric oxide synthase activity in human astrocytes by interleukin-1β and interferon-γ. *J. Neuroimmunol.* **46,** 19–24.

Lemire, J. M., and Weigle, W. O. (1986). Myelin basic protein-specific T cell clones and experimental allergic encephalomyelitis. *Pathol. Immunopathol. Res.* **5,** 248–252.

Levine, S. M., and Warren, D. L. (1998). Chemical analysis of multiple sclerosis lesions by FT-IR microspectroscopy. *Free Radicals Biol. Med.* **25,** 33–41.

López-Moratalla, N., González, A., Soledad Aymerich, M., López-Zabalza, M. J., Pio, R., de Castro, P., and Santiago, E. (1997). Monocyte inducible nitric oxide synthase in multiple sclerosis: Regulatory role of nitric oxide. *Nitric Oxide: Biol. Chem.* **1,** 95–104.

Mackenzie-Graham, A. J., Mitrovic, B., Smoll, A., and Merrill, J. E. (1994). Differential sensitivity to nitric oxide in immortalized, cloned murine oligodendrocyte cell lines. *Dev. Neurosci.* **16,** 162–171.

Maggi, E., Parronchi, P., Manetti, R., and Romagnani, S. (1992). Reciprocal regulatory effect of IFNγ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* **148,** 2142–2147.

Magrinat, G., Mason, S. N., Shami, P. J., and Weinberg, J. B. (1992). Nitric oxide modulation of human leukemia cell differentiation and gene expression. *Blood* **80,** 1880–1884.

Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W., and Medford, R. M. (1993). Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* **92,** 1866–1874.

Masuyama, J.-I., Minato, N., and Kano, S. (1986). Mechanisms of lymphocyte adhesion to human vascular endothelial cells in culture. *J. Clin. Invest.* **77,** 1596–1605.

Matthys, P., Froyen, G., Verdot, L., Huang, S., Sobis, H., Van Damme, J., Vray, B., Aguet, M., and Billiau, A. (1995). IFN-γ receptor-deficient mice are hypersensitive to the anti-CD3-induced cytokine release syndrome and thymocyte apoptosis. Protective role of endogenous nitric oxide. *J. Immunol.* **155,** 3823.

Merrill, J. E., and Benveniste, E. N. (1996). Cytokines in inflammatory brain lesions: Helpful and harmful. *Trends Neurosci.* **19,** 331–338.

Merrill, J. E., and Murphy, S. (1996). Nitric oxide. *In* ''The Role of Glia in Neurotoxicity'' (M. Aschner and H. K. Kimelberg, eds.), pp. 263–281. CRC Press, New York.

Merrill, J. E., Ignarro, L. J., Sherman, M. P., Melinek, J., and Lane, T. E. (1993). Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J. Immunol.* **151,** 2132–2141.

Merrill, J. E., Murphy, S. P., Mitrovic, B., Mackenzie-Graham, A., Dopp, J. C., Ding, M., Griscavage, J., Ignarro, L. J., and Lowenstein, C. J.

(1997). Inducible nitric oxide synthase and nitric oxide production by oligodendrocytes. *J. Neurosci. Res.* **48,** 372–384.

Merrill, J. E., De Rosa, M., and Franklin, R. J. M. (1998). New concepts in nitric oxide in demyelination and remyelination. *J. Neuroimmunol.* **90,** 50 (Abstract 272).

Meyer, M. R., Schreck, R., and Baeuerle, P. A. (1993). H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* **12,** 2005–1013.

Mills, C. D. (1991). Molecular basis of ''suppressor'' macrophages. Arginine metabolism via the nitric oxide synthetase pathway. *J. Immunol.* **146,** 2719–2723.

Misko, T. P., Trotter, J. L., and Cross, A. H. (1995). Mediation of inflammation by encephalitogenic cells: Interferon γ induction of nitric oxide synthase and cyclooxygenase 2. *J. Neuroimmunol.* **61,** 195.

Mitrovic, B., Ignarro, L. J., Montestruque, S., Smoll, A., and Merrill, J. E. (1994). Nitric oxide as a potential pathological mechanism in demyelination: Its differential effects on primary glial cells in vitro. *Neuroscience* **61,** 575–585.

Mitrovic, B., Ignarro, L. J., Vinters, H. V., Akers, M.-A., Schmid, I., Uittenbogaart, C., and Merrill, J. E. (1995). Nitric oxide induces necrotic but not apoptotic cell death in oligodendrocytes. *Neuroscience* **65,** 531–539.

Mitrovic, B., Parkinson, J., and Merrill, J. E. (1996). An in vitro model of oligodendrocyte destruction by nitric oxide and its relevance to multiple sclerosis. *Methods* (*San Diego*) **10,** 501–513.

Mokhtarian, V. F., McFarlin, D. E., and Raine, C. S. (1984). Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature* **309,** 356–358.

Morrow, J. D., Awad, J. A., Boss, H. J., Blair, I. A., and Roberts II, L. J. (1992). Noncyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10721–10725.

Mosmann, T. R. (1991). Cytokine secretion patterns and cross-regulation of T-cell subsets. *Immunol. Res.* **10,** 183–188.

Mosmann, T. R., and Coffman, R. L. (1989). Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7,** 145–150.

Muñoz, C., Castellanos, M. C., Alfranca, A., Vara, A., Esteban, M. A., Redondo, J. M., and de Landázuri, M. O. (1996). Transcriptional up-regulation of intercellular adhesion molecule-1 in human endothelial cells by the antioxidant pyrolidine dithiocarbamate involves the activation of activating protein-1. *J. Immunol.* **157,** 3587–3597.

Murphy, S., Grzybicki, D. M., and Simmons, M. L. (1995). Glial Cells as nitric oxide sources and targets. *In* ''Nitric Oxide in the Nervous System'' (S. Vincent, ed.), pp. 164–190. Academic Press, San Diego.

Murray, H. W., and Teitelbaum, R. F. (1992). L-Arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. *J. Infect. Dis.* **165,** 513–517.

Nagra, R. M., Becher, B., Tourtellotte, W. W., Antel, J. P., Gold, D., Paladino, T., Smith, R. A., Nelson, J. R., and Reynolds, W. F. (1997). Immunohistochemical and genetic evidence of myeloperoxidase involvement in multiple sclerosis. *J. Neuroimmunol.* **78,** 97–107.

Newcombe, J., Li, H., and Cuzner, M. L. (1994). Low density lipoprotein uptake by macrophages in multiple sclerosis plaques: Implications for pathogenesis. *Neuropathol. Appl. Neurobiol.* **20,** 152–162.

Nicholson, S., da Gloria Bonecini-Almeida, M., Lapa e Silva, J. R., Nathan, C., Xie, Q.-W., Mumford, R., Weidner, J. R., Calaycay, J., Geng, J., Boechat, M., Linhares, C., Rom, W., and Ho, J. L. (1996). Indicible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J. Exp. Med.* **183,** 2293–2302.

Nukaya, I., Takaji, K., Kawabe, T., and Suketa, Y. (1995). Suppression of cytokine production in T helper type 2 cells by nitric oxide in comparison with T helper type 1 cells. *Microbiol. Immunol.* **39,** 709–714.

Ohkuma, S., Narihara, H., Katsura, M., Hasegawa, T., and Kuriyama, K. (1995). Nitric oxide-induced [^{3}H]GABA release from cerebral cortical neurons is mediated by peroxynitrite. *J. Neurochem.* **65,** 1109–1114.

Oka, A., Belliveau, M. J., Rosenberg, P. A., and Volpe, J. J. (1998). Vulnerability of oligodendroglia to glutamate: Pharmacology, mechanisms, and prevention. *J. Neurosci.* **13,** 1441–1453.

Okyda, Y., Nakatsuji, Y., Fujimura, H., Esumi, H., Ogura, T., Yanagihara, T., and Sakoda, S. (1995). Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis. *J. Neuroimmunol.* **62,** 103–108.

Ottnad, E., Parthasarathy, S., Sambrano, G. R., Ramprasad, M. P., Quehenberger, O., Kondratenko, N., Green, S., and Steinberg, D. (1995). A macrophage receptor for oxidized low density lipoprotein distinct from the receptor for acetyl low density lipoprotein: Partial purification and role in recognition of oxidatively damaged cells. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1391–1395.

Padgett, E. L., and Pruett, S. B. (1992). Evaluation of nitrite production by human monocyte-derived macrophages. *Biochem. Biophy. Res. Commun.* **186,** 775–781.

Parkinson, J. F., Mitrovic, B., and Merrill, J. E. (1997). The role of nitric oxide in multiple sclerosis. *J. Mol. Med.* **75,** 174–186.

Peng, H., Libby, P., and Liao, J. K. (1995). Induction and stabilization of IκB by nitric oxide mediates inhibition of NF-κB. *J. Biol. Chem.* **270,** 14214–14219.

Powrie, F., Menom, S., and Coffman, R. L. (1993). Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *Eur. J. Immunol.* **23,** 3043–3049.

Qi, Y., and Dawson, G. (1993). Effects of hypoxia on oligodendrocyte signal transduction. *J. Neurochem.* **61,** 1097–1104.

Racke, M. K., Bonomo, A., Scott, D. E., Cannella, B., Levine, A., Raine, C. S., Shevach, E. M., and Rocken, M. (1994). Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J. Exp. Med.* **180,** 1961–1966.

Raes, M., Renard, P., and Remacle, J. (1995). Free radicals as second messengers. *C. R. Soc. Biol.* **189,** 355–366.

Ransohoff, R. M., Glabinski, A., and Tani, M. (1996). Chemokines in immune-mediated inflammation of the central nervous system. *Cytokine Growth Factor Rev.* **7,** 35–46.

Redford, E. J., Kapoor, R., and Smith, K. J. (1997). Nitric oxide donors reversibly block axonal conduction: Demyelinated axons are especially susceptible. *Brain* **120,** 2149–2157.

Remick, D. G., and Villarete, L. (1996). Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. *J. Leukocyte Biol.* **59,** 471–475.

Renno, T., Krakowski, M., Piccirillo, C., Lin, J.-Y., and Owens, T. (1995). TNF-α expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. *J. Immunol.* **154,** 944–953.

Rice, J. E., Vannucci, R. C., and Brierly, J. B. (1980). The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.* **9,** 131–141.

Rothe, H., Hartmann, B., Geerlings, P., and Kolb, H. (1996). Interleukin-12 gene expression of macrophages is regulated by nitric oxide. *Biochem. Biophys. Res. Commun.* **224,** 159–163.

Rott, O., Fleischer, B., and Cash, E. (1994). Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur. J. Immunol.* **24,** 1434–1440.

Ruuls, S. R., Bauer, J., Sontrop, K., Huitinga, I., Hart, B. A., and Dijkstra, C. D. (1995). Reactive oxygen species are involved in the pathogenesis of experimental allergic encephalomyelitis in Lewis rats. *J. Neuroimmunol.* **56,** 207–215.

Ruuls, S. R., van der Linden, S., Sontrop, K., Huitinga, I., and Dijkstra, C. D. (1996). Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors. *Clin. Exp. Immunol.* **103,** 467–474.

Sahrbacher, U. C., Lechner, F., Egster, H. P., Frei, K., Lassmann, H., and Fontana, A. (1998). Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **28,** 1332–1339.

Sarchielli, P., Orlacchio, A., Vicinanza, F., Pelliccioli, G., Tognoloni, M., Saccardi, C., and Gallai, V. (1997). Cytokine secretion and nitric oxide production by mononuclear cells of patients with multiple sclerosis. *J. Neuroimmunol.* **80,** 76–86.

Satriano, J. A., Shuldiner, M., Hora, K., Xing, Y., Shan, Z., and Schlondorff, D. (1993). Oxygen radicals as second messengers for expression of the

monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor a and immunoglobulin G. *J. Clin. Invest.* **92,** 1564–1571.

Schenk, H., Vogt, M., Dröge, W., and Schulze-Osthoff, K. (1996). Thioredoxin as a potent costimulus of cytokine expression. *J. Immunol.* **156,** 765–771.

Schneemann, M., Schoedon, G., Frei, K., and Schaffner, A. (1993). Immunovascular communication: Activation and deactivation of murine endothelial cell nitric oxide synthase by cytokines. *Immunol. Lett.* **35,** 159–162.

Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G., and Fiers, W. (1993). Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J.* **12,** 3095–3104.

Segal, B. M., and Shevach, E. M. (1996). IL-12 unmasks latent autoimmune disease in resistant mice. *J. Exp. Med.* **184,** 771–775.

Shin, W. S., Hong, Y.-H., Peng, H.-B., De Caterina, R., Libby, P., and Liao, J. K. (1996). Nitric oxide attenuates vascular smooth muscle cell activation by interferon γ. *J. Biol. Chem.* **271,** 11317–11324.

Sicher, S. C., Vazquez, M. A., and Lu, C. Y. (1994). Inhibition of macrophage Ia expression by nitric oxide. *J. Immunol.* **153,** 1293–1300.

Sicher, S. C., Chung, G. W., Vazquez, M. A., and Lu, C. Y. (1995). Augmentation or inhibition of IFN-γ-induced MHC class II expression by lipopolysaccharides. *J. Immunol.* **155,** 5826–5834.

Simmons, M. L., and Murphy, S. (1992). Induction of nitric oxide synthase in glial cells. *J. Neurochem.* **59,** 897–905.

Smith, M. A., Harris, P. L. R., Sayre, L. M., Beckman, J. S., and Perry, G. (1997). Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17,** 2653–2657.

St. Pierre, B., Wong, J. L., and Merrill, J. E. (1996). Inhibition of human and rat glial cell function by antiinflammatory cytokines, antioxidants, and elevators of cAMP. *In* ''Cell Biology and Pathology of Myelin: Evolving Biological Concepts and Therapeutic Approaches'' (R. M. Devon, R. Doucette, B. H. J. Juurlink, A. J. Nazarali, D. J. Schreye, and V. M. K. Verge, eds.), pp. 265–276. Plenum, New York.

Stadler, J., Billiar, T. R., Curran, R. D., Stuehr, D. J., Ochoa, J. B., and Simmons, R. L. (1991). Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. *Am. J. Physiol.* **260,** C910–C916.

Stewart, V. C., Land, J. M., Clark, J. B., and Heales, S. J. R. (1998). Pretreatment of astrocytes with interferon-α/β prevents neuronal mitochondrial respiratory chain damage. *J. Neurochem.* **70,** 432–434.

Streilein, J. W. (1993). Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr. Opin. Immunol.* **5,** 428–432.

Taga, K., and Tosato, G. (1992). IL-10 inhibits human T cell proliferation and IL-2 production. *J. Immunol.* **148,** 1143–1148.

Takahashi, K., Nammour, T. M., Fukunaga, M., Ebert, J., Morrow, J. D., Roberts II, L. J., Hoover, R. L., and Badr, K. F. (1992). Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin F2 alpha, in the rat: Evidence for interaction with thromboxane A2 receptors. *J. Clin. Invest.* **90,** 136–141.

Tanaka, C., Kamata, H., Takeshita, H., Yagisawa, H., and Hirata, H. (1997). Redox regulation of LPS-induced IL-8 gene expression mediated by NF-κB and AP-1 in human astrocytoma U373 cells. *Biochem. Biophys. Res. Commun.* **232,** 568–573.

Taylor-Robinson, A. W., Liew, F. Y., Severn, A., Xu, D., McSorley, S. J., Garside, P., Padron, J., and Phillips, R. S. (1994). Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *Eur. J. Immunol.* **24,** 980–984.

Thorburne, S. K., and Juurlink, B. H. J. (1996). Low glutahione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. *J. Neurochem.* **67,** 1014–1022.

Togashi, H., Sasaki, M., Frohman, E., Taira, E., Ratan, R. R., Dawson, T. M., and Dawson, V. L. (1997). Neuronal (type 1) nitric oxide synthase regulates nuclear NFκB activity and immunologic (type II) nitric oxide synthase expression. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2676–2680.

Tomioka, H., and Saito, H. (1991). Characterization of immunosuppressive functions of murine peritoneal macrophages induced with various agents. *J. Leukocyte Biol.* **51,** 24–31.

Tracey, W. R., Nakane, M., Pollock, J. S., and Förstermann, U. (1993). Nitric oxide synthases in neuronal cells, macrophages, and endothelium are NADPH diaphorases, but represent only a fraction of total cellular NADPH diaphorase activity. *Biochem. Biophys. Res. Commun.* **195,** 1035–1040.

Tzeng, E., Billiar, T. R., Robbins, P. D., Loftus, M., and Stuehr, D. J. (1995). Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11771–11775.

Ulvestad, E., Williams, K., Matre, R., Nyland, H., Olivier, A., and Antel, J. (1994). Fc receptors on cultured human microglia mediate cytotoxicity and phagocytosis of antibody-coated targets. *J. Neuropathol. Exp. Neurol.* **53,** 27–36.

van der Veen, R. C., Trotter, J. L., Hickey, W. F., and Kapp, J. A. (1990). The development and characterization of encephalitogenic cloned T cells specific for myelin proteolipid protein. *J. Neuroimmunol.* **26,** 139–146.

van der Veen, R. C., Hinton, D. R., Incardonna, F., and Hofman, F. M. (1997). Extensive peroxynitrite activity during progressive stages of central nervous system inflammation. *J. Neuroimmunol.* **77,** 1–7.

van der Veen, R. C., and Roberts, J. L. (1999). Contrasting roles for nitric oxide and peroxynitrite in the peroxidation of myelin lipids. *J. Neuroimmunol.* **95,** 1–7.

Vodovotz, Y., Scott Lucia, M., Flanders, K. C., Chesler, L., Xie, Q.-W., Smith, T. W., Weidner, J., Mumford, R., Webber, R., Nathan, C., Roberts, A. B., Lippa, C. F., and Sporn, M. B. (1996). Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. *J. Exp. Med.* **184,** 1425–1433.

Voskuhl, R. R., Martin, R., Bergmann, C., Dalal, M., Ruddle, N. H., and McFarland, H. F. (1993). T helper 1 (Th1) functional phenotype of human myelin basic protein-specific T lymphocytes. *Autoimmunity* **15,** 137–143.

Vouldoukis, I., Riveros-Moreno, V., Dugas, B., Ouaaz, F., Becheral, P., Debre, P., Moncada, S., and Mossalayi, M. D. (1995). The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the FceRII/CD23 surface antigen. *Proc. Natl. Acad. Sci.* **92,** 7804–7808.

Wei, X.-Q., Charles, I. G., Smith, A., Ure, J., Feng, G.-J., Huang, F.-P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375,** 408–411.

Weinberg, J. B., Misukonis, M. A., Shami, P. J., Mason, S. N., Sauls, D. L., Dittman, W. A., Wood, E. R., Smith, G. K., McDonald, B., Bachus, K. E., *et al.* (1995). Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): Analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood* **86,** 1184–1195.

Wesselingh, S. L., Levine, B., Fox, R. J., Choi, S., and Griffin, D. E. (1992). Intracerebral cytokine mRNA expression during fatal and nonfatal alphavirus encephalitis suggests a predominant type 2 T cell response. *J. Immunol.* **152,** 1289–1297.

Winrow, V. R., Winyard, P. G., Morris, C. J., and Blake, D. R. (1993). Free radicals in inflammation: Second messengers and mediators of tissue destruction. *Br. Med. Bull.* **49,** 506.

Xiao, B.-G., Zhang, G.-X., Ma, C.-G., and Link, H. (1996). The cerebrospinal fluid from patients with multiple sclerosis promotes neuronal and oligodendrocyte damage by delayed production of nitric oxide in vitro. *J. Neurol. Sci.* **142,** 114–120.

Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science,* **256,** 225–228.

Yamashita, T., Ando, Y., Obayashi, K., Uchino, M., and Ando, M. (1997). Changes in nitrite and nitrate (NO2-/NO3-) levels in cerebrospinal fluid of patients with multiple sclerosis. *J. Neurol. Sci.* **153,** 32–34.

Yonezawa, M., Back, S. A., Gan, X., Rosenberg, P. A., and Volpe, J. J. (1996). Cystine deprivation induces oligodendroglial death: Rescue by free radical scavengers and a diffusible glial factor. *J. Neurochem.* **67,** 566–573.

Zajicek, J. P., Wing, M., Scolding, N. J., and Compston, D. A. S. (1992). Interactions between oligodendrocytes and microglia. *Brain* **115,** 1611–1631.

Zamvil, S. S., Nelson, P., Trotter, J., Mitchell, D. J., Knobler, R., Fritz, R. B., and Steinman, L. (1985). T-cell clones specific for MBP induce chronic relapsing paralysis and demyelination. *Nature* **317,** 355–361.

Zeiher, A. M., Fisslthaler, B., Schray-Utz, B., and Busse, R. (1995). Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ. Res.* **76,** 980–986.

Zhao, W., Tilton, R. G., Corbett, J. A., McDaniel, M. L., Misko, T. P., Williamson, J. R., Cross, A. H., and Hickey, W. F. (1996). Experimental allergic encephalomyelitis in the rat is inhibited by aminoguanidine, an inhibitor of nitric oxide synthase. *J. Neuroimmunol.* **64,** 123–133.

Ziegler-Heitbrock, H. W. L., Sternsdorf, T., Liese, J., Belohradsky, B., Weber, C., Wedel, A., Schreck, R., Bäuerle, P., and Ströbel, M. (1993). Pyrrolidine dithiocarbamate inhibits NF-κB mobilization and TNF production in human monocytes. *J. Immunol.* **151,** 6986–6993.

Zielasek, J., Jung, S., Gold, R., Liew, F. Y., Toyka, K. V., and Hartung, H. P. (1995). Administration of nitric oxide synthase inhibitors in experimental autoimmune neuritis and experimental autoimmune encephalitis. *J. Neuroimmunol.* **58,** 81–88.

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CHAPTER 31

Role of Nitric Oxide in the Regulation of Blood Flow

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LOCAL HEMODYNAMIC FORCES ACTIVELY REGULATE VASCULAR SMOOTH MUSCLE TONE THROUGH THE PHENOMENON OF FLOW-DEPENDENT DILATION, WHICH IS PRINCIPALLY MEDIATED VIA NITRIC OXIDE (NO) RELEASED BY THE ENDOTHELIUM. THIS DYNAMIC RESPONSE CONTRIBUTES TO THE HYDRAULIC EFFICIENCY OF THE CIRCULATION AND OPTIMIZES PERFUSION BY INTERACTING WITH MYOGENIC AND METABOLIC CONTROL MECHANISMS. THE ABILITY OF THE ENDOTHELIAL CELL TO SENSE SHEAR STRESS INVOLVES AN ARRAY OF SIGNALING PATHWAYS AND CELLULAR COMPONENTS, INCLUDING G PROTEINS, CAVEOLAE, INTEGRINS, AND THE CYTOSKELETON, WHICH MAY ALL PARTICIPATE IN THE MECHANOTRANSDUCTION MECHANISMS THAT LEAD TO THE ACTIVATION OF THE ENDOTHELIAL NO SYNTHASE (eNOS). ON A LONG-TERM BASIS, REGULATION OF eNOS BY SHEAR FORCES IS THOUGHT TO CONTRIBUTE TO THE ABILITY OF THE VESSEL WALL TO REMODEL AND THEREBY NORMALIZE SHEAR STRESS FOLLOWING SUSTAINED CHANGES IN FLOW. FURTHERMORE, MOMENT-TO-MOMENT RELEASE OF NO AND EXPRESSION OF eNOS ITSELF ARE DIFFERENTIALLY REGULATED BY STEADY, PULSATILE, AND TURBULENT SHEAR FORCES. THIS SENSITIVITY TO DIFFERENT FLOW PATTERNS MAY EXPLAIN WHY CERTAIN SITES IN THE CIRCULATION ARE PREDISPOSED TO THE DEVELOPMENT OF DISEASE.

Introduction

Schretzenmayr first described flow-dependent increases in the diameter of the canine femoral artery as early as 1933, but nearly 50 years were to elapse before Furchgott and Zawadzki (1980) ''serendipitously'' discovered the importance of the endothelium in the regulation of vascular tone—a pivotal observation that has provided the key to understanding how hemodynamic forces influence local vascular resistance. It is now well established that nitric oxide (NO) synthesis by the constitutive endothelial NO synthase (eNOS or NOS type III) is stimulated by longitudinal shear forces acting on the endothelium as a result of blood flow, as well as by agonist occupation of endothelial membrane receptors. Flow-dependent release of NO allows efficient and dynamic coupling of downstream metabolic demands with upstream resistance, and its interaction with pressure-sensitive and metabolic mechanisms of regulating smooth muscle tone contributes to the integration of vascular control mechanisms and the optimization of organ perfusion. Many aspects of the flow-dependent response nevertheless remain poorly understood, and the mechanotransduction pathways that allow the endothelial cell to sense and respond to physical forces remain a focus of active research. Indeed, there are major differences in the pathways that lead to NO synthesis in response to pharmacological and to mechanical stimuli, as agonist-stimulated responses are allosterically regulated by the association of eNOS with the Ca^{2+}/calmodulin complex, whereas shear forces can also activate eNOS in the absence of extracellular Ca^{2+}. This overview will attempt to summa-

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rize the current state of knowledge and highlight growing points in this major area of NO research.

Shear Forces and the Endothelium

In steady laminar flow the longitudinal shear stress τ acting at any point in the fluid is the product of the shear rate $\dot{\gamma}$ (the velocity gradient) and the coefficient of viscosity μ such that

$$\tau = \mu\dot{\gamma} = \mu \frac{dv}{dy} \quad (1)$$

where v is the local flow velocity and y is distance from the wall. For steady flow in a cylindrical tube the velocity profile is parabolic, and the velocity gradient steepens toward the wall, where the velocity of the fluid is zero if there is no slip (Fig. 1). Shear stress is consequently greatest at the wall and is given by

$$\tau = \frac{4\mu\dot{Q}}{\pi r^3} = \frac{4\mu\, v_{mean}}{r} \quad (2)$$

where $\dot{Q}$ is the volumetric flow rate, r is the tube radius, and v_{mean} is the mean flow velocity. *In vivo,* the time-averaged shear forces experienced by the endothelium of conduit arteries are of the order of 10–30 dynes/cm^2, but in venules and veins they may be as low as 1–6 dynes/cm^2 (Olesen *et al.,* 1988; Taber, 1998). In arterioles >20 μm in size, estimates are comparable with large arteries, and in the smallest arterioles and capillaries levels may be as high as 100 dynes/cm^2 (Pries *et al.,* 1995). Since NO production increases monotonically with time-averaged shear stress until it approaches a plateau at 10–30 dynes/cm (Fig. 2) (Hecker *et al.,* 1993; Hutcheson and Griffith, 1994; Posch *et al.,* 1999), basal NO release (Griffith *et al.,* 1984; Martin *et al.,* 1986) will be continuously amplified by blood flow, thus explaining the often large (up to 30%) increases in systemic pressure that follow inhibition of NO production *in vivo* (see Moncada *et al.,* 1991 for review). Shear stress also stimulates endothelial uptake of L-arginine, the physiological substrate for eNOS, by increasing the velocity but not the affinity of the L-arginine transport system, thereby enhancing NO production (Posch *et al.,* 1999).

Influence of Flow

Equation (2) implies that NO release will be sensitive to flow velocity, blood viscosity, and alterations in vascular caliber, but under physiological conditions it may be difficult to discriminate between the effects of changes in these interdependent parameters. Arguably, however, acute changes in flow are the most important physiological determinant of changes in endothelial shear stress (Fig. 1). Flow-induced NO release and dilation have been demonstrated in conduit arteries (Pohl *et al.,* 1986; Rubanyi *et al.,* 1986), resistance arteries (Griffith *et al.,* 1987, 1990a; Pohl *et al.,* 1991), and

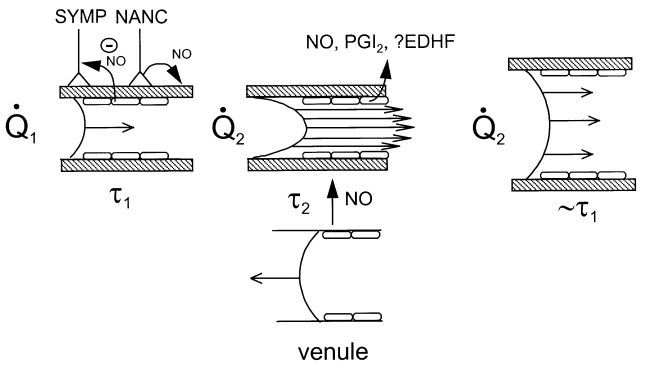


Figure 1 Elevations in arterial flow rate ($\dot{Q}_2 > \dot{Q}_1$) increase endothelial shear stress (τ), the principal hemodynamic stimulus to the endothelium, resulting in NO release and an increase in vascular caliber that restores τ back toward baseline. Shear stress also promotes NO release from venules, from which it diffuses and dilates adjacent arterioles, thereby permitting cross talk between the inflow and outflow of blood. Endothelium-derived NO inhibits sympathetic nerves (SYMP) that promote constriction, and in some vessels NO is itself released from nonadrenergic noncholinergic (NANC) nerves. Prostanoids such as prostacyclin (PGI_2) may also contribute to flow-dependent dilation, but a flow-dependent role for endothelium-derived hyperpolarizing factor (EDHF) remains to be demonstrated.

arterioles (Kuo *et al.,* 1990, 1991) from a wide variety of vascular beds. The response is well suited to respond to changes in organ function on a dynamic basis, as it is activated within seconds and maximal within 1 to 2 min. NO may also participate in the regulation of flow on subacute time scales. For example, perfusion of a segment of rabbit central ear artery isolated between two ligatures increases to ~50% of its preocclusion control level over 90 min as a consequence of slow NO-dependent diversion of flow through preexisting collateral pathways (Randall and Griffith, 1992). NO-mediated, flow-dependent dilatation has been demonstrated in isolated venules (Kuo *et al.,* 1993; Koller *et al.,* 1998), and the associated diffusion of NO to nearby arterioles may contribute to the coordination of tissue perfusion. Increases in venular flow velocity have thus been shown to promote arteriolar dilation in the skeletal muscle microvasculature via this mechanism (Boegehold, 1996).

Equation (2) defines shear stress for steady laminar flow, but physiological NO release is also highly sensitive to flow pulsatility (Pohl *et al.,* 1986), being maximal at a similar pulse frequency of 5 Hz in conduit arteries from the rabbit, rat, and pig (Fig. 2) (Griffith *et al.,* 1991a; Hutcheson and Griffith, 1991, 1994). Vascular caliber consequently increases if steady flow becomes pulsatile at the same mean flow rate in an NO-dependent fashion (Shimoda *et al.,* 1998). In the isolated rabbit heart, NO release is enhanced by the periodic compression imposed by contraction (Lamontagne *et al.,* 1992), and in conscious dogs epicardial coronary dilation in response to changes in pacing frequency is mediated by NO (Canty and Schwartz, 1994). Changes in heart rate may thus directly modulate organ perfusion via the endothelium.

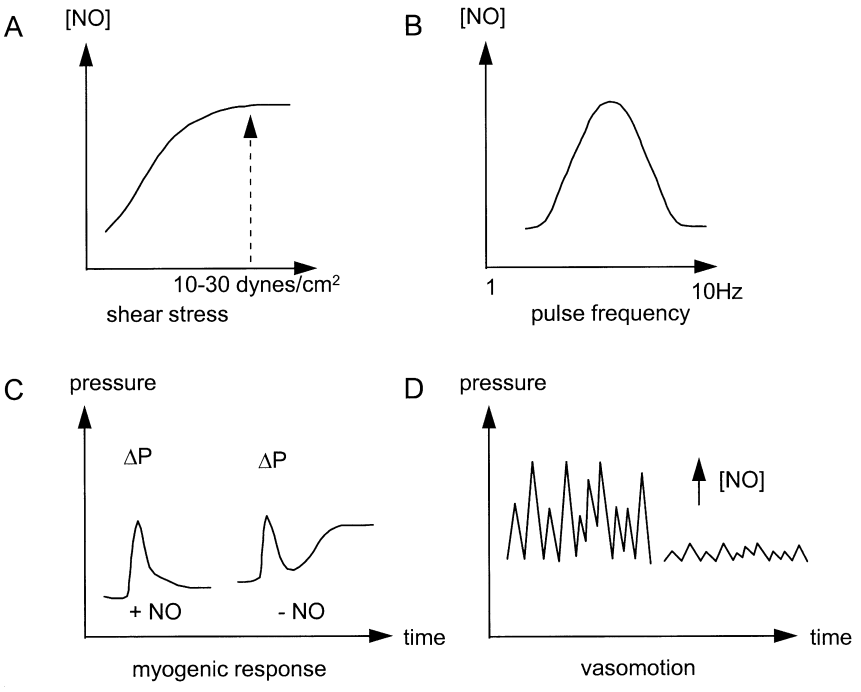


Figure 2 Key mechanisms involved in the regulation of perfusion by NO. (A) Endothelial NO production increases monotonically with time-averaged shear stress, reaching a plateau at 10 to 30 dynes/cm^2. (B) NO release from the endothelium is sensitive to flow pulsatility, being maximal at pulse frequencies of ~5 Hz. (C) In isolated vessels acute increases in transmural pressure (ΔP) promote a myogenic constrictor response, normally attenuated by NO. Loss of NO activity also elevates basal constrictor tone. (D) The amplitude of spontaneous oscillations in vascular caliber (vasomotion) is damped by NO.

Another way in which flow may influence NO production is through the release of agonists such as ACh, ATP, bradykinin, and substance P from endothelial cells themselves (Fig. 3) (Milner *et al.,* 1990). In the isolated rat lung, for example, perfusion pressure is elevated by P_2 purinoceptor antagonism, suggesting the participation of flow-dependent release of ATP (Hassessian *et al.,* 1993). The potential clinical importance of such autocrine mechanisms has been confirmed in the human myocardium, in which bradykinin B_2 antagonists promote constriction and reduce basal flow (Groves *et al.,* 1995), and in the human forearm, in which flow-dependent dilation is enhanced by angiotensin-converting enzyme (ACE) inhibitors that decrease the metabolism of bradykinin (Hornig *et al.,* 1997). As ATP and bradykinin are rapidly degraded by ectoenzymes, their delivery to receptors will be governed by diffusive and convective mass transport processes in the intimal hydrodynamic boundary layer. Dull and co-workers (1992) thus demonstrated that a stepwise increment in flow produced rapid elevations in endothelial $[Ca^{2+}]_i$ that decayed over minutes in the presence of either ATP or its nonhydrolysable analog ADPβS, but that additional increases in flow evoked further Ca^{2+} transients only in the presence of ATP. This is consistent with rapid equilibration of boundary layer and bulk concentrations of ADPβS, but not ATP, because of dynamic clearance by the 5'-nucleotidase.

In contrast to the effects of flow, NO release from conduit arteries is depressed by acute increases in mean transmural pressure (Rubanyi, 1988) and is inversely related to the amplitude of the pressure pulse during pulsatile flow (Hutcheson and Griffith, 1991). Agonist-stimulated NO release from cultured endothelial cells grown on a flat surface also appears to be susceptible to the effects of pressure, exhibiting a progressive but reversible ~sixfold decrease over the range 0–160 mm Hg (Hishikawa *et al.,* 1992), whereas NO release from cells grown on microcarrier beads was found to be insensitive to changes in pressure (Kelm *et al.,* 1991). Because the direct effects of pressure on NO release are quantitatively smaller than those of flow, they are likely to be of less physiological importance. Indeed, in a study of 500 clinically fit adults, no correlation was found between mean blood pressure and the magnitude of flow-dependent dilation measured ultrasonically in the brachial or femoral arteries following release of distal limb occlusion (Celermajer *et al.,* 1994).

Influence of Constrictor Tone

The second factor determining shear stress is vascular diameter. For a given flow rate, shear forces are necessarily lower when diameter is greater, so that local constrictor mechanisms may exert an important influence on NO re-

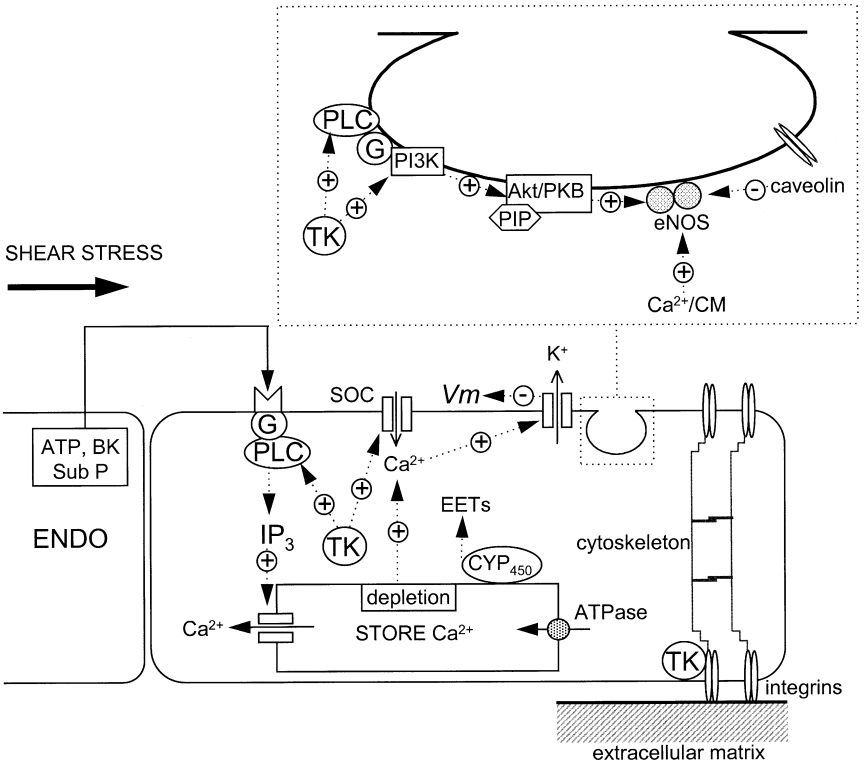


Figure 3 Pathways involved in the activation of eNOS by shear forces. G protein–phospholipase C interactions that involve tyrosine kinase (TK)-dependent phosphorylation result in transient $InsP_3$ (IP_3)-evoked Ca^{2+} release from internal stores. The subsequent depletion of stores then promotes influx of Ca^{2+} via a store-operated channel (SOC) that appears to be activated by mechanisms involving TK-dependent phosphorylation and epoxyeicosatrienoic acids (EETs) derived from arachidonic acid via the cytochrome P-450 monooxygenase (CYP_{450}). Shear stress and elevations in $[Ca^{2+}]_i$ also stimulate K^+ channel activity, and the associated decrease in membrane potential (V_m) promotes Ca^{2+} entry by increasing the electrochemical gradient for influx. Elevations in $[Ca^{2+}]_i$ activate the Ca^{2+}/calmodulin (CM)-dependent eNOS that is located in membrane caveolae, where it is negatively regulated by the structural coat protein caveolin (inset). TK-dependent phosphorylation and G proteins may stimulate phosphatidylinositol 3-kinase (PI3K), which is an upstream activator of protein kinase B (Akt/PKB) that in turn phosphorylates eNOS to increase its activity in a Ca^{2+}-independent fashion in response to fluid shear stress. Shear forces may also increase NO production by releasing endothelium-dependent agonists such as ATP, bradykinin, and substance P.

lease. *In vivo,* ambient vascular tone is largely determined by the intrinsic myogenic behavior of smooth muscle, with a contribution from sympathetically mediated constriction. Increases in local transmural pressure lead to constriction (and decreases in pressure to dilation) through the ability of vascular smooth muscle to sense and respond to changes in circumferential wall stress—the so-called myogenic response (Fig. 2) (for review, see Davis and Hill, 1999). In isolated perfused vessels, myogenic tone is potentiated by endothelial denudation and attenuated by basal NO release, the inhibitory role of the endothelium predictably being greater in the presence of shear stress, such that myogenic constriction is most pronounced at low flow rates and attenuated by up to ~50% as flow is increased (Griffith and Edwards, 1990a; Pohl *et al.,* 1991; Kuo *et al.,* 1991; Sun *et al.,* 1995a; Juncos *et al.,* 1995). In the converse sense, flow-dependent dilation is maximal at transmural pressures equivalent to those found under normal physiological conditions when myogenic tone is at an intermediate level (Kuo *et al.,* 1990, 1991). *In vivo,* eNOS inhibitors tend to unmask or enhance myogenic constriction in large arterioles that normally react little to increases in pressure, either having little effect on smaller downstream arterioles, or causing them to dilate as compensatory mechanisms may be activated by upstream constriction (Hester *et al.,* 1993; Jones *et al.,* 1995). Loss of NO activity thus causes a shift in the major site of vascular resistance upstream to vessels which are less tightly controlled by metabolites.

Theoretically, the myogenic response and flow-dependent dilation may be considered positive feedback mechanisms, with high pressure promoting progressively greater constriction and flow-dependent dilation further enhancing flow if the supply pressure remains unchanged. However, myogenic constriction will increase shear stress, thereby promoting NO release that opposes the initial constrictor stimulus and confers circulatory stability. Sympathetically mediated con-

striction will similarly increase endothelial shear stress and NO production, but in addition to directly decreasing smooth muscle tone, NO exerts an inhibitory prejunctional effect on catecholamine release, thereby contributing to a negative feedback loop that diminishes the original constrictor stimulus (Fig. 1) (Tesfamariam and Cohen, 1988). Pressure-dependent, neurogenic, and flow-dependent control mechanisms may thus coexist in a state of dynamic equilibrium.

Adventitial nerves may themselves attenuate constrictor tone through NO-dependent mechanisms (Fig. 1). For example, NO is synthesized by nonadrenergic, noncholinergic (NANC) nerves in the corpus cavernosum, thereby mediating penile erection (Rajfer *et al.,* 1992). A similar mechanism contributes to dilatation in cerebral and mesenteric arteries (Toda and Okamura, 1990; Ahlner *et al.,* 1991). It has also been suggested that acetylcholine (ACh) released from parasympathetic adventitial nerves diffuses to the endothelium and stimulates NO synthesis, on the basis that vasodilatation induced by vagal stimulation is blocked by atropine and eNOS inhibitors (Broten *et al.,* 1992; McMahon *et al.,* 1992). In analogous fashion, neurotransmitter peptides such as vasoactive intestinal polypeptide (VIP) and substance P mediate endothelium-dependent vasodilation in salivary gland and skeletal muscle (Persson *et al.,* 1991; Edwards and Garrett, 1993).

Influence of Viscosity

The third factor determining shear stress is viscosity, whose importance in the regulation of NO production has been confirmed by the demonstration that solutions containing high-molecular-weight dextran enhance NO release from isolated vessels through a mechanism that is independent of flow rate (Tesfamariam and Cohen, 1988; Hutcheson and Griffith, 1991). Dextran has also been shown to evoke NO-dependent increases in arteriolar diameter in the microcirculation *in vivo* (de Wit *et al.,* 1997). These observations suggest that the hemodynamic stimulus to the endothelium is shear stress, rather than flow velocity. Indeed, Ando and colleagues (1993) found that elevations in $[Ca^{2+}]_i$ evoked by flow are amplified by increased perfusate viscosity, but that their magnitude correlates with absolute shear stress ($\tau = \mu \times \dot{\gamma}$), rather than viscosity, μ, or shear rate, $\dot{\gamma}$, when these factors were manipulated separately. Viscosity-related increases in NO production are likely to contribute to the increased organ blood flow found in high viscosity states such as polycythemia (Wilcox *et al.,* 1993).

NO and Cardiovascular Optimization

Pressure Gradients

Flow, resistance, and the pressure gradient driving flow (i.e., pressure drop per unit length) in a cylindrical tube of length L and radius r under steady laminar conditions are related according to the Poiseuille equation

$$\frac{dP}{dL} = \frac{(P_1 - P_2)}{L} = \frac{8\,\mu\dot{Q}}{\pi\, r^4} = R \times \dot{Q} \qquad (3)$$

where P_1 and P_2 are the supply and output pressures, respectively, and R denotes hydraulic resistance. It is readily appreciated from this formula that if the vascular tree consisted simply of a system of rigid tubes, left ventricular pressure would need to rise to physiologically unacceptable levels to sustain the approximately fivefold increase in cardiac output that occurs during maximal exercise. In practice, however, flow-dependent dilation can readily restore the pressure gradient, dP/dL, toward its control value because of the fourth power dependence on radius but direct relationship with flow. This ability to stabilize pressure gradients has been confirmed experimentally in the rat saphenous artery, in which the pressure drop remains virtually unchanged over a fivefold range of flow rates if the endothelium is intact (Khayutin *et al.,* 1993a,b). Calculations suggest that in the rabbit ear, the pressure gradient driving flow asymptotically approaches a constant value at high flow rates in arterial branch generations with diameters ranging from 70 to 800 μm as a direct consequence of NO release (Griffith *et al.,* 1987). In human conduit arteries flow-dependent dilation can vary between 5 and 15% of control diameter and could thus in theory accommodate up to an ~60% increase in flow with no change in pressure gradient (Celermajer *et al.,* 1994). However, small resistance arteries and arterioles may be considerably more sensitive to changes in flow. Mesenteric and coronary arterioles have thus been shown to exhibit increases in diameter in response to increments in flow of ~70 and ~30%, respectively (Smiesko *et al.,* 1989; Kuo *et al.,* 1990). Since small vessels are the major site of systemic resistance, this is likely to explain why mean arterial pressure increases by only 10–20% during maximal exercise.

Stabilization of pressure gradients by the endothelium may confer other physiological advantages. First, it will contribute to hydraulic efficiency, particularly in the microcirculation where flow is viscous rather than inertial. Energy losses are directly proportional to the product of pressure gradient and flow and therefore are proportional to $\dot{Q}$ if flow-dependent dilation maintains a constant pressure gradient, rather than to $\dot{Q}^2$ as in a rigid tube (Griffith *et al.,* 1987). During maximal exercise, for example, this would represent a fivefold reduction in hydraulic energy expenditure. Second, stabilization of pressure gradients will help to prevent a microvascular ''steal'' phenomenon that would otherwise result from the release of vasodilator metabolites. Metabolic dilation will cause a local fall in resistance and an increase in flow. Consequently, if the supply pressure remains constant [P_1 in Eq. (3)] and if the resistance of proximal feed arteries remains fixed, Poiseuille's law implies that there will be a fall in distal pressure [P_2 in Eq. (3)] which could decrease the perfusion of adjacent tissue elements. A steal effect will nevertheless be offset by the upstream dilation of proximal arterioles and distributive arteries through both myogenic and flow-dependent mechanisms, thereby normalizing distal pressures. This will have obvious benefits in

terms of the delivery of oxygen and nutrients, as well as contributing to the physiological regulation of hydrostatic pressure and microcirculatory fluid exchange. Direct visualization of the microcirculation of the rabbit ear also suggests that NO importantly helps to maintain spatially homogeneous patterns of flow distribution by preserving similar relative resistances in different vascular segments under altered conditions of flow and constriction (Griffith *et al.,* 1987).

Relationship between ''Constant'' Shear Stress and Minimization of Hydraulic Work

Although there may be an approximately tenfold difference in shear stress between the arterial and venous circulations, there is a surprising similarity in its magnitude across species and vessel size given that flow rates in different vessels may vary by a factor of $\sim 10^4$ (Pries *et al.,* 1995; Taber, 1998). Furthermore, long-term increases or decreases in flow over days or weeks result in adaptations of local arterial caliber through a strictly endothelium-dependent mechanism that restores shear stress to its previous control level (Langille and O'Donnell, 1986; Tronc *et al.,* 1996). A simple theoretical approach devised by Murray (1926) provides insights into the design principles that could underlie these observations. Murray postulated that the sum of the work and energy losses incurred through viscous friction and through maintaining a finite circulatory volume ought in theory to be as small as possible for optimum economy. For an artery of unit length, total energy losses, *W,* can be written as

$$W = \frac{8\,\mu\dot{Q}^2}{\pi\, r^4} + br^2 \tag{4}$$

where b is a constant reflecting the metabolic cost of blood. It can readily be shown that there is a minimum in this combined term (i.e., $dW/dr = 0$) when $\dot{Q} \propto r^x$, where the exponent $x = 3$. One immediate theoretical corollary of this result is that shear stress, which is proportional to $\dot{Q}/r^3$ [Eq. (2)], should be constant throughout the circulation. Values of x close to 3 have been obtained for arteries in the dog lung and in the human cerebral and coronary circulations, and for arterioles in the rat microcirculation (Hutchins *et al.,* 1976; Mayrovitz and Roy, 1983; Liu and Ritman, 1986; Rossitti and Löfgren, 1993). In the rabbit ear median values of x remain close to the Murray optimum over an approximately tenfold range of flow rates at bifurcations of arteries with a wide range of diameters, provided that there is normal NO activity (Griffith and Edwards, 1990b). High levels of pharmacological constriction cause deviation from the optimum, but x may be restored toward 3 by agonists that dilate via the endothelium (Griffith *et al.,* 1991b). Adaptive control mechanisms also restore optimality in the sense postulated by Murray in the human retinal circulation following chronic reductions in flow caused by head trauma (Rossitti and Frisen, 1994).

Murray's hypothesis is clearly a simplification of the physiological situation, as it ignores the pulsatile nature of blood flow and the non-Newtonian properties of blood. Furthermore, there may be a relatively broad statistical spread in the values of x obtained experimentally; in the rabbit ear microvasculature, for example, the frequency distribution of the exponent x is skewed to the right with a significant number of estimates $\geqslant 3$ (Griffith and Edwards, 1990b). Calculations nevertheless show that the mathematical optimum is ''shallow,'' in that the energetic cost penalty incurred by departing from $x = 3$ is only a few percent over a wide range of exponents ($x = 1$ to 10) (Sherman *et al.,* 1989). Although the agreement between experimental data and theory is approximate rather than exact, it is nevertheless consistent with the view that flow-dependent dilation contributes to cardiovascular design by optimizing tissue perfusion for a given level of cardiac work and finite blood volume. A simple modification of Eq. (4) incorporating an extra term to take into account the energy expended in maintaining the vessel wall in an active state of myogenic constriction has predicted that average shear stress will be correlated with ambient pressure at different levels of the circulation, and will therefore be lowest in veins (Taber, 1998).

Interactions of NO with Other Dynamic Control Mechanisms

Conducted Responses

Localized dilator responses, including those induced by ACh, can propagate electrotonically along the arterial wall with a velocity and amplitude space constant similar to that in other tissues (Segal *et al.,* 1989; Frame and Sarelius, 1995). The myogenic response also propagates electronically, with the magnitude of the conducted constriction correlating with the locally sensed pressure change (Rivers, 1995). Such conducted responses may interact synergistically with flow-dependent dilation to minimize local perfusion heterogeneity, by integrating the intensity and nature of stimuli arriving from downstream sites. Dilation of a daughter vessel at a branch point will increase flow in the parent vessel, thereby decreasing local pressure and flow through the nondilated daughter branch, but steal effects may be offset by propagation of the dilator response into the nondilated daughter branch (Segal *et al.,* 1989). Much remains to be learned about the interaction between flow-dependent and conducted control mechanisms. Topical application of L-arginine or ACh to distal microvessels, for example, enhances remote upstream propagated responses through an unknown mechanism that is sensitive to inhibition of eNOS (Frame and Sarelius, 1995).

Vasomotion

Cyclic fluctuations in perfusion with a wide range of frequencies and amplitudes can be observed in myocardial subregions as large as 1 mm^3 in size, and at segmental and lobar levels in the pulmonary circulation (Sestier *et al.,* 1978; Glenny *et al.,* 1995). This temporal variability is largely generated by oscillations in vascular diameter, a phenomenon known as vasomotion (Fig. 2). Although the patterns of vasomotion observed in isolated vessels are often highly irregular, they are not random, but rather are generated by

deterministic nonlinear control mechanisms that can be classified as ''chaotic'' (see Griffith, 1996, for review). In resistance arteries, fluctuations in pressure and flow are generated by a relatively small number of dominant control variables (probably four) and involve nonlinearity in the coupling between two distinct intracellular and membrane Ca^{2+} oscillators located at the level of the smooth muscle cell. Although NO profoundly affects the visible form and amplitude of vasomotion, which can be completely suppressed by micromolar concentrations of ACh (Griffith and Edwards, 1994), nonlinear analysis confirms that the mathematical complexity of vasomotion (assessed as a fractal dimension) is insensitive to changes in pressure, flow, and NO activity (Griffith, 1996). This supports the view that mechanical forces and their influence on NO release do not control the cellular mechanisms that underlie vasomotion in a fundamental dynamical sense (Parthimos *et al.,* 1999). Nevertheless, the response of chaotic systems to perturbation is highly unpredictable, a property that may explain why NO can also ''paradoxically'' promote rhythmic activity in otherwise quiescent arteries under certain experimental conditions (Jackson *et al.,* 1991).

Vasomotion is thought to limit extravascular fluid filtration by reducing hydrostatic pressure during periods of low flow, to enhance lymphatic drainage through a pumping action, and to represent a beneficial hemodynamic response during hypoxia and ischemia by decreasing local vascular resistance. One implication of the demonstration of nonlinear behavior is that vascular control possesses greater flexibility than is possible in a linear system: in chaotic systems minute perturbations can effect changes in state with minimal energy expenditure, thereby readily permitting escape from patterns of behavior that have become disadvantageous. NO may consequently exert subtle effects on many aspects of microvascular function. Present evidence suggests that NO activity normally suppresses the 0.2–0.6 Hz frequency component of blood pressure variability *in vivo,* and experiments with eNOS knockout mice confirm that this is specifically attributable to NO derived from the endothelium (Nafz *et al.,* 1997; Stauss *et al.,* 1999).

Role of Ca^{2+} in the Activation of eNOS by Shear Forces

Ca^{2+}-Dependent Mechanisms

As discussed elsewhere in this volume, binding of the Ca^{2+}/calmodulin complex to the eNOS dimer is central to its catalytic activity. This interaction is likely to underlie the endothelial response to acute increases in shear stress, as exposure to laminar flow initiates a rise in $[Ca^{2+}]_i$ within 1 to 3 s, which is followed by NO release within 2 to 3 s (Kanai *et al.,* 1995). Such $[Ca^{2+}]_i$ transients may be monophasic, biphasic, or oscillatory, and high-resolution imaging has demonstrated elevations in $[Ca^{2+}]_i$ in distinct nuclear and subplasmalemmal pools, whose magnitude and speed of onset are directly related to the shear stress applied (Geiger *et al.,* 1992; Shen *et al.,* 1992; Falcone *et al.,* 1993; Kanai *et al.,* 1995). Some reports suggest preferential elevations in $[Ca^{2+}]_i$ at the downstream edge of the endothelial cell (Geiger *et al.,* 1992; Falcone *et al.,* 1993), whereas others demonstrate a repetitive Ca^{2+} wave that originates at the upstream edge and propagates downstream (Yoshikawa *et al.,* 1997). Repeat exposure to flow does not always activate the same subpopulation of cells, suggesting the existence of a refractory period (Falcone *et al.,* 1993; James *et al.,* 1995). Theoretical computations based on the irregular surface geometry of endothelial monolayers suggest large cell-to-cell variations in stress fields and an uneven distribution over the surface of individual cells due to their nonuniform thickness (Davies, 1995). This could explain why the number of cells responding to flow increases with the magnitude of the applied shear stress in a graded fashion (Geiger *et al.,* 1992; Falcone *et al.,* 1993; James *et al.,* 1995; Yoshikawa *et al.,* 1997).

The Ca^{2+}-dependent pathways through which shear stress stimulates NO synthesis are similar to those activated by agonists, which transiently (~2 min) activate the PLC-γ_1 isoform of phospholipase C via tyrosine phosphorylation, resulting in parallel formation of inositol 1,4,5-trisphosphate ($InsP_3$) and Ca^{2+} release from the endoplasmic reticulum (ER) (Fleming *et al.,* 1996). Tyrosine phosphorylation of PLC is rapidly reversed by the activation of Ca^{2+}-dependent tyrosine phosphatases, thus explaining the transitory nature of the $InsP_3$ response (Fleming *et al.,* 1996). Stepwise changes in flow similarly stimulate $InsP_3$ synthesis, with levels exhibiting a peak at 15–30 s (Nollert *et al.,* 1990; Prasad *et al.,* 1993). Endothelial $InsP_3$ turnover is also modulated by oscillatory mechanical forces, with both increases and decreases in the frequency of cyclical stretch stimulating a transient rise (Rosales and Sumpio, 1992; Brophy *et al.,* 1993). It is currently unknown if this mechanism contributes to the sensitivity of NO production to flow pulsatility.

After the initial peak in $[Ca^{2+}]_i$ induced by agonists or stepwise changes in shear, there may be a sustained elevation above baseline which is maintained by influx of extracellular Ca^{2+}. This occurs principally via a ''store-operated channel'' regulated by the Ca^{2+} status of the ER stores themselves. Indeed, basal NO production is markedly enhanced by depletion of the ER with compounds that inhibit its Ca^{2+}-ATPase pump, such as cyclopiazonic acid (Moritoki *et al.,* 1994), although the mechanism exhibits tachyphylaxis and after prolonged depletion neither agonists nor acute changes in flow elicit NO release (Hutcheson and Griffith, 1997). Only a small subcomponent of the ER is involved in regulating the store-operated channel, because ryanodine, which locks a specific ER Ca^{2+}-activated Ca^{2+} release channel in an open subconductance state, depletes the ER more rapidly than cyclopiazonic acid, but does not activate Ca^{2+} influx (Sasajima *et al.,* 1997). Functional compartmentalization may explain why ryanodine does not significantly modulate shear stress- or ACh-induced NO release in cascade bioassay (Hutcheson and Griffith, 1997).

The mode of activation of the endothelial store-operated channel is controversial, and it is unknown whether it requires direct mechanical coupling with $InsP_3$ receptors present in the peripheral ER, as suggested in other cell types (Kiselyov *et al.,* 1998). It is established, however, that Ca^{2+} entry via this channel is attenuated by tyrosine kinase inhibitors and prolonged by inhibitors of tyrosine phosphatase, and it may therefore involve a protein tyrosine phosphorylation transduction cascade (Fleming *et al.,* 1996; Davis and Sharma, 1997). Store depletion also promotes the synthesis from arachidonic acid of epoxyeicosatrienoic acids (EETs), which may function as a ''Ca^{2+} influx factor'' that promotes Ca^{2+} entry (Graier *et al.,* 1995; Hoebel *et al.,* 1997; Rzigalinski *et al.,* 1999). These microsomal P-450 monooxygenase metabolites activate tyrosine kinase in endothelial cell homogenates (Hoebel *et al.,* 1998) and can directly stimulate NO synthesis in intact arteries (Tan *et al.,* 1997; Hutcheson *et al.,* 1999). The rate at which Ca^{2+} is sequestered within stores by the ER Ca^{2+}-ATPase pump also influences NO production, as agonist-induced relaxations are attenuated in phospholamban gene-ablated mice in which there is enhanced uptake by the ER (Sutliff *et al.,* 1999).

In marked contrast to vascular smooth muscle, the endothelium is devoid of voltage-operated Ca^{2+} channels, and membrane hyperpolarization promotes, rather than inhibits, Ca^{2+} entry by increasing the electrochemical gradient for Ca^{2+} influx (Lückhoff and Busse, 1990). Agonists may stimulate endothelial hyperpolarization by increasing the open-state probability of Ca^{2+}-activated K^+ (K_{Ca}) channels following elevations in $[Ca^{2+}]_i$, a mechanism that may consequently be closely coupled with store depletion and subsequent Ca^{2+} influx (Davis and Sharma, 1997). EETs may amplify this hyperpolarization as they activate K_{Ca} channels (Graier *et al.,* 1995; Hoebel *et al.,* 1997). Several studies have confirmed that K^+ channel activation may contribute to NO release stimulated both by increases in time-averaged shear stress and by the frequency-related component of pulsatile flow, with subtypes involved including high-, medium-, and low-conductance K_{Ca} channels and ATP-sensitive K^+ channels (Ohno *et al.,* 1993; Hutcheson and Griffith, 1994). Flow also activates an inwardly rectifying K^+ channel that mediates hyperpolarization and is fully activated at physiological levels of shear stress, desensitizes slowly, and recovers rapidly on cessation of flow (Olesen *et al.,* 1988). However, when such channels are enclosed by a micropipette within a cell-attached patch, they respond to flow after a delay of several minutes, thus implying that gating does not require direct exposure to shear stress (Jacobs *et al.,* 1995). In cultured endothelial cells K^+ channel activity and membrane potential may not always be coupled to NO release stimulated by shear stress or agonists (O'Neill, 1995; Gooch and Frangos, 1996).

Ca^{2+}-Independent Mechanisms

Shear stress may also enhance the catalytic activity of eNOS through a Ca^{2+}-independent mechanism that is distinct from the effects of agonists. During the initial phase of exposure of cultured endothelium to flow, NO production exhibits mixed Ca^{2+} dependence and Ca^{2+} independence, but over time eNOS activity becomes Ca^{2+}/calmodulin independent while nevertheless permitting continuous production of NO (Kuchan and Frangos, 1994; O'Neill, 1995). This Ca^{2+}-independent NO production requires tyrosine phosphorylation of eNOS or an associated regulatory protein, and it can therefore be attenuated by tyrosine kinase inhibitors and be mimicked by protein tyrosine phosphatase inhibitors, which induce sustained NO release in the absence of extracellular Ca^{2+} (García-Cardeña *et al.,* 1996; Fleming *et al.,* 1996, 1998). Following exposure to shear stress, eNOS is also rapidly phosphorylated on serine residues (Corson *et al.,* 1996; Fleming *et al.,* 1998). Although serine phosphorylation at specific positions, for example, by protein kinase C, may be inhibitory (Hirata *et al.,* 1995), more recent studies have shown that shear stress increases the catalytic activity of eNOS in a Ca^{2+}-independent fashion following serine phosphorylation by the serine/threonine kinase Akt (protein kinase B) (Fulton *et al.,* 1999; Dimmeler *et al.,* 1999). Activation of Akt is downstream of phosphatidylinositol 3-kinase (PI3K), as increases in NO production are abolished by the PI3K inhibitor wortmannin (Fulton *et al.,* 1999; Dimmeler *et al.,* 1999). Agonists and shear stress also cause eNOS to associate with the heat-shock protein Hsp 90 (Garciá-Cardeña *et al.,* 1998). This molecular chaperone regulates protein folding, and its recruitment to eNOS results in an increase in activity that may be effected by allosteric modulation or stabilization of the eNOS dimer in a Ca^{2+}/calmodulin-independent fashion.

How Are Shear Forces Sensed?

Fluid shear stress stimulates membrane mechanoreceptors that subsequently lead to the activation of a wide spectrum of intracellular signaling pathways (Fig. 3). In the context of eNOS, the physical structure and fluidity of the endothelial cell membrane are both important determinants of the transduction of shear forces, since cleavage of sialic acid residues from the endothelial cell glycocalyx with neuraminidase and ''stiffening'' of the endothelial cell membrane with cholesteryl hemisuccinate selectively impair flow—but not agonist-induced NO synthesis (Hecker *et al.,* 1993; Knudsen and Frangos, 1997). As discussed above, ion channels are also activated by shear stress, but none has convincingly emerged as the primary mechanosensor coupled to NO production. Indeed, a more likely scenario is that the modulation of transmembrane Ca^{2+} and K^+ fluxes by increases in flow is secondary to the activation of a highly complex network of signaling mechanisms.

G Proteins

Activation of endothelial PLC by agonists involves G proteins coupled to the NO synthetic pathway that include a

pertussis toxin (PTX)-sensitive G_i protein linked to serotonergic, α_2-adrenergic, and muscarinic receptors, and a PTX-insensitive G_q protein linked to bradykinin and ATP receptors (Boulanger and Vanhoutte, 1997). G proteins also participate in the earliest transduction events associated with flow, as immunoprecipitation techniques demonstrate activation of heterotrimeric G-protein subunits within 1 s of exposure to shear stress (Gudi *et al.,* 1996), and G_i and G_q subtypes have both been reported to contribute to the NO response to flow (Kuchan *et al.,* 1994). Comparison of step, ramp, and impulse changes in flow on NO production suggests the existence of a rapid G-protein-dependent response that is sensitive to the rate of change of shear stress and a G-protein-independent response to steady shear forces (Frangos *et al.,* 1996). The general G-protein inhibitor GDPβS thus blocks NO production during the first hour of exposure of cultured human umbilical vein endothelial cells (HUVECs) to flow, but significant attenuation is no longer apparent after 1 hour (Kuchan *et al.,* 1994). Membrane fluidity may be an important determinant of the protein receptor-independent GTPase activity of G proteins reconstituted into phospholipid vesicles and subjected to shear stress (Sivaramaprasad *et al.,* 1998). Liposomes containing lysophosphatidylcholine or treated with benzyl alcohol to increase bilayer fluidity exhibit substantial enhancement of basal GTPase activity, whereas incorporation of cholesterol has the opposite effect.

Caveolae

Cholesterol-rich invaginations of the endothelial cell membrane known as caveolae serve as specialized signaling domains whose particular structural properties confer sensitivity to membrane tension and allow the rapid transduction of mechanical forces into biochemical events (Fig. 3). Uniquely among the NOS isoforms, eNOS possesses a substrate site for acyl transferase at its amino terminus that permits sequential N-myristoylation and palmitoylation and subcellular localization within caveolae and in the region of the Golgi apparatus (Robinson and Michel, 1995; Liu *et al.,* 1996). Caveolin-1, the key structural coat protein of caveolae in endothelial cells, interacts with eNOS to form an inactive heteromeric complex that is quantitatively dissociated by Ca^{2+}/calmodulin, leading to NO production (Michel *et al.,* 1997; Ju *et al.,* 1997). Acute changes in flow cause rapid dissociation of eNOS from caveolin and binding to calmodulin, high levels of which are also found in caveolae (Rizzo *et al.,* 1998a).

Other signaling molecules involved in the response to shear forces—including G proteins, PLC, and tyrosine kinases—are also localized in caveolae (Shaul and Anderson, 1998). Shear stress rapidly activates members of the Src tyrosine kinase family (Takahashi and Berk, 1996), and a wide spectrum of caveolar proteins become tyrosine phosphorylated following acute increases in flow (Rizzo *et al.,* 1998b). The same proteins are also phosphorylated following stimulation by agonists (Liu *et al.,* 1997), suggesting the existence of common pathways for receptor- and shear-mediated events, although a greater fraction of the caveolae present in subcellular fractions respond to flow than agonists (Rizzo *et al.,* 1998b). More recent evidence suggests that colocalization of eNOS with an upstream activator of protein kinase B (Akt/PKB) in the cell membrane is obligatory for Ca^{2+}-independent synthesis of NO (Fulton *et al.,* 1999). Its upstream activator PI3K is found in caveolae (Liu *et al.,* 1997) and is regulated by caveolar signaling proteins that are sensitive to shear stress, including G proteins, Ras, and Src-family tyrosine kinases (Katada *et al.,* 1999; Shepherd *et al.,* 1998).

Cytoskeleton and Integrins

The cytoskeleton is supported by actin in its polymeric or filamentous (F-actin) form, which is organized into microfilament bundles that confer tension to the cell and a lattice of microtubules formed by polymerization of tubulin; which in conjunction with intermediate filaments, provides cell rigidity (Davies, 1995; Banes *et al.,* 1995). In rabbit aorta, endothelial NO release evoked by acute changes in shear stress is attenuated by cytochalasin B (an F-actin depolymerizing agent), phalloidin (an F-actin stabilizing agent), and colchicine (an inhibitor of tubulin dimerization), whereas none of these agents affects the response to ACh (Hutcheson and Griffith, 1996). Nocodazole, an agent that interferes with microtubule polymerization, similarly attenuates flow-dependent dilation in isolated skeletal muscle arterioles, without affecting the response to ACh (Sun *et al.,* 1995b), and in mice lacking vimentin, a major intermediate filament protein, NO-mediated flow-dependent dilation is impaired in mesenteric resistance arteries (Henrion *et al.,* 1997). Cytochalasins also prevent the rise in $[Ca^{2+}]_i$ evoked by direct mechanical perturbation of the endothelial membrane (Diamond *et al.,* 1994). By contrast, in cultured endothelial cells neither disruption nor stabilization of actin microfilaments affect the NO response to shear stress or bradykinin, and disruption of microtubules may actually enhance the response to shear (Knudsen and Frangos, 1997). Shear-induced elevations in $[Ca^{2+}]_i$ are unaffected by pharmacological dissolution or stabilization of the microtubule network in cultured cells (Malek *et al.,* 1999). It remains to be determined whether these apparently conflicting findings reflect differences in cellular architecture resulting from culture in the absence of flow, for endothelial cells then lack stress fibers formed from actin microfilaments and an organized lattice of microtubules and intermediate filments (Davies, 1995; Banes *et al.,* 1995).

The cytoskeleton interacts with other elements that are involved in the sensing of shear stress, perhaps allowing them to function as an integrated dynamic unit. Cytoskeletal components are linked to stretch-activated ion channels, G proteins, and $InsP_3$-sensitive stores (Banes *et al.,* 1995). They are also involved in the maintenance of caveolae, with caveolin normally undergoing microtubule-dependent cycling between the plasma membrane and the Golgi (Conrad

et al., 1995). Microtubular disruption thus causes accumulation of caveolin in the ER/Golgi intermediate compartment (Conrad *et al.,* 1995), whereas disruption of actin microfilaments causes caveolae to aggregate (Fujimoto *et al.,* 1995). Importantly, physical forces acting on the endothelial cell surface are transmitted via the cytoskeleton to integrins, which are heterodimeric transmembrane glycoproteins that bind to the extracellular matrix on the abluminal side of the cell (Davies, 1995). At such sites of endothelial anchorage there are focal adhesion complexes that contain tyrosine kinases which are rapidly activated by shear stress, including Src (Hamasaki *et al.,* 1996; Takahashi and Berk, 1996). In isolated coronary arterioles in which NO-mediated flow-dependent dilation involves tyrosine kinase-mediated protein phosphorylation, dilatations evoked by flow are attenuated by a blocking antibody targeted at the integrin β_3 chain and by a peptide containing an RGD sequence that specifically inhibits integrin binding to matrix proteins (Muller *et al.,* 1997). This peptide attenuates the endothelial response to flow but does not affect endothelium-dependent relaxations to agonists (Muller *et al.,* 1997). Focal adhesion complexes may therefore be involved in the activation of eNOS by shear forces, although the downstream transduction pathways are presently unknown.

NO-Independent Mechanisms of Endothelial Control

Prostanoids

In some vessels endothelium-derived prostanoids may also contribute to flow-dependent dilation (e.g., Koller and Kaley, 1990a; Friebel *et al.,* 1995), although this is by no means a universal finding (e.g., Holtz *et al.,* 1984; Kuo *et al.,* 1991). Synthesis of prostacyclin, like that of NO, is enhanced both by increases in laminar shear stress and by pulsatile flow through a G-protein-dependent pathway (Pohl *et al.,* 1986; Berthiaume and Frangos, 1992; Onohara *et al.,* 1991). In skeletal muscle arterioles of wild-type mice, NO and prostanoids contribute to flow-dependent dilation to an approximately equal extent, whereas in eNOS knockouts the magnitude of the phenomenon, though attributable entirely to prostanoids, is unchanged because this pathway is upregulated (Sun *et al.,* 1999). There is growing evidence for interactions between the two mediators. Generation of the superoxide anion ($O_2^{\cdot-}$) by cyclooxygenase may thus attenuate functional NO activity (Tesfamariam and Cohen, 1992), whereas the peroxynitrite ion, $ONOO^-$, formed by the combination of these radicals directly stimulates prostaglandin H synthase (Goodwin *et al.,* 1999). The latter mechanism could explain why prostacyclin synthesis by cultured endothelial cells exposed to shear stresses of 25 dynes/cm^2 is attenuated by ~50% following inhibition of eNOS (Wang and Diamond, 1997). In some models, however, $ONOO^-$ inhibits prostacyclin synthase by causing tyrosine nitration, leaving unmetabolized prostaglandin H_2 to promote vasoconstriction (Zou and Bachschmid, 1999).

EDHF and Gap Junctional Communication

An endothelium-derived hyperpolarizing factor (EDHF) is widely recognized as mediating endothelium-dependent vascular relaxations that are independent of NO and prostanoid synthesis (see Mombouli and Vanhoutte, 1997 for review). There is now compelling evidence that this agent preferentially diffuses from the endothelium to smooth muscle via myoendothelial gap junctions (Chaytor *et al.,* 1998). Chemical signals may also transfer via gap junctions in the opposite direction from smooth muscle to endothelium, as arteriolar constriction by agonists or high extracellular $[K^+]$ may cause secondary elevations in endothelial $[Ca^{2+}]_i$ and thereby stimulate NO production (Dora *et al.,* 1997). Gap junctions consist of two hemichannels contributed by opposing cells, each formed by six connexin protein subunits arranged around a central pore that permits electrical continuity and intercellular transfer of molecules <1 kDa in size. EDHF-type relaxations and hyperpolarizations are markedly attenuated by inhibitory synthetic peptides that possess sequence homology with regions of the extracellular loops of connexins found in the vascular wall (Chaytor *et al.,* 1998; Hutcheson *et al.,* 1999; Dora *et al.,* 1999), and also by 18α- and 18β-glycyrrhetinic acids, which are lipophilic aglycones that disrupt gap junctional communication (Taylor *et al.,* 1998; Yamamoto *et al.,* 1999). EDHF-type responses are initiated by mobilization of arachidonic acid by a Ca^{2+}-dependent phospholipase A_2, but the mechanisms involved distal to this step have not been resolved (Hutcheson *et al.,* 1999). Mediators proposed to be EDHFs include arachidonic acid metabolites such as the endocannabinoid anandamide and epoxyeicosatrienoic acids (EETs) (see Randall and Kendall, 1998 and Campbell and Harder, 1999, for reviews). However, anandamide and EETs can stimulate endothelium-dependent relaxations that involve myoendothelial gap junctions and/or NO, thus questioning their equivalence with EDHF (Hutcheson *et al.,* 1999; Tan *et al.,* 1997; Chaytor *et al.,* 1999).

Transfer of EDHF via gap junctions may explain the difficulty experienced in detecting downstream relaxations mediated by EDHF in cascade bioassay (Mombouli and Vanhoutte, 1997), though under some conditions there may be a small ''overspill'' of EDHF from intact vessels. The Ca^{2+} ionophore A23187 thus promotes release of EDHF directly into the extracellular space (Hutcheson *et al.,* 1999). In cascade bioassay it is also possible to detect transient hyperpolarization of vascular myocytes under conditions of combined eNOS and cyclooxygenase blockade (Popp *et al.,* 1996). Increases in transmural pressure in the donor vessel enhance the duration but not the amplitude of this downstream hyperpolarizing response, suggesting that mechanical forces modulate EDHF release (Popp *et al.,* 1996). It nevertheless remains to be established whether shear forces activate EDHF/gap junction-dependent mechanisms, a possibility suggested by NO- and prostanoid-independent flow-dependent dilation of cat femoral and rat basilar arteries (Fujii *et al.,* 1991; Melkumyants *et al.,* 1992). Although

endothelium-dependent agonists and shear stress induce endothelial hyperpolarization directly (Olesen *et al.,* 1988; Mehrke and Daut, 1990), there is as yet little evidence that electrotonic transmission of a hyperpolarizing electrical current from the endothelium to smooth muscle contributes significantly to EDHF-mediated responses, perhaps reflecting the relatively small mass of the endothelium relative to the medium (Beny, 1999).

EDHF-type relaxations are particularly prominent in small arteries, including those obtained from the human myocardium, as might be expected when the average diffusion distance for a chemical mediator within the media is small and the endothelial:smooth muscle cell ratio is high (Berman and Griffith, 1998; Miura *et al.,* 1999). Gap junctional communication may accordingly be particularly important in the regulation of microcirculatory flow. In the rabbit central ear artery, experiments with connexinmimetic peptides have shown that NO-dependent relaxations dominate the response to ACh, whereas the gap junctional component dominates in vessels <100 μm in diameter (Berman *et al.,* 2000). This finding is reinforced by observations that there is a strong negative correlation between the absolute magnitudes of NO- and EDHF-dependent relaxations in rings of superior mesenteric artery from different rabbits (Hutcheson *et al.,* 1999), and that long-term blockade of endothelial NO synthesis potentiates relaxations to EDHF (Kessler *et al.,* 1999). Conversely, EDHF activity is downregulated in sepsis as a direct consequence of the overproduction of NO (Kessler *et al.,* 1999). The nature of the inhibitory action of NO against the EDHF pathway is unknown. It also remains to be determined whether EDHF can function as a ''back-up'' mechanism that compensates for loss of NO activity, a possibility suggested by impaired NO- and prostanoid-relaxations but preservation of EDHF-type responses in arteries from human subjects subjected to radiation therapy (Sugihara *et al.,* 1999).

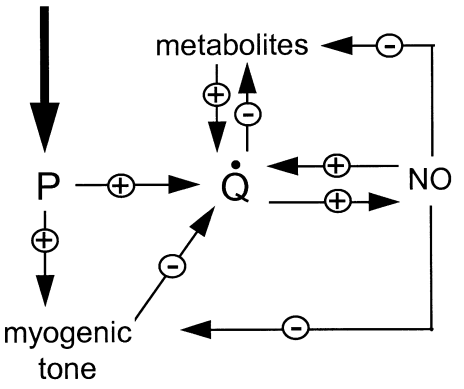


Figure 4 Schematic illustrating the complex network of interactions linking changes in microvascular pressure, flow, and metabolite release with NO production. NO may directly modulate parenchymal metabolism in addition to exerting effects which are secondary to changes in flow.

Integrated Control of Organ Perfusion

Autoregulation

In many organs, flow is regulated so tightly that it remains virtually constant when the supply pressure varies over the range of ~50 to ~150 mm Hg. This phenomenon is known as autoregulation and depends on negative feedback interactions between myogenic and metabolic control mechanisms. Increases in supply pressure will initially increase flow, but this will rapidly be restored toward baseline following myogenic constriction caused by the increase in pressure itself, and by secondary constriction due to washout of vasodilator metabolites (Fig. 4). In general, loss of NO activity enhances autoregulation in beds where the ability to control flow is relatively weak. For example, in the isolated rabbit ear—a cutaneous circulation that normally does not autoregulate—the capacity for autoregulation becomes demonstrable following inhibition of eNOS or scavenging of NO with hemoglobin (Griffith and Edwards, 1990a). eNOS inhibition similarly extends the weak autoregulatory range of coronary vessels in isolated guinea pig and rabbit hearts (Ueeda *et al.,* 1992; Pohl *et al.,* 1994). In such beds diminished NO activity unmasks or enhances myogenic constriction, thereby attenuating pressure-induced increases in flow. Indeed, metabolic activity in the rabbit ear is so low that the contribution of metabolites can be discounted: myogenic constriction alone appears sufficient to mediate autoregulation (Griffith and Edwards, 1990a). Enhanced myogenic constriction following loss of NO activity may override metabolic factors, as in the isolated rabbit heart autoregulation occurs in the face of increased lactate release and O_2 extraction (Pohl *et al.,* 1994). Although control of local resistance by washout of the dilator metabolite adenosine is thought to contribute to the ability of the mesenteric circulation to autoregulate flow, eNOS inhibition still enhances the normally weak autoregulation found in this bed (Macedo and Lautt, 1996).

NO plays a less prominent role in the coronary, cerebral, and renal circulations *in vivo,* where there is normally a high intrinsic autoregulatory gain. The dominant effects of eNOS inhibition are then an increase in vascular resistance and an associated reduction in baseline flow such that autoregulation is ''reset'' with the flow plateau of the pressure–flow relationship depressed by up to 30% (Fig. 5). Although there may be no change in the pressure at which autoregulation first becomes evident or in its physiological efficacy (Wang *et al.,* 1992; Beierwaltes *et al.,* 1992; Baumann *et al.,* 1992; Thompson *et al.,* 1996), there may be a 10–20 mm Hg increase in the pressure that defines the lower autoregulatory break point, for example, in canine kidney and myocardium (Smith and Canty, 1993; Majid and Navar, 1992) and in rat brain (Jones *et al.,* 1999). Some reports also suggest that the upper end of the autoregulated range may be extended to higher pressures (Wang *et al.,* 1992; Talman and Dragon, 1995). This rightward shift in the position of the autoregulatory range along the pressure axis may simply reflect attenuated myogenic dilation at low perfusion pressures and

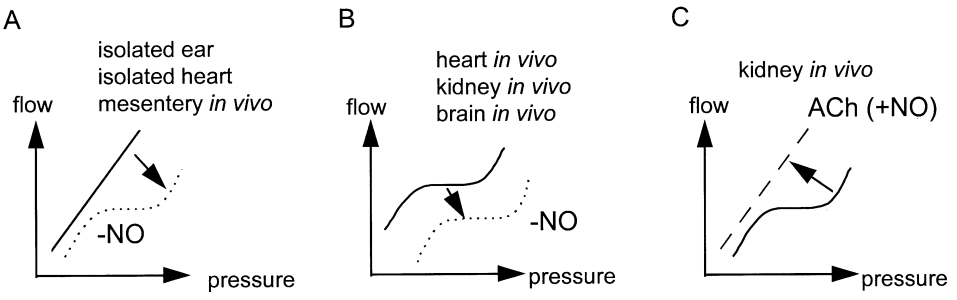


Figure 5 The effects of eNOS inhibition on autoregulation depend on the balance between myogenic and metabolic mechanisms. (A) In beds where autoregulatory gain is weak, autoregulation is enhanced following loss of NO activity. (B) When there is intrinsically strong autoregulation the plateau in the pressure–flow curve may simply be depressed and shifted to the right. (C) In such beds endothelium-dependent agonists may suppress the ability to autoregulate.

attenuated passive distension at high pressures as a result of the exaggerated constrictor tone that follows loss of NO activity. In beds with high autoregulatory gain such as the kidney, increased endothelium-dependent vasodilation by agonists such as ACh abolishes autoregulation, so that pressure–flow relationships exhibit purely passive characteristics (Gross *et al.,* 1976).

A general picture emerges in which apparent differences in the effects of eNOS inhibition may be explained by differences in the contributions of myogenic and metabolic control relative to the ambient level of NO production (Fig. 5). Interactions between these mechanisms could also underlie regional variations in autoregulation within the same organ. In the rat, for example, eNOS inhibition abolishes autoregulation in cerebral and cerebellar cortices, but not in all deep brain structures (Wang *et al.,* 1992; Tanaka *et al.,* 1993). NO synthesis by neurons and other cell types could contribute to autoregulation in the cerebral circulation in addition to endothelial production (Jones *et al.,* 1999).

Hyperemias

Reactive hyperemia is the term used to denote the dilation that follows transient vascular occlusion. It is particularly pronounced in organs with high metabolic demands. Experimentally, it is usually characterized by three parameters: the peak flow achieved after the release of the occlusion, the duration of the increase in flow, and the flow excess, that is, the product of basal flow and occlusion time subtracted from the total hyperemic flow. Flow-dependent dilation of proximal conduit arteries becomes evident in canine epicardial coronary arteries and the human forearm on restoration of flow, and it may be observed proximal to the occlusion (Hintze and Vatner, 1984; Hayashi *et al.,* 1988; Celermajer *et al.,* 1994). There is an emerging consensus that eNOS inhibition or endothelial impairment reduces the duration of the hyperemic response, although the peak flow and flow excess may not always be affected (Yamabe *et al.,* 1992; Altman *et al.,* 1994; O'Leary *et al.,* 1994; Engelke *et al.,* 1996; Puybasset *et al.,* 1996; Dakak *et al.,* 1998). This is perhaps to be expected, as reactive flow-dependent dilation is maximal only after 1 to 2 min, whereas peak hyperemic flow generally occurs within 15 s (Hintze and Vatner, 1984). An endothelium-independent myogenic dilation that is evoked by the mechanical unloading that occurs during occlusion may also contribute to the peak flow (Koller and Kaley, 1990b).

The overall duration of the hyperemic response increases greatly as occlusion times are extended from periods of seconds to minutes as metabolites accumulate in direct proportion to the length of the occlusion. Flow repayment after coronary occlusion in the dog involves both NO and adenosine, with each mediator separately accounting for up to 30% of the resulting reactive hyperemia (Yamabe *et al.,* 1992). In the isolated guinea pig heart the decrease in flow caused by inhibition of eNOS may actually enhance adenosine formation, which partially compensates for loss of NO activity (Kostic and Schrader, 1992). In some species endothelial prostanoids also contribute to reactive hyperemia in skeletal muscle (Koller and Kaley 1990b), and in the human forearm total excess flow is much lower after combined eNOS and cyclooxygenase inhibition than after eNOS inhibition alone (Engelke *et al.,* 1996). Like adenosine, prostanoids may act in a compensatory fashion. In conscious dogs they normally do not contribute to reactive hyperemia, but prolonged inhibition of eNOS leads to upregulation of prostanoid-dependent dilator mechanisms, so that cyclooxygenase inhibitors then significantly reduce the flow excess after coronary occlusion (Puybasset *et al.,* 1996).

In general, organ blood flow increases in direct proportion to tissue activity, a response that is known as functional or active hyperemia and is dominated by the dilator effects of metabolites on distal arterioles. In some beds NO activity can enhance this hyperemic flow by up to 50%, for example, during cardiac pacing in canine and human myocardium (Jones *et al.,* 1995; Quyyumi *et al.,* 1995), during motor nerve or direct field stimulation in skeletal muscle (Hussain *et al.,* 1992; Hester *et al.,* 1993), and during exercise in rat

hind limb (Hirai *et al.,* 1994). Indeed, in humans, NO is almost entirely responsible for the epicardial coronary dilation observed during pacing (Quyyumi *et al.,* 1995). Intravital microscopy has demonstrated that there may be substantial longitudinal gradients within the same vascular bed in the relative contribution of NO and of metabolic factors. In hamster cremaster muscle, eNOS inhibitors constrict larger arterioles and prevent their dilation during functional hyperemia, whereas the responses of the smallest arterioles are relatively unaffected (Hester *et al.,* 1993). In contrast, in canine myocardium small feed arteries constrict following administration of eNOS inhibitors, whereas distal arterioles may dilate secondary to autoregulatory mechanisms, leading to a reduction in metabolic reserve during pacing (Jones *et al.,* 1995). Perfusion studies with microspheres have shown that the attenuation of functional hyperemia by eNOS inhibitors is most pronounced in muscles with high oxidative capacity during exercise, suggesting that flow-dependent coordination of upstream and downstream responses through NO-dependent mechanisms is particularly important in beds with high perfusion requirements (Hirai *et al.,* 1994). In other beds, enhanced metabolic dilatation of small arterioles compensates for impaired flow-dependent upstream dilation: many studies have failed to demonstrate a role for NO in overall functional hyperemia during exercise, for example, in the human forearm (Endo *et al.,* 1994; Wilson and Kapoor, 1993) and in canine skeletal muscle (O'Leary *et al.,* 1994; Shen *et al.,* 1994) and myocardium (Altman *et al.,* 1994).

Direct Metabolic Effects of NO

NO released from the microvascular endothelium may depress mitochondrial respiration in adjacent parenchymal cells, thus explaining the respective increases and decreases in skeletal and cardiac muscle O_2 consumption that follow administration of eNOS inhibitors or endothelium-dependent agonists (Shen *et al.,* 1995; Xie *et al.,* 1996). This action of NO may involve direct inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, which functions as an NO-controlled O_2 sensor regulating the rate of O_2 consumption according to the ambient O_2 concentration (Clementi *et al.,* 1999). In endothelial cells the control of O_2 uptake by NO requires influx of extracellular Ca^{2+}, but it is unknown if the Ca^{2+}-dependent NOS enzyme involved is eNOS or a mitochondrial isoform located in the inner mitochondrial membrane (Giulivi, 1998; Clementi *et al.,* 1999). The effects of NO on mitochondrial respiration have not been investigated in the context of autoregulation and the hyperemias, although flow-induced increases in NO production might be expected to decrease the formation of dilator metabolites by depressing metabolism, thereby enhancing autoregulation through negative feedback and attenuating hyperemic responses (Fig. 4). The relative lack of effect of eNOS inhibition on hyperemic responses reported in some vascular beds could also reflect a compensatory increase in metabolite formation following an increase in mitochondrial respiration.

Long-Term Regulation and Vascular Disease

eNOS Expression

Expression of the constitutive endothelial NO synthase (eNOS) increases within hours of exposure of endothelial cells to fluid shear stress, thus explaining the enhancement of NO-dependent responses to flow and agonists by exercise training in dogs and rats (Sessa *et al.,* 1994; Uematsu *et al.,* 1995). Upregulation may extend to all beds in which exercise affects flow, as increases in eNOS expression are found in epicardial arteries and coronary/skeletal muscle arterioles, but not in mesenteric arterioles which do not experience increased flow during exercise (Sessa *et al.,* 1994; Sun *et al.,* 1998). Analogously, in high output states induced by creation of an aorto-caval fistula, NO activity is enhanced only proximal to the fistula (Arnal *et al.,* 1993). Cyclic strain at levels comparable to those generated by the pulsatility of blood flow *in vivo* also upregulates eNOS in the absence of flow, so that fluid shear stress may not be the only mechanical force regulating eNOS expression (Awolesi *et al.,* 1995). Long-term changes in ambient flow also modulate the transduction pathways that couple shear forces to the activation of eNOS, as exercise enhances agonist-induced responses by increasing either the number or affinity of the endothelial receptors (Cheng *et al.,* 1999), and G-protein expression and GTPase activity are enhanced in cultured endothelial cells exposed to high levels of shear stress (Redmond *et al.,* 1998).

The mechanisms involved in the upregulation of eNOS by shear stress have some features in common with the acute NO response to flow. Flow-enhanced expression of eNOS mRNA involves a PTX-sensitive G-protein-coupled pathway (Malek *et al.,* 1999), is attenuated by K^+ channel inhibition (Uematsu *et al.,* 1995), and is Ca^{2+} dependent with the requirement for Ca^{2+} being specific for influx triggered by store depletion (Xiao *et al.,* 1997; Malek *et al.,* 1999). In other respects, however, differences are apparent. eNOS contains a shear stress responsive element (SSRE) that is present in other endothelial genes such as platelet-derived growth factor β chain, in which it is thought to regulate expression by mechanical forces (Marsden *et al.,* 1993). Upregulation of eNOS, however, does not appear to depend on PLC, calmodulin, and tyrosine kinase-dependent mechanisms that affect shear-induced NO production and transcription of such SSRE-containing genes (Uematsu *et al.,* 1995; Xiao *et al.,* 1997; Malek *et al.,* 1999). There is evidence that protein kinase C (PKC) may enhance transcription of eNOS under specific experimental conditions (Li *et al.,* 1998), but this mechanism does not appear to operate under increased shear stress (Ranjan *et al.,* 1995; Uematsu *et al.,* 1995). Nor is the integrity of the endothelial

microtubular network essential for the upregulation of eNOS in response to flow, at least in cultured cells (Malek *et al.,* 1999).

Remodeling

The alterations in vascular wall structure and composition induced by long-term changes in blood flow that lead to normalization of shear stress are multifactorial in etiology, as the endothelium can regulate cell proliferation and extracellular matrix production through both NO-dependent and -independent mechanisms (Garg and Hassid, 1989; De Mey *et al.,* 1991; Tronc *et al.,* 1996). Creation of an arteriovenous fistula in the rabbit carotid circulation leads to increases in carotid artery diameter and remodeling of the media that normalizes wall shear stress, and these adaptive changes are partially attenuated by eNOS inhibition (Tronc *et al.,* 1996). In marked contrast to wild-type mice, remodeling of the carotid artery is prevented in knockout mice with targeted disruption of eNOS (Rudic *et al.,* 1998). eNOS mutants instead display a paradoxical hyperplastic increase in arterial wall thickness, suggesting that NO activity prevents pathological changes in vessel wall morphology (Rudic *et al.,* 1998). Analogously, long-term pharmacological inhibition of eNOS leads to medial thickening and perivascular fibrosis in the rat microvasculature even when the associated hypertension is offset by long-term hypotensive therapy (Numaguchi *et al.,* 1995).

Shear Stress, Superoxide Anions, and Atherosclerosis

Local hemodynamic factors predispose to the development of atheroma on the inner wall of curved arterial segments where time-averaged shear stress is low, and at arterial bifurcations where flow separation and reattachment may lead to oscillatory fluctuations in shear stress (Caro *et al.,* 1971). Observations that high levels of flow prevent or reverse smooth muscle proliferation and neointimal thickening (Kohler *et al.,* 1991; Mattsson *et al.,* 1997), that endothelial dysfunction occurs first at bifurcations (McLenachan *et al.,* 1990), and that NO modulates mass transport processes in the arterial wall (Forster and Weinberg, 1997) suggest that local variations in NO activity could contribute to the focal nature of atherosclerosis. Flow also stimulates production of the $\cdot O_2^-$ anion by the endothelium (Laurindo *et al.,* 1994), and there is growing evidence that its reaction with NO is of major importance in the genesis of vascular dysfunction. Superoxide thus attenuates NO activity relative to NO production, and their reaction product $ONOO^-$ is a potent oxidant whose toxic effects may contribute to vascular pathology in hypercholesterolemia, hypertension, and diabetes (for review, see Kojda and Harrison, 1999). Short-term elevations in transmural pressure, for example, stimulate $\cdot O_2^-$ production in isolated rat arterioles, thereby acutely impairing flow-dependent and agonist-induced dilations (Huang *et al.,* 1998a); on the other hand, sustained hypertension can increase both $\cdot O_2^-$ production and eNOS expression, and endothelial dysfunction may then be associated with $ONOO^-$-mediated protein nitration (Bouloumie *et al.,* 1997). Importantly, endothelial dysfunction can be reversed by cell-permeant forms of superoxide dismutase (SOD) and antioxidants in a variety of disease states (Kojda and Harrison, 1999).

Different temporal patterns of shear stress may cause specific effects on NO production and endothelial redox state. Steady and periodic laminar shear forces increase the expression of both eNOS and SOD, which can be considered as an antioxidant defense enzyme that adapts to changes in oxidative stress, whereas turbulent flow characterized by high-frequency fluctuations in amplitude and direction is without effect (Noris *et al.,* 1995; Topper *et al.,* 1996). In cells exposed to oscillatory shear forces with zero mean stress, there is a sustained increase in the expression of NADH oxidase, the major source of endothelial $\cdot O_2^-$ production, whereas SOD levels remain unchanged (De Keulenaer *et al.,* 1998). In contrast, laminar shear stress induces transient increases in NADH oxidase activity, but increases in SOD expression are sustained. Intracellular levels of $\cdot O_2^-$ are consequently lower in endothelial cells exposed to steady laminar shear stress than to purely oscillatory shear forces, a finding which may further help to explain the development of atheroma at specific sites (De Keulenaer *et al.,* 1998). Additional links between hemodynamic forces and endothelial production of NO and $\cdot O_2^-$ are suggested by the demonstration that reactive oxygen species generated in response to shear stress regulate protein tyrosine phophorylation (Yeh *et al.,* 1999) and may also modulate the association of eNOS and caveolin within caveolae (Peterson *et al.,* 1999).

It remains to be determined whether gender-related differences in the incidence of cardiovascular disease involve differences in NO and $\cdot O_2^-$ production. NO-mediated flow-dependent dilation is greater in arterioles from female than male rats—a difference that is lost following oophorectomy but restored by estrogen replacement (Huang *et al.,* 1998b). Similar sex-related differences in endothelial function are found clinically (Pinto *et al.,* 1997). Although multiple mechanisms may underlie the vascular effects of estrogens, estrogens have been reported to decrease endothelial $\cdot O_2^-$ and $ONOO^-$ production, without change in eNOS expression (Barbacanne *et al.,* 1999).

Conclusions

It is now well established that endothelial NO synthesis plays a pivotal role in the control of blood flow, thereby helping to optimize the distribution of microcirculatory flow and to maintain the hydraulic efficiency of the circulation. Hemodynamic forces activate a highly complex array of signaling pathways within the endothelial cell, and the mechanisms through which these regulate NO production and eNOS expression in response to pulsatile blood flow are incompletely understood. Much is also yet to be learned about the interaction of NO with other endothelium-derived mediators, including the superoxide anion, prostanoids, and

EDHF, whose formation may be influenced by mechanical forces. NO participates in long-term remodeling of the vasculature following chronic changes in flow and is now thought to exert an important ''anti-inflammatory'' action against the development of atherogenesis. However, the extent to which impaired endothelial function contributes directly to the development of disease or is a secondary phenomenon remains to be fully delineated. Although significant advances have been made, further insights are likely to lead to a more precise understanding of acute and chronic vascular responses to local hemodynamic factors, better clinical diagnostic characterization of vascular states, and ultimately, it is hoped, more effective prevention and treatment of vascular disease.

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References

Ahlner, J., Ljusegren, M. E., Grundstrom, N., and Axelsson, K. L. (1991). Role of nitric oxide and cyclic GMP as mediators of endothelium-independent neurogenic relaxation in bovine mesenteric artery. *Circ. Res.* **68,** 756–762.

Altman, J. D., Kinn, J., Duncker, D. J., and Bache, R. J. (1994). Effect of inhibition of nitric oxide formation on coronary blood flow during exercise in the dog. *Cardiovasc. Res.* **28,** 119–124.

Ando, J., Ohtsuka, A., Korenaga, R., Kawamura, T., and Kamiya, A. (1993). Wall shear stress rather than shear rate regulates cytoplasmic Ca^{++} responses to flow in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **190,** 716–723.

Arnal, J.-F., Schott, C., and Stoclet, J.-C. (1993). Vascular relaxation and cyclic guanosine monophosphate in a rat model of high output heart failure. *Cardiovasc. Res.* **27,** 1651–1656.

Awolesi, M. A., Sessa, W. C., and Sumpio, B. E. (1995). Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. *J. Clin. Invest.* **96,** 1449–1454.

Banes, A. J., Tsuzaki, M., Yamamoto, J., Fischer, T., Brigman, B., Brown, T., and Miller, L. (1995). Mechanoreception at the cellular level: The detection, interpretation, and diversity of responses to mechanical signals. *Biochem. Cell Biol.* **73,** 349–365.

Barbacanne, M. A., Rami, J., Michel, J. B., Souchard, J. P., Philippe, M., Besombes, J. P., Bayard, F., and Arnal, J. F. (1999). Estradiol increases rat aorta endothelium-derived relaxing factor (EDRF) activity without changes in endothelial NO synthase gene expression: Possible role of decreased endothelium-derived superoxide anion production. *Cardiovasc. Res.* **41,** 672–681.

Baumann, J. E., Persson, P. B., Ehmke, H., Nafz, B., and Kirchheim, H. R. (1992). Role of endothelium-derived relaxing factor in renal autoregulation in conscious dogs. *Am. J. Physiol.* **263,** F208–F213.

Beierwaltes, W. H., Sigmon, D. H., and Carretero, O. A. (1992). Endothelium modulates renal blood flow but not autoregulation. *Am. J. Physiol.* **262,** F943–949.

Beny, J.-L. (1999). Information networks in the arterial wall. *NIPS* **14,** 68–73.

Berman, R. S., and Griffith, T. M. (1998). Spatial heterogeneity in the mechanisms contributing to acetylcholine-induced dilatation in the rabbit isolated ear. *Br. J. Pharmacol.* **124,** 1245–1253.

Berman, R. S., Martin, P. E. M., Evans, W. H., and Griffith, T. M. (2000). EDHF-type relaxations are abolished by peptides homologous to extracellular loops 1 and 2 of connexin 43 in rabbit resistance arteries of different sizes. *J. Vasc. Res.* (submitted).

Berthiaume, F., and Frangos, J. A. (1992). Flow-induced prostacyclin production is mediated by a pertussis toxin-sensitive G protein. *FEBS Lett.* **308,** 277–279.

Boegehold, M. A. (1996). Shear-dependent release of venular nitric oxide: Effect on arteriolar tone in rat striated muscle. *Am. J. Physiol.* **271,** H387–H395.

Boulanger, C. M., and Vanhoutte, P. M. (1997). G proteins and endothelium-dependent relaxations. *J. Vasc. Res.* **34,** 175–185.

Bouloumie, A., Bauersachs, J., Linz, W., Scholkens, B. A., Wiemer, G., Fleming, I., and Busse, R. (1997). Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production. *Hypertension* **30,** 934–941.

Brophy, C. M., Mills, I., Rosales, O., Isales, C., and Sumpio, B. E. (1993). Phospholipase C: A putative mechanotransducer for endothelial cell response to acute hemodynamic changes. *Biochem. Biophys. Res. Commun.* **190,** 576–581.

Broten, T. P., Miyashiro, J. K., Moncada, S., and Feigl, E. O. (1992). Role of endothelium-derived relaxing factor in parasympathetic coronary vasodilation. *Am. J. Physiol.* **264,** H1579–H1584.

Campbell, W. B., and Harder, D. R. (1999). Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circ. Res.* **84,** 484–488.

Canty, J. M., and Schwartz, J. S. (1994). Nitric oxide mediates flow-dependent epicardial coronary vasodilation to changes in pulse frequency but not mean flow in conscious dogs. *Circulation* **89,** 375–384.

Caro, C. G., Fitz-Gerald, J. M., and Schroter, R. C. (1971). Atheroma and arterial wall shear. Observation, correlation and proposal of a shear dependent mass transfer mechanism for atherogenesis. *Proc. R. Soc. London B. Biol. Sci* **177,** 109–159.

Celermajer, D. S., Sorensen, K. E., Bull, C., Robinson, J., and Deanfield, J. E. (1994). Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J. Am. Coll. Cardiol.* **24,** 1468–1474.

Chaytor, A. T., Evans, W. H., and Griffith, T. M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.* **508,** 561–573.

Chaytor, A. T., Martin, P. E. M., Evans, W. H., Randall, M. D., and Griffith, T. M. (1999). The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. *J. Physiol.* **520,** 539–550.

Cheng, L.-J., Yang, C.-C., Hsu, L.-Y., Lin, M. T., Jen, C. J., and Chen, H.-I. (1999). Acute exercise enhances receptor-mediated endothelium-dependent vasodilation by receptor upregulation. *J. Biomed. Sci.* **6,** 22–27.

Clementi, E., Brown, C. G., Foxwell, N., and Moncada, S. (1999). On the mechanism by which vascular endotelial cells regulate their oxygen consumption. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 1559–1562.

Conrad, P. A., Smart, E. J., Ying, Y. S., Anderson, R. G., and Bloom, G. S. (1995). Caveolin cycles between plasma membrane caveolae and the Golgi complex by microtubule-dependent and microtubule-independent steps. *J. Cell Biol.* **131,** 1421–1433.

Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C., and Harrison, D. G. (1996). Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ. Res.* **79,** 984–991.

Dakak, N., Husain, S., Mulcahy, D., Andrews, N. P., Panza, J. A., Waclawiw, M., Schenke, W., and Quyyumi, A. A. (1998). Contribution of nitric oxide to reactive hyperemia impact of endothelial dysfunction. *Hypertension* **32,** 9–15.

Davies, P. F. (1995). Flow-mediated endothelial mechanotransduction. *Physiol. Rev.* **75,** 519–560.

Davis, M. J., and Hill, M. A. (1999). Signalling mechanisms underlying the vascular myogenic response. *Physiol. Rev.* **79,** 387–423.

Davis, M. J., and Sharma, N. R. (1997). Calcium-release-activated calcium influx in endothelium. *J. Vasc. Res.* **34,** 186–195.

De Keulenaer, G. W., Chappell, D. C., Ishizaka, N., Nerem, R. M., Alexander, R. W., and Griendling, K. K. (1998). Oscillatory and steady laminar shear stress differentially affect human endothelial redox state. *Circ. Res.* **82,** 1094–1101.

De Mey, J. G. R., Dijkstra, E. H., and Vrijdag, M. J. J. F. (1991). Endothelium reduces DNA synthesis in isolated arteries. *Am. J. Physiol.* **260,** H1128–H1134.

de Wit, C., Schafer, C., von Bismarck, P., Bolz, S. S., and Pohl, U. (1997). Elevation of plasma viscosity induces sustained NO-mediated dilation in the hamster cremaster microcirculation in vivo. *Pfluegers Arch.* **434,** 354–361.

Diamond, S. L., Sachs, F., and Sigurdson, W. J. (1994). Mechanically induced calcium mobilization in cultured endothelial cells is dependent on actin and phospholipase. *Arterioscler. Thromb.* **14,** 2000–2006.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399,** 601–605.

Dora, K. A., Doyle, M. P., and Duling, B. R. (1997). Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 6529–6534.

Dora, K. A., Martin, P. E. M., Chaytor, A. T., Evans, W. H., and Griffith, T. M. (1999). Role of heterocellular gap junctional communication in endothelium-dependent smooth muscle hyperpolarization: Inhibition by a connexin-mimetic peptide. *Biochem. Biophys. Res. Commun.* **254,** 27–31.

Dull, R. O., Tarbell, J. M., and Davies, P. F. (1992). Mechanisms of flow-mediated signal transduction in endothelial cells: Kinetics of ATP surface concentrations. *J. Vasc. Res.* **29,** 410–419.

Edwards, A. V., and Garrett, J. R. (1993). Nitric oxide-related vasodilator responses to parasympathetic stimulation of the submandibular gland in the cat. *J. Physiol.* **464,** 379–392.

Endo, T., Imaizumi, T., Tagawa, T., Shiramoto, M., Ando, S.-I., and Takeshita, A. (1994). Role of nitric oxide in exercise-induced vasodilation of the forearm. *Circulation* **90,** 2886–2890.

Engelke, K. A., Halliwill, J. R., Proctor, D. N., Dietz, N. M., and Joyner, M. J. (1996). Contribution of nitric oxide and prostaglandins to reactive hyperemia in the human forearm. *J. Appl. Physiol.* **81,** 1807–1814.

Falcone, J. C., Kuo, L., and Meininger, G. A. (1993). Endothelial cell calcium increases during flow-induced dilation in isolated arterioles. *Am. J. Physiol.* **264,** H653–H659.

Fleming, I., Fisslthaler, B., and Busse, R. (1996). Interdependence of calcium signaling and protein tyrosine phosphorylation in human endothelial cells. *J. Biol. Chem.* **271,** 11009–11015.

Fleming, I., Bauersachs, J., Fisslthaler, B., and Busse, R. (1998). Ca^{2+}-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ. Res.* **82,** 686–695.

Forster, B. A., and Weinberg, P. D. (1997). Changes with age in the influence of endogenous nitric oxide on transport properties of the rabbit aortic wall near branches. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1361–1368.

Frame, M. D. S., and Sarelius, I. H. (1995). L-Arginine-induced conducted signals alter upstream arteriolar responsibility to L-arginine. *Circ. Res.* **77,** 695–701.

Frangos, J. A., Huang, T. Y., and Clark, C. B. (1996). Steady shear and step changes in shear stimulate endothelium via independent mechanisms—superposition of transient and sustained nitric oxide production. *Biochem. Biophys. Res. Commun.* **224,** 660–665.

Friebel, M., Klotz, K. F., Ley, K., Gaehtgens, P., and Pries, A. R. (1995). Flow-dependent regulation of arteriolar diameter in rat skeletal muscle in situ: Role of endothelium-derived relaxing factor and prostanoids. *J. Physiol.* **483,** 715–726.

Fujii, K., Heistad, D. D., and Faraci, F. M. (1991). Flow-mediated dilatation of the basilar artery *in vivo. Circ. Res.* **69,** 697–705.

Fujimoto, T., Miyawaki, A., and Mikoshiba, K. (1995). Inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae is linked to actin filaments. *J. Cell Sci.* **108,** 7–15.

Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399,** 597–601.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetycholine. *Nature* **288,** 373–376.

García-Cardeña, G., Fan, R., Stern, D. F., Liu, J., and Sessa, W. C. (1996). Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.* **271,** 27237–27240.

García-Cardeña, G., Fan, R., Shah, V., Sorrention, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392,** 821–824.

Garg, U. C., and Hassid, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83,** 1774–1777.

Geiger, R. V., Berk, B. C., Alexander, R. W., and Nerem, R. M. (1992). Flow-induced calcium transients in single endothelial cells: Spatial and temporal analysis. *Am. J. Physiol.* **262,** C1411–C1417.

Giulivi, C. (1998). Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. *Biochem. J.* **332,** 673–679.

Glenny, R. W., Polissar, N. L., McKinney, S., and Robertson, H. T. (1995). Temporal heterogeneity of regional pulmonary perfusion is spatially clustered. *J. Appl. Physiol.* **79,** 986–1001.

Gooch, K. J., and Frangos, J. A. (1996). Flow- and bradykinin-induced nitric oxide production by endothelial cells is independent of membrane potential. *Am. J. Physiol.* **270,** C546–C551.

Goodwin, D. C., Landino, L. M., and Marnett, L. J. (1999). Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. *FASEB J.* **13,** 1121–1136.

Graier, W. F., Simecek, S., and Sturek, M. (1995). Cytochrome P450 monooxygenase-regulated signalling of Ca^{2+} entry in human and bovine endothelial cells. *J. Physiol.* **482,** 259–274.

Griffith, T. M. (1996). Temporal chaos in the microcirculation. *Cardiovasc. Res.* **31,** 342–358.

Griffith, T. M., and Edwards, D. H. (1990a). Myogenic autoregulation of flow may be inversely related to endothelium-derived relaxing factor activity. *Am. J. Physiol.* **258,** H1171–H1180.

Griffith, T. M., and Edwards, D. H. (1990b). Basal NO activity helps to keep the geometrical configuration of arterial bifurcations close to the Murray optimum. *J. Theor. Biol.* **146,** 545–573.

Griffith, T. M., and Edwards, D. H. (1994). NO suppresses chaotic pressure oscillations in an isolated resistance artery without influencing their intrinsic complexity. *Am. J. Physiol.* **266,** H1786–H1800.

Griffith, T. M., Edwards, D. H., Lewis, M. J., Newby, A. C., and Henderson, A. H. (1984). The nature of endothelium-derived vascular relaxant factor. *Nature* **308,** 645–647.

Griffith, T. M., Edwards, D. H., and Davies, R. L., Harrison, T. J., and Evans, K. T. (1987). NO coordinates the behaviour of vascular resistance vessels. *Nature* **329,** 442–445.

Griffith, T. M., Hutcheson, I. R., Randall, M. D., and Edwards, D. H. (1991a). Role of flow in endothelium-mediated responses. *In* ''Resistance Arteries, Structure and Function'' (M. J. Mulvany, R. C. Aalkjaer, A. M. Heagerty, N. C. B. Nyborg, and S. Strandgaard, eds.), pp. 204–207. Excerpta Medica, Amsterdam.

Griffith, T. M., Edwards, D. H., and Randall, M. D. (1991b). Blood flow and optimal vascular topography: Role of the endothelium. *Basic Res. Cardiol.* **86**(Suppl. 2), 89–96.

Gross, R., Kirchheim, H., and Brandstetter, K. (1976). Basal vascular tone in the kidney. Evaluation from the static pressure–flow relationship under normal autoregulation and at maximal dilation in the dog. *Circ. Res.* **38,** 525–531.

Groves, P., Kurz, S., and Drexler, H. (1995). Role of endogenous bradykinin in human coronary vasomotor control. *Circulation* **92,** 3424–3430.

Gudi, S. R. P., Clark, C. B., and Frangos, J. A. (1996). Fluid flow rapidly activates G proteins in human endothelial cells. Involvement of G proteins in mechanochemical signal transduction. *Circ. Res.* **79,** 834–839.

Hamasaki, K., Mimura, T., Morino, N., Furuya, H., Nakamoto, T., Aizawa, S., Morimoto, C., Yazaki, Y., Hirai, H., and Nojima, Y. (1996). Src kinase plays an essential role in integrin-mediated tyrosine phosphorylation of Crk-associated substrate p130Cas. *Biochem. Biophys. Res. Commun.* **222,** 338–343.

Hassessian, H., Bodin, P., and Burnstock, G. (1993). Blockade by glibenclamide of the flow-evoked endothelial release of ATP that contributes to vasodilatation in the pulmonary vascular bed of the rat. *Br. J. Pharmacol.* **109,** 466–472.

Hayashi, Y., Tomoike, H., Nagasawa, K., Yamada, A., Nishijima, H., Adachi, H., Nakamura, M. (1988). Functional and anatomical recovery of endothelium after denudation of coronary artery. *Am. J. Physiol.* **254,** H1081–H1090.

Hecker, M., Mülsch, A., Bassenge, E., and Busse, R. (1993). Vasoconstriction and increased flow: Two principal mechanisms of shear stress-dependent endothelial autacoid release. *Am. J. Physiol.* **265,** H828–H833.

Henrion, D., Terzi, F., Matrougui, K., Duriez, M., Boulanger, C. M., Colucci-Guyon, E., Babinet, C., Briand, P., Friedlander, G., Poitevin, P., and Levy, B. I. (1997). Impaired flow-induced dilation in mesenteric resistance arteries from mice lacking vimentin. *J. Clin. Invest.* **100,** 2909–2914.

Hester, R. L., Eraslan, A., and Saito, Y. (1993). Differences in EDNO contribution to arteriolar diameters at rest and during functional dilation in striated muscle. *Am. J. Physiol.* **265,** H146–H151.

Hintze, T. H., and Vatner, S. F. (1984). Reactive dilation of large coronary arteries in conscious dogs. *Circ. Res.* **54,** 50–57.

Hirai, T., Visneski, M. D., Kearns, K. J., Zelis, R., and Musch, T. I. (1994). Effects of NO synthase inhibition on the muscular blood flow response to treadmill exercise in rats. *J. Appl. Physiol.* **77,** 1288–1293.

Hirata, K., Kuroda, R., Sakoda, T., Katayama, M., Inoue, N., Suematsu, M., Kawashima, S., and Yokoyama, M. (1995). Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension* **25,** 180–185.

Hishikawa, K., Nakaki, T., Suzuki, H., Saruta, T., and Kato, R. (1992). Transmural pressure inhibits nitric oxide release from human endothelial cells. *Eur. J. Pharmacol.* **215,** 329–331.

Hoebel, B. G., and Graier, W. F. (1998). 11,12-Epoxyeicosatrienoic acid stimulates tyrosine kinase activity in procine aortic endotehlial cells. *Eur. J. Pharmacol.* **346,** 115–117.

Hoebel, B. G., Kostner, G. M., and Graier, W. F. (1997). Activation of microsomal cytochrome P450 mono-oxygenase by Ca^{2+} store depletion and its contribution to Ca^{2+} entry in porcine aortic endothelial cells. *Br. J. Pharmacol.* **121,** 1579–1588.

Holtz, J., Förstermann, U., Pohl, U., Giesler, M., and Bassenge, E. (1984). Flow-dependent, endothelium-mediated dilation of epicardial coronary arteries in conscious dogs: Effects of cyclooxygenase inhibition. *J. Cardiovasc. Pharmacol.* **6,** 1161–1169.

Hornig, B., Kohler, C., and Drexler, H. (1997). Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. *Circulation* **95,** 1115–1118.

Huang, A., Sun, D., Kaley, G., and Koller, A. (1998a). Superoxide released to high intra-arteriolar pressure reduces nitric oxide-mediated shear stress- and agonist-induced dilations. *Circ. Res.* **83,** 960–965.

Huang, A., Sun, D., Koller, A., and Kaley, G. (1998b). Gender differences in flow-induced dilation and regulation of shear stress: Role of estrogen and nitric oxide. *Am. J. Physiol.* **275,** R1571–R1577.

Hussain, S. N. A., Stewart, D. J., Ludemann, J. P., and Madger, S. (1992). Role of endothelium-derived relaxing factor in active hyperemia of the canine diaphragm. *J. Appl. Physiol.* **72,** 2393–2401.

Hutcheson, I. R., and Griffith, T. M. (1991). Release of endothelium-derived relaxing factor is modulated both by frequency and amplitude of pulsatile flow. *Am. J. Physiol.* **261,** H257–H262.

Hutcheson, I. R., and Griffith, T. M. (1994). Heterogeneous populations of K^+ channels mediate NO release to flow but not agonists in rabbit aorta. *Am. J. Physiol.* **266,** H590–H596.

Hutcheson, I. R., and Griffith, T. M. (1996). Mechanotransduction through the endothelial cytoskeleton: Mediation of flow- but not agonist-induced EDRF release. *Br. J. Pharmacol.* **118,** 720–726.

Hutcheson, I. R., and Griffith, T. M. (1997). Central role of intracellular calcium stores in acute flow- and agonist-evoked endothelial nitric oxide release. *Br. J. Pharmacol.* **122,** 117–125.

Hutcheson, I. R., Chaytor, A. T., Evans, W. H., and Griffith, T. M. (1999). Nitric oxide-independent relaxations to acetylcholine and A23187 involve different routes of heterocellular communication. Role of Gap junctions and phospholipase A2. *Circ. Res.* **84,** 53–63.

Hutchins, G. M., Miner, M. M., and Boitnott, J. K. (1976). Vessel caliber and branch-angle of human coronary artery branch-points. *Circ. Res.* **38,** 572–576.

Jackson, W. F., Mülsch, A., and Busse, R. (1991). Rhythmic smooth muscle activity in hamster aortas is mediated by continuous release of NO from the endothelium. *Am. J. Physiol.* **260,** H248–H253.

Jacobs, E. R., Cheliakine, C., Gebremedhin, D., Birks, E. K., Davies, P. F., and Harder, D. R. (1995). Shear activated channels in cell-attached patches of cultured bovine aortic endothelial cells. *Pfluegers Arch.* **431,** 129–131.

James, N. L., Harrison, D. G., and Nerem, R. M. (1995). Effects of shear on endothelial cell calcium in the presence and absence of ATP. *FASEB J.* **9,** 968–973.

Jones, C. J. H., Kuo, L., Davis, M. J., DeFily, D. V., and Chilian, W. M. (1995). Role of nitric oxide in the coronary microvascular responses to adenosine and increased metabolic demand. *Circulation* **91,** 1807–1813.

Jones, S. C., Radinsky, C. R., Furlan, A. J., Chyatte, D., and Perez-Trepichio, A. D. (1999). Cortical NOS inhibition raises the lower limit of cerebral blood flow-arterial pressure autoregulation. *Am. J. Physiol.* **276.** H1253–H1262.

Ju, H., Zou, R., Venema, V. J., and Venema, R. C. (1997). Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J. Biol. Chem.* **272,** 18522–18525.

Juncos, L. A., Garvin, J., Carretero, O. A., and Sadayoshi, I. (1995). Flow modulates myogenic responses in isolated microperfused rabbit afferent arterioles via endothelium-derived nitric oxide. *J. Clin. Invest.* **95,** 2741–2748.

Kanai, A. J., Strauss, H. C., Truskey, G. A., Crews, A. L., Grunfeld, S., and Malinski, T. (1995). Shear stress induces ATP-independent transient nitric oxide release from vascular endothelial cells, measured directly with a porphyrinic microsensor. *Circ. Res.* **77,** 284–293.

Katada, T., Kurosu, H., Okada, T., Tsujimoto, N., Takasuga, S., Kontani, K., Hazeki, O., and Ui, M. (1999). Synergistic activation of a family of phosphoinositide 3-kinase via G-protein coupled and tyrosine kinase-related receptors. *Chem. Phys. Lipids.* **98,** 79–86.

Kelm, M., Feelisch, M., Deussen, A., Strauer, B. E., and Schrader, J. (1991). Release of endothelium derived nitric oxide in relation to pressure and flow. *Cardiovasc. Res.* **25,** 831–836.

Kessler, P., Popp, R., Busse, R., and Schini-Kerth, V. B. (1999). Proinflammatory mediators chronically downregulate the formatin of the endothelium-derived hyperpolarizing factor in arteries via a nitric oxide/cyclic GMP-dependent mechanism. *Circulation* **99,** 1878–1884.

Khayutin, V. M., Nikolsky, V. P., Rogoza, A. N., and Lukoshkova, E. V. (1993a). The effect of pressure drop stabilization in arteries during variations in blood flow rate. *Acta Physiol. Scand.* **148,** 285–294.

Khayutin, V. M., Nikolsky, V. P., Rogoza, A. N., and Lukoshkova, E. V. (1993b). Endothelium determines stabilization of the pressure drop in arteries. *Acta Physiol. Scand.* **148,** 295–304.

Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L., and Muallem, S. (1998). Functional interaction between $InsP_3$ receptors and store-operated Htrp3 channels. *Nature* **396,** 478–482.

Knudsen, H. L., and Frangos, J. A. (1997). Role of cytoskeleton in shear stress-induced endothelial nitric oxide production. *Am. J. Physiol.* **273,** H347–H355.

Kohler, T. R., Kirkman, T. R., Kraiss, L. W., Zierler, B. K., and Clowes, A. W. (1991). Increased flow inhibits neointimal hyperplasia in endothelialized vascular grafts. *Circ. Res.* **69,** 1557–1565.

Kojda, G., and Harrison, D. (1999). Interactions between NO and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc. Res.* **43,** 562–571.

Koller, A., and Kaley, G. (1990a). Prostaglandins mediate arteriolar dilation to increased blood flow velocity in skeletal muscle microcirculation. *Circ. Res.* **67,** 529–534.

Koller, A., and Kaley, G. (1990b). Role of endothelium in reactive dilation of skeletal muscle arterioles. *Am. J. Physiol.* **259,** H1313–H1316.

Koller, A., Dornyei, G., and Kaley, G. (1998). Flow-induced responses in skeletal muscle venules: Modulation by nitric oxide and prostaglandins. *Am. J. Physiol.* **275,** H831–H836.

Kostic, M. M., and Schrader, J. (1992). Role of nitric oxide in reactive hyperemia of the guinea pig heart. *Circ. Res.* **70,** 208–212.

Kuchan, M. J., and Frangos, J. A. (1994). Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am. J. Physiol.* **266,** C628–C636.

Kuchan, M. J., Jo, H., and Frangos, J. A. (1994). Role of G proteins in shear stress-mediated nitric oxide production by endothelial cells. *Am. J. Physiol.* **267,** C753–C758.

Kuo, L., Davies, M. J., and Chilian, W. M. (1990). Endothelium-dependent, flow-induced dilation of isolated coronary arterioles. *Am. J. Physiol.* **259,** H1063–H1070.

Kuo, L., Chilian, W. M., and Davis, M. J. (1991). Interaction of pressure- and flow-induced responses in porcine coronary resistance vessels. *Am. J. Physiol.* **261,** H1706–H1715.

Kuo, L., Arko, F., Chilian, W. M., and Davis, M. J. (1993). Coronary venular responses to flow and pressure. *Circ. Res.* **72,** 607–615.

Lamontagne, D., Pohl, U., and Busse, R. (1992). Mechanical deformation of vessel wall and shear stress determine the basal release of endothelium-derived relaxing factor in the intact rabbit coronary vascular bed. *Circ. Res.* **70,** 123–130.

Langille, B. L., and O'Donnell, F. (1986). Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. *Science* **231,** 405–407.

Laurindo, F. R., Pedro, M de A., Barbeiro, H. V., Pileggi, F., Carvalho, M. H., Augusto, O., and da Luz, P. L. (1994). Vascular free radical release. *Ex vivo* and *in vivo* evidence for a flow-dependent endothelial mechanism. *Circ. Res.* **74,** 700–709.

Li, H., Oehrlein, S. A., Wallerath, T., Ihrig-Biedert, I., Wohlfart, P., Ulshöfer, T., Jessen, T., Herget, T., Förstermann, U., and Kleinert, H. (1998). Activation of protein kinase Cα and/or ϵ enhances transcription of the human endothelial nitric oxide synthase gene. *Mol. Pharmacol.* **53,** 630–637.

Liu, J., García-Cardeña, G., and Sessa, W. C. (1996). Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: Implications for caveolae localization. *Biochemistry* **35,** 13277–13281.

Liu, J., Oh, P., Horner, T., Rogers, R. A., and Schnitzer, J. E. (1997). Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. *J. Biol. Chem.* **272,** 7211–7222.

Liu, Y. H., and Ritman, E. L. (1986). Branching pattern of pulmonary arterial tone in anaesthetized dogs. *J. Biomech. Eng.* **108,** 289–293.

Lückhoff, A., and Busse, R. (1990). Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pfluegers Arch.* **416,** 305–311.

Macedo, M. P., and Lautt, W. W. (1996). Autoregulatory capacity in the superior mesenteric artery is attenuated by nitric oxide. *Am. J. Physiol.* **271,** G400–G404.

McLenachan, J. M., Vita, J., Fish, D. R., Treasure, C. B., Cox, D. A., Ganz, P., and Selwyn, A. P. (1990). Early evidence of endothelial vasodilator dysfunction at coronary branch points. *Circulation* **82,** 1169–1173.

McMahon, T. J., Hood, J. S., and Kadowitz, P. J. (1992). Pulmonary vasodilator response to vagal stimulation is blocked by N^{ω}-nitro-L-arginine methyl ester in the cat. *Circ. Res.* **70,** 364–369.

Majid, D. S. A., and Navar, L. G., (1992). Suppression of blood flow autoregulation plateau during nitric oxide blockade in canine kidney. *Am. J. Physiol.* **262,** F40–F46.

Malek, A. M., Jiang, L., Lee, I., Sessa, W. C., Izumo, S., and Alper, S. L. (1999). Induction of nitric oxide synthase mRNA by shear stress requires intracellular calcium and G-protein signals and is modulated by PI 3 kinase. *Biochem. Biophys. Res. Commun.* **254,** 231–242.

Marsden, P. A., Heng, H. H. Q., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J. Biol. Chem.* **268,** 17478–17488.

Martin, W., Furchgott, R. F., Villani, G. M., and Jothianandan, D. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.* **237,** 529–538.

Mattsson, E. J. R., Kohler, T. R., Vergel, S. M., and Clowes, A. W. (1997). Increased blood flow induces regression of intimal hyperplasia. *Arterioscler. Thromb. Vasc. Biol.* **17,** 2245–2249.

Mayrovitz, H. N., and Roy, J. (1983). Microvascular blood flow: Evidence indicating a cubic dependence on arteriolar diameter. *Am. J. Physiol.* **245,** H1031–H1038.

Mehrke, G., and Daut, J. (1990). The electrical response of cultured guinea-pig coronary endothelial cells to endothelium-dependent vasodilators. *J. Physiol.* **430,** 251–272.

Melkumyants, A. M., Balashov, S. A., and Klimachev, A. N., Kartamyshev, S. P., and Khayutin, V. M. (1992). Nitric oxide does not mediate flow induced endothelium dependent arterial dilatation in the cat. *Cardiovasc. Res.* **26,** 256–260.

Michel, J. B., Feron, O., Sacks, D., and Michel, T. (1997). Reciprocal regulation of endothelial nitric-oxide synthase by Ca^{2+}-calmodulin and caveolin. *J. Biol. Chem.* **272,** 15583–15586.

Milner, P., Kirkpatrick, K. A., Ralevic, V., Toothill, V., Pearson, J., and Burnstock, G. (1990). Endothelial cells cultured from human umbilical vein release ATP, substance P and ACh in response to increased flow. *Proc. R. Soc. London (B)* **241,** 245–248.

Miura, H., Liu, Y., and Gutterman, D. D. (1999). Human coronary arteriolar dilation to bradykinin depends on membrane hyperpolarization: Contribution of nitric oxide and Ca^{2+}-activated K^+ channels. *Circulation* **99,** 3132–3138.

Mombouli, J.-V., and Vanhoutte, P. M. (1997). Endothelium-derived hyperpolarizing factor(s): Updating the unknown. *Trends Pharmacol. Sci.* **18,** 252–256.

Moncada, S., Palmer, R. M., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Moritoki, H., Hisayama, T., Takeuchi, S., Kondoh, W., and Imagawa, M. (1994). Relaxation of rat thoracic aorta induced by the Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, possibly through nitric oxide formation. *Br. J. Pharmacol.* **111,** 655–662.

Muller, J. M., Chilian, W. M., and Davis, M. J. (1997). Integrin signaling transduces shear stress-dependent vasodilation of coronary arterioles. *Circ. Res.* **80,** 320–326.

Murray, C. D. (1926). The physiological principle of minimum work applied to the angle of branching of arteries. *J. Gen. Physiol.* **9,** 835–841.

Nafz, B., Wagner, C. D., and Persson, P. B. (1997). Endogenous nitric oxide buffers blood pressure variability between 0.2 and 0.6 Hz in the conscious rat. *Am. J. Physiol.* **272,** H632–H637.

Nollert, M. U., Eskin, S. G., and McIntire, L. V. (1990). Shear stress increases inositol trisphosphate levels in human endothelial cells. *Biochem. Biophys. Res. Commun.* **170,** 281–287.

Noris, M., Morigi, M., Donadelli, R., Aiello, S., Foppolo, M., Todeschini, M., Orisio, S., Remuzzi, G., and Remuzzi, A. (1995). Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ. Res.* **76,** 536–543.

Numaguchi, K., Egashira, K., Takemoto, M., Kadokami, T., Shimokawa, H., Sueishi, K., and Takeshita, A. (1995). Chronic inhibition of nitric oxide synthesis causes coronary microvascular remodeling in rats. *Hypertension* **26,** 957–962.

O'Leary, D. S., Dunlap, R. C., and Glover, K. W. (1994). Role of endothelium-derived relaxing factor in hindlimb reactive and active hyperemia in conscious dogs. *Am. J. Physiol.* **266,** R1213–R1219.

Ohno, M., Gibbons, G. H., Dzau, V. J., and Cooke, J. P. (1993). Shear stress elevates endothelial cGMP. Role of a potassium channel and G protein coupling. *Circulation* **88,** 193–197.

Olesen, S.-P., Clapham, D. E., and Davies, P. F. (1988). Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. *Nature* **331,** 168–170.

O'Neill, W. C. (1995). Flow-mediated NO release from endothelial cells is independent of K^+ channel activation or intracellular Ca^{2+}. *Am. J. Physiol.* **269,** C863–C869.

Onohara, T., Okadome, K., and Yamamura, S., Mii, S., and Sugimachi, K. (1991). Simulated blood flow and the effects on prostacyclin production in the dog femoral artery. *Circ. Res.* **68,** 1095–1099.

Parthimos, D., Edwards, D. H., and Griffith, T. M. (1999). Minimal model of arterial chaos generated by coupled intracellular and membrane Ca^{2+} oscillators. *Am. J. Physiol.* **277,** H1119–H1144.

Persson, M. G., Hedqvist, P., and Gustafsson, L. E. (1991). Nerve-induced tachykinin-mediated vasodilatation in skeletal muscle is dependent on nitric oxide formation. *Eur. J. Pharmacol.* **205,** 295–301.

Peterson, T. E., Poppa, V., Ueba, H., Wu, A., Yan, C., and Berk, B. C. (1999). Opposing effects of reactive oxygen species and cholesterol on endothelial nitric oxide synthase and endothelial cell caveolae. *Circ. Res.* **85,** 29–37.

Pinto, S., Virdis, A., Ghiadoni, L., Bernini, G., Lombardo, M., Petraglia, F., Genazzani, A. R., Taddei, S., and Savetti, A. (1997). Endogenous estrogen and acetylcholine-induced vasodilation in normotensive women. *Hypertension* **29,** 268–273.

Pohl, U., Busse, R., Kuon, E., and Bassenge, E. (1986). Pulsatile perfusion stimulates the release of endothelial autoacoids. *J. Appl. Cardiol.* **1,** 215–235.

Pohl, U., Herlan, K., Huang, A., and Bassenge, E. (1991). NO-mediated, shear-induced dilation opposes myogenic vasoconstriction in small rabbit arteries. *Am. J. Physiol.* **261,** H2016–H2023.

Pohl, U., Lamontagne, D., Bassenge, E., and Busse, R. (1994). Attenuation of coronary autoregulation in the isolated rabbit heart by endothelium derived nitric oxide. *Cardiovasc. Res.* **28,** 414–419.

Popp, R., Bauersachs, J., Hecker, M., Fleming, I., and Busse, R. (1996). A transferable, β-naphthoflavone-inducible, hyperpolarizing factor is synthesized by native and cultured porcine coronary endothelial cells. *J. Physiol.* **497,** 699–709.

Posch, K., Schmidt, K., and Graier, W. F. (1999). Selective stimulation of L-arginine uptake contributes to shear stress-induced formation of nitric oxide. *Life Sci.* **64,** 663–670.

Prasad, A. R., Logan, S. A., Nerem, R. M., Schwartz, C. J., and Sprague, E. A. (1993). Flow-related responses of intracellular inositol phosphate levels in cultured aortic endothelial cells. *Circ. Res.* **72,** 827–836.

Pries, A. R., Secomb, T. W., and Gaehtgens, P. (1995). Design principles of vascular beds. *Circ. Res.* **77,** 1017–1023.

Puybasset, L., Béa, M.-L., Ghaleb, B., Giudicelli, J.-F., and Berdeaux, A. (1996). Coronary and systemic hemodynamic effects of sustained inhibition of nitric oxide synthesis in conscious dogs. Evidence for cross talk between nitric oxide and cyclooxygenase in coronary vessels. *Circ. Res.* **79,** 343–357.

Quyyumi, A. A., Dakak, N., Andrews, N. P., Gilligan, D. M., Panza, J. A., and Cannon, R. O. (1995). Contribution of nitric oxide to metabolic coronary vasodilation in the human heart. *Circulation* **92,** 320–326.

Rajfer, J., Aronson, W. J., Bush, P. A., Dorey, F. J., and Ignarro, L. J. (1992). Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N. Engl. J. Med.* **326,** 90–94.

Randall, M. D., and Griffith, T. M. (1992). NO plays a central role in collateral perfusion following arterial occlusion. *Am. J. Physiol.* **263,** H752–H760.

Randall, M. D., and Kendall, D. A. (1998). Endocannabinoids: A new class of vasoactive substances. *Trends Pharmacol. Sci.* **19,** 55–58.

Ranjan, V., Xiao, Z., and Diamond, S. L. (1995). Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. *Am. J. Physiol.* **269,** H550–H555.

Redmond, E. M., Cahill, P. A., and Sitzmann, J. V. (1998). Flow-mediated regulation of G-protein expression in cocultured vascular smooth muscle and endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18,** 75–83.

Rivers, R. J. (1995). Remote effects of pressure changes in arterioles. *Am. J. Physiol.* **268,** H1379–H1382.

Rizzo, V., McIntosh, D. P., Oh, P., and Schinitzer, J. E. (1998a). *In situ* flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. *J. Biol. Chem.* **273,** 34724–34729.

Rizzo, V., Sung, A., Oh, P., and Schnitzer, J. E. (1998b). Rapid mechanotransduction *in situ* at the luminal cell surface of vascular endothelium and its caveolae. *J. Biol. Chem.* **273,** 26323–26329.

Robinson, L. J., and Michel, T. (1995). Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targeting. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11776–11780.

Rosales, O. R., and Sumpio, B. E. (1992). Changes in cyclic strain increase inositol trisphosphate and diacylglycerol in endothelial cells. *Am. J. Physiol.* **262,** C956–C962.

Rossitti, S., and Frisen, L. (1994). Remodelling of the retinal arterioles in descending optic atrophy follows the principle of minimum work. *Acta Physiol. Scand.* **152,** 333–340.

Rossitti, S., and Löfgren, J. (1993). Vascular dimensions of the cerebral arteries follow the principle of minimum work. *Stroke* **24,** 371–377.

Rubanyi, G. M. (1988). Endothelium-dependent pressure-induced contraction of isolated canine carotid arteries. *Am. J. Physiol.* **255,** H783–H788.

Rubanyi, G. M., Romero, J. C., and Vanhoutte, P. M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.* **250,** H1145–H1149.

Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O., Segal, S. S., and Sessa, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J. Clin. Invest.* **101,** 731–736.

Rzigalinski, B. A., Willoughby, K. A., Hoffman, S. W., Falck, J. R., and Ellis, E. F. (1999). Calcium influx factor, further evidence it is 5,6-epoxyeicosatrienoic acid. *J. Biol. Chem.* **274,** 175–182.

Sasajima, H., Wang, X., and van Breemen, C. (1997). Fractional Ca^{2+} release from the endoplasmic reticulum activates Ca^{2+} entry in freshly isolated rabbit aortic endothelial cells. *Biochem. Biophys. Res. Commun.* **241,** 471–475.

Schretzenmayr, A. (1933). Uber kreislaufregulatorische Vorgange an den groβen Arterien bei der Muskelarbeit. *Pfluegers Arch. Ges. Physiol.* **232,** 743–748.

Segal, S. S., Damon, D. N., and Duling, B. R. (1989). Propagation of vasomotor responses coordinates arteriolar resistances. *Am. J. Physiol.* **256,** H822–H837.

Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J., and Hintze, T. H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* **74,** 349–353.

Sestier, F. J., Mildenberger, R. R., and Klassen, G. A. (1978). Role of autoregulation in spatial and temporal perfusion heterogeneity of canine myocardium. *Am. J. Physiol.* **235,** H64–H71.

Shaul, P. W., and Anderson, R. G. W. (1998). Role of plasmalemmal caveolae in signal transduction. *Am. J. Physiol.* **275,** L843–L851.

Shen, J., Luscinskas, F. W., Connolly, A., Dewey, C. F., Jr., and Grimbrone, M. A. (1992). Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. *Am. J. Physiol.* **262,** C384–C390.

Shen, W., Lundborg, M., Wang, J., Stewart, J. M., Xu, X., Ochoa, M., and Hintze, T. H. (1994). Role of EDRF in the regulation of regional blood flow and vascular resistance at rest and during exercise in conscious dogs. *J. Appl. Physiol.* **77,** 165–172.

Shen, W., Hintze, T. H., and Wolin, M. S. (1995). Nitric oxide an important signalling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation* **92,** 3505–3512.

Shepherd, P. R., Withers, D. J., and Siddle, K. (1998). Phosphoinositide 3-kinase: The key switch mechanism in insulin signalling. *Biochem. J.* **333,** 471–490.

Sherman, T. F., Popel, A. S., Koller, A., and Johnson, P. C. (1989). The cost of departure from optimal radii in microvascular networks. *J. Theor. Biol.* **136,** 245–265.

Shimoda, L. A., Norins, N. A., and Madden, J. A. (1998). Responses to pulsatile flow in piglet isolated cerebral arteries. *Pediatr. Res.* **43,** 514–520.

Sivaramaprasad, G., Nolan, J. P., and Frangos, J. A. (1998). Modulation of GTPase activity of G proteins by fluid shear stress and phospholipid composition. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2515–2519.

Smiesko, V., Lang, D. J., and Johnson, P. C. (1989). Dilator response of rat mesenteric arcading arterioles to increased blood flow velocity. *Am. J. Physiol.* **257,** H1958–H1965.

Smith, T. P., Jr., and Canty, J. M., Jr. (1993). Modulation of coronary autoregulatory responses by nitric oxide: Evidence for flow-dependent resistance adjustments in conscious dogs. *Circ. Res.* **73,** 232–240.

Stauss, H. M., Godecke, A., Mrowka, R., Schrader, J., and Persson, P. B. (1999). Enhanced blood pressure variability in eNOS knockout mice. *Hypertension* **33,** 1359–1369.

Sugihara, T., Hattori, Y., Yamamoto, Y., Qi, F., Ichikawa, R., Sato, A., Liu, M. Y., Abe, K., and Kanno, M. (1999). Preferential impairment of nitric oxide-mediated endothelium-dependent relaxation in human cervical arteries after irradiation. *Circulation* **100,** 635–641.

Sun, D., Huang, A., and Kaley, G. (1995a). Flow-dependent dilation and myogenic constriction interact to establish the resistance of skeletal muscle arterioles. *Microcirculation* **2,** 289–295.

Sun, D., Huang, A., Koller, A., and Kaley, G. (1995b). Flow-dependent dilation of arterioles is abolished by blockade of endothelial microtubule assembly. *Circulation* **92,** I-629.

Sun, D., Huang, A., Koller, A., and Kaley, G. (1998). Adaptation of flow-induced dilation of arterioles to daily exercise. *Microvasc. Res.* **56,** 54–61.

Sun, D., Huang, A., Smith, C. J., Stackpole, C. J., Connetta, J. A., Shesely, E. G., Koller, A., and Kaley, G. (1999). Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. *Circ. Res.* **85,** 288–293.

Sutliff, R. L., Hoying, J. B., Kadambi, V. J., Kranias, E. G., and Paul, R. J. (1999). Phospholamban is present in endothelial cells and modulates endothelium-dependent relaxation. *Circ. Res.* **84,** 360–364.

Taber, L. A. (1998). An optimization principle for vascular radius including the effects of smooth muscle tone. *Biophys. J.* **74,** 109–114.

Takahashi, M., and Berk, B. C. (1996). Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J. Clin. Invest.* **98,** 2623–2631.

Talman, W. T., and Dragon, D. N. (1995). Inhibition of nitric oxide synthesis extends cerebrovascular autoregulation during hypertension. *Brain Res.* **672,** 48–54.

Tan, J. Z., Kaley, G., and Gurtner, G. H. (1997). Nitric oxide and prostaglandins mediate vasodilation to 5,6-EET in rabbit lung. *Adv. Exp. Med. Biol.* **407,** 561–566.

Tanaka, K., Fukuuchi, Y., Gomi, S., Mihara, B., Shirai, T., Nogawa, S., Nozaki, H., and Nagata, E. (1993). Inhibition of nitric oxide synthesis impairs autoregulation of local blood flow in the rat. *Neuroreport* **4,** 267–270.

Taylor, H. J., Chaytor, A. T., Evans, W. H., and Griffith, T. M. (1998). Inhibition of the gap junctional component of endothelium-dependent relaxations in rabbit iliac artery by 18-alpha glycyrrhetinic acid. *Br. J. Pharmacol.* **125,** 1–3.

Tesfamariam, B., and Cohen, R. A. (1988). Inhibition of adrenergic vasoconstriction by endothelial cell shear stress. *Circ. Res.* **63,** 720–725.

Tesfamariam, B., and Cohen, R. A. (1992). Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am. J. Physiol.* **263,** H321–H326.

Thompson, B. G., Pluta, R. M., Girton, M. E., and Oldfield, E. H. (1996). Nitric oxide mediation of chemoregulation but not autoregulation of cerebral blood flow in primates. *J. Neurosurg.* **84,** 71–78.

Toda, N., and Okamura, T. (1990). Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebroarterial muscle. *Biochem. Biophys. Res. Commun.* **170,** 308–313.

Topper, J. N., Cai, J., Falb, D., and Gimbrone, M. A. (1996). Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: Cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 10417–10422.

Tronc, F., Wassef, M., Esposito, B., Henrion, D., Glagov, S., and Tedgui, A. (1996). Role of NO in flow-induced remodeling of the rabbit common carotid artery. *Arterioscler. Thromb. Vasc. Biol.* **16,** 1256–1262.

Ueeda, M., Silvia, S. K., and Olsson, R. A. (1992). Nitric oxide modulates coronary autoregulation in the guinea pig. *Circ. Res.* **70,** 1296–1303.

Uematsu, M., Ohara, Y., Navas, J. P., Nishida, K., Murphy, T. J., Alexander, R. W., Nerem, R. M., and Harrison, D. G. (1995). Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am. J. Physiol.* **269,** C1371–C1378.

Wang, Q., Paulson, O. B., and Lassen, N. A. (1992). Is autoregulation of cerebral blood flow in rats influenced by nitro-L-arginine, a blocker of the synthesis of nitric oxide? *Acta Physiol. Scand.* **145,** 297–298.

Wang, W., and Diamond, S. L. (1997). Does elevated nitric oxide production enhance the release of prostacyclin from shear stressed aortic endothelial cells? *Biochem. Biophys. Res. Commun.* **233,** 748–751.

Wilcox, C. S., Deng, X., Doll, A. H., Snellen, H., and Welch, W. J. (1993). Nitric oxide mediates renal vasodilation during erythropoietin-induced polycythemia. *Kidney Int.* **44,** 430–435.

Wilson, J. R., and Kapoor, S. (1993). Contribution of endothelium-derived relaxing factors to exercise-induced vasodilation in humans. *J. Appl. Physiol.* **75,** 2740–2744.

Xiao, Z., Zhang, Z., and Diamond, S. L. (1997). Shear stress induction of the endothelial nitric oxide synthase gene is calcium-dependent but not calcium-activated. *J. Cell. Physiol.* **171,** 205–211.

Xie, Y., Shen, W., Zhao, G., Xu, X., Wolin, M. S., and Hintze, T. H. (1996). Role of endothelium-derived nitric oxide in the modulation of canine myocardial mitochondrial respiration in vitro. *Circ. Res.* **79,** 381–387.

Yamabe, H., Okumura, K., Ishizaka, H., Tsuchiya, T., and Yasue, H. (1992). Role of endothelium-derived nitric oxide in myocardial reactive hyperemia. *Am. J. Physiol.* **263,** H8–H14.

Yamamoto, Y., Imaeda, K., and Suzuki, H. (1999). Endothelium-dependent hyperpolarization and intercellular electrical coupling in guinea-pig mesenteric arterioles. *J. Physiol.* **514,** 505–513.

Yeh, L. H., Park, Y. J., Hansalia, R. J., Ahmed, I. S., Deshpande, S. S., Goldschmidt-Clermont, P. J., Irani, K., and Alevriadou, B. R. (1999). Shear-induced tyrosine phosphorylation in endothelial cells requires Rac1-dependent production of ROS. *Am. J. Physiol.* **276,** C838–C847.

Yoshikawa, N., Ariyoshi, H., Ikeda, M., Sakon, M., Kawasaki, T., and Monden, M. (1997). Shear-stress causes polarized change in cytoplasmic calcium concentration in human umbilical vein endothelial cells (HUVECs). *Cell Calcium* **22,** 189–194.

Zou, B., M.-H., and Bachschmid, M. (1999). Hypoxia-reoxygenation triggers coronary vasospasm in isolated bovine coronary arteries via tyrosine nitration of prostacyclin synthase. *J. Exp. Med.* **190,** 135–139.

CHAPTER 32

Nitric Oxide Synthase Gene Transfer

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OWING TO ITS DIVERSE BIOLOGICAL EFFECTS, NITRIC OXIDE (NO) WAS ONE OF THE MOST STUDIED MOLECULES IN THE 1980S AND 1990S. NO HAS COMPLEX FUNCTIONS AND IS A POTENTIAL MEDIATOR IN MANY DISEASE PROCESSES. IT HAS BEEN LINKED TO THE DEVELOPMENT OF VASOMOTOR DYSFUNCTION, INTIMAL HYPERPLASIA, IMPOTENCE, WOUND HEALING, AND TUMORS. WITH THE ADVENT OF RECOMBINANT DNA AND GENE TRANSFER TECHNOLOGIES, DELIVERY OF THE NITRIC OXIDE SYNTHASE (NOS) GENE MAKES LOCAL OVEREXPRESSION OF NO FEASIBLE. THIS IS POTENTIALLY THERAPEUTIC IN A VARIETY OF DISEASES WHERE ADDITIONAL NO MAY BE BENEFICIAL. IN THIS REVIEW, WE DISCUSS THE MERITS AND DRAWBACKS OF DIFFERENT GENE TRANSFER VECTORS INCLUDING RETROVIRUS, ADENOVIRUS, AND ADENO-ASSOCIATED VIRUS AS WELL AS OTHER NONVIRAL GENE TRANSFER METHODS SUCH AS THE USE OF CATIONIC LIPOSOMES AND THE HEMAGGLUTINATING VIRUS OF JAPAN. WE THEN PROVIDE A DETAILED REVIEW OF THE ROLE OF NO IN THE PATHOPHYSIOLOGY OF THE AFOREMENTIONED DISEASES AND THE BENEFITS OF NOS GENE TRANSFER.

Introduction

Historically, nitric oxide (NO) was regarded with little interest beyond its function in ozone destruction and as a by-product of microbial nitrogen metabolism. More interest in NO developed in the late 1970s and early 1980s, when mammalian NO production was recognized and linked to potential roles in inflammation, tumor surveillance, neurotransmission and memory formation, and vascular physiology. In a landmark paper in 1980, Furchgott and Zawadzki reported that acetylcholine, a potent vasodilator, required an intact endothelial cell layer to promote vascular smooth muscle relaxation. They hypothesized that endothelial cells released a labile and diffusible factor in response to acetylcholine that mediated vasorelaxation through cGMP production. This endothelium-derived relaxing factor (EDRF) was subsequently identified as NO or an intermediate derivative thereof (Ignarro *et al.,* 1987; Palmer *et al.,* 1987). Since the discovery of NO, its diverse properties and functions have driven the development of NO-based therapies targeting the regulation of vasomotor tone, prevention of arterial injury and transplantation vasculopathy, apoptosis, wound healing, and tumor chemotherapy.

Since the 1980s and 1990s, the development of genetic engineering and gene transfer as modes of investigation and treatment of a variety of disease processes has also been occurring. There has always been a human curiosity to better understand the basic building blocks of living organisms. With the discovery of DNA as the information-bearing molecule and the elucidation of the genetic code, the recombinant DNA era was born. Technologies were developed to purify, sequence, cleave, and paste DNA that allowed the identification of genes and their coding sequences. Armed

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Table I Targets for Nitric Oxide Synthase Gene Therapy

Vasomotor function
Hypertension
Systemic
Pulmonary
Portal
Arterial injury
Vein graft intimal hyperplasia
Transplant vasculopathy
Lung transplant rejection
Erectile dysfunction
Wound healing
Cancer

with these tools, it was inevitable that genetic engineering would become a reality and gene therapy a goal for the present and the future.

As the studies into the properties of NO progressed, it became clear that NO may have many therapeutic roles that range from preventing vascular pathophysiology to promoting wound healing to tumoricidal actions. Methods of delivering NO to target tissues and organs include systemic administration and local gene therapies. The cloning of the three different NO synthase (NOS) isoforms permitted the development of NOS gene therapies (Table I). Shortcomings of systemic NO delivery include the risk of severe hypotension and potential toxicity to sensitive tissues. NOS gene therapies permit delivery of NO locally to defined target areas, thereby limiting the risk of potential adverse effects of NO and making this mode of NO delivery attractive. The duration of NO production can also be tailored to what is needed for a particular disease process.

Nitric Oxide Synthases

NO biosynthesis begins with a family of enzymes that catalyzes the conversion of L-arginine in a reaction with molecular oxygen to yield NO and the by-product L-citrulline. There are three distinct NOS isoforms that can carry out this synthetic pathway. Endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) have all been cloned from a variety of species as well as from human tissues. Characterization of these genes revealed that the three isoforms are unique products of three different genes. cDNAs for each of these isoforms have been cloned, sequenced, and demonstrated to encode functional enzymes. The availability of the cDNAs permits the use of NOS gene transfer to investigate the effect of NO on a variety of tissues and organs *in vivo.* The unique characteristics of each NOS isoform, including the enzymatic specific activity, cofactor requirement, and posttranslational regulation, can influence the selection process of which NOS would function in any particular gene therapy protocol. In order to perform NOS gene transfer, the design and selection of the most appropriate gene transfer vectors must be carefully considered.

Gene Transfer Vectors

The study of gene therapy and the ultimate use of gene transfer for the treatment of genetic or acquired diseases rely on efficient and safe vectors with which to deliver a gene of interest. The urgency to develop human gene therapy provides the greatest incentive for rapid innovations in this area. A number of vectors are currently available, but each has advantages as well as limitations that must be acknowledged because of the impact these limitations have on the outcome of experiments and therapies (Table II). For this purpose, several of the most commonly used vectors will be reviewed.

Retrovirus

Retroviruses are RNA viruses that integrate into the normal life cycles of the eukaryotic host cell and harness the host cellular machinery for viral propagation (Tzeng *et al.,* 1996a). The retrovirus has a very simple genome consisting of long terminal repeats (LTRs) and *gag, pol,* and *env* regions measuring 8 to 10 kb in length (Miller, 1992). The

Table II Vectors for Vascular Gene Therapy

Vector	Target cell type	Target receptor	Transfer efficiency	Duration of expression	Safety issues
Viral					
Retrovirus	Replicating	Multiple	Low	Months–Years	Insertional mutagenesis
Adenovirus	Replicating and nonreplicating	Coxsackievirus–adenovirus receptor (CAR) and α_v-integrin receptors	High	Weeks	Inflammatory response
Adeno-associated virus (AAV)	Replicating and nonreplicating	Heparan sulfate proteoglycan receptor	High	Months–Years	Insertional mutagenesis, Rep protein toxicity
Other					
Cationic liposomes	All	Cell membrane	Low	Days	None
Hemagglutinating virus of Japan (HVJ)	All	Sialoglycoprotein receptor	Low	Days	None

gag and *pol* regions encode core proteins, a protease, reverse transcriptase, and an integrase. The *env* region encodes the envelope proteins (Varmus, 1988). A single promoter, the LTR, regulates the transcription of viral RNA molecules that can be spliced to yield two different transcripts. Only the full-length transcript carries the packaging signal (Ψ) located upstream of the *gag* region. This packaging signal is essential to the encapsidation of only the appropriate viral RNAs into mature virions (Linial and Miller, 1990).

Retroviruses contain two single-stranded RNA molecules and viral proteins in an icosahedral viral core that is encapsulated by a host cell membrane lipid bilayer and viral glycoproteins (Miller, 1992). Retroviral infection is a receptor-mediated process. Following cell recognition and attachment, viral internalization occurs with release of the viral transcripts, reverse transcriptase, integrase, and protease into the cell. Reverse transcription generates a double-stranded DNA provirus intermediate from the two RNA molecules. The provirus enters the nucleus where viral integration proceeds in a semirandom fashion. The LTRs located at both 5' and 3' ends of the provirus are essential for the integration process. The integrated provirus is then transcribed and translated by the host cell machinery. Progeny viral particles are formed by the packaging of RNA transcripts possessing the Ψ signal, followed by viral release through a budding process of the cell membrane that does not result in cell lysis.

In order to safely use the retrovirus for gene transfer, the virus must be rendered replication incompetent while remaining infectious. The simplicity of the retroviral genome permits the deletion of large segments of the coding sequence without interfering with the packaging and assembly of infectious virions. The only components of the retroviral genome that are essential for successful genomic integration and virion formation are the LTRs and Ψ (Miller, 1992). The *gag, pol,* and *env* genes are dispensable as long as an alternate source of the viral proteins and enzymes are provided during viral assembly. The deleted viral genes can be easily replaced by foreign genes measuring up to 8 kb in size. The LTR may serve as the promoter for this foreign coding sequence, or an exogenous promoter may be concurrently inserted. The recombinant retroviral plasmid is then introduced using nonviral transfection methods into packaging cell lines that have been engineered to constitutively synthesize viral proteins necessary for assembly, packaging, and encapsulation of the viral RNA.

The primary advantage of retrovirus-mediated gene transfer is that the transferred genetic material becomes integrated into the host genome in a semipermanent fashion, permitting prolonged gene expression and becoming an inheritable trait. Nabel *et al.* (1990) reported expression of retrovirally transferred marker gene products for up to 5 months after viral exposure in arteries. The theoretical potential for permanent gene transfer using retroviral vectors make them well suited for gene therapies for diseases stemming from genetic disorders such as cystic fibrosis and adenosine deaminase deficiency. Another advantage of retroviral vectors is the simplicity of the retroviral genome. The deletion of essentially all of the coding sequence of the viral genome results in the elimination of viral protein production. Viral antigens can be recognized by the host immune system as foreign and can generate a substantial inflammatory response that would result in the elimination of cells expressing the viral products. Retroviral vectors are associated with little if any inflammatory response and contributes to the longevity of transgene expression.

Even though retroviral vectors were initially looked on as an ideal vehicle for gene delivery, there are some limitations of retroviral gene transfer. The retroviral life cycle requires genomic integration prior to expression of viral gene products, and this integration process requires active cellular proliferation (Miller *et al.,* 1990). Cells that undergo slow turnover or that are quiescent are therefore very poor targets for retroviral gene transfer; this limits the utility of retroviruses to only cells undergoing rapid replication. Additionally, retroviral infection requires specific cell surface receptors that are not abundant on most cell types. This paucity of receptors results in low gene transfer efficiency, especially *in vivo.* For example, retroviral infection in the arterial wall results in transfer efficiency of only 0.1–1.0% (Flugelman *et al.,* 1992; Nabel *et al.,* 1990). Finally, a theoretic concern is that retroviral integration into the host genome may result in the activation of an oncogene, leading to malignant transformation, or may disrupt a vital gene. Such events are semirandom, with some sites preferred over others, but no known events have occurred with retroviral gene transfer that has been associated with oncogenesis or an acquired genetic disorder.

Adenovirus

Human adenoviruses were discovered in 1953 after an exhaustive search for the cause of the common cold. Currently, there are more than 100 different adenoviral serotypes known, of which there are 47 distinct human serotypes (Ad1–Ad47) that are subclassified into six groups (A through F) according to tissue specificity, hemagglutination, oncogenic properties, DNA homology, and virulence (Hierholzer *et al.,* 1988). The two best-characterized and most widely used adenovirus serotypes for gene transfer studies are Ad2 and Ad5, which belong to subgroup C and are primarily respiratory pathogens (Straus, 1984).

The adenovirus is a DNA virus characterized by a complex, nonenveloped, icosahedral outer protein capsid (Stratford-Perricaudet and Perricaudet, 1994; Straus, 1984). The genome consists of a double-stranded linear DNA molecule measuring 36 kb in length with inverted terminal repeats (ITRs) at either end. The genome is packaged within an icosahedral capsid (Hitt *et al.,* 1997) composed of virally encoded capsomers, including 240 hexons and 12 penton bases (Perricaudet and Stratford-Perricaudet, 1995). From each penton base protrudes the fiber coat proteins. Adenoviral infection is a receptor–ligand interaction and, therefore, is limited to cells that express the receptor. Adenovirus fiber coat protein attaches with high affinity to the coxsackievirus–

adenovirus receptor (CAR) (Roelvink *et al.,* 1998; Tomko *et al.,* 1997). After viral attachment, internalization occurs through binding of the adenoviral penton base via an RGD motif to an α_v integrin receptor which, in turn, binds the extracellular matrix protein vitronectin (Wickham *et al.,* 1997). Receptor-mediated endocytosis ensues with release of the contents of the endosome into the cytoplasm of the cell (Seth *et al.,* 1984). The DNA then migrates to the nucleus where it remains as an episome.

In sharp contrast to that of retrovirus, the adenoviral genome is extremely complex with overlapping coding sequences. The adenoviral genome has four early genes that are expressed within 6–8 hours after infection. These are the early region 1 (E1A and E1B), E2 (E2A and E2B), E3, and E4. These genes encode proteins that initiate host cell DNA replication, shut off host protein synthesis, promote viral DNA replication, and protect the adenovirus-infected cell from recognition by host immune surveillance (Babiss *et al.,* 1985). Following viral DNA replication, the late viral genes become activated. Once the late genes are activated, host protein synthesis has essentially been halted, and the focus of the cells is centered on viral replication. The late genes encode structural viral proteins that are required for assembly of the viral core and outer capsid. The portions of the adenoviral genome that encode for the penton base, hexon, and fiber coat protein are the L2, L3, and L5 transcripts, respectively (Hitt *et al.,* 1997). By 30–40 hours postinfection, viral production is nearly complete and these daughter adenoviral particles are released through host cell lysis (Hitt *et al.,* 1997).

The complexity of the adenoviral genome has hindered the development of adenoviral gene transfer vectors because of the difficulty in creating deletional mutants that continue to possess the ability to infect and express a foreign transgene. However, such vectors have been successfully constructed. Adenoviral serotypes 2 and 5 are the most widely used adenoviral vectors because they are the best characterized and their complete DNA sequences are available. They are also nononcogenic in rodents, which is an important safety issue. The majority of the early vectors are E1 region deleted, thereby rendering these vectors replication incompetent. These E1-deleted adenoviral vectors, along with E3-deleted vectors, are termed first-generation recombinant Ad vectors. In place of the E1 gene, a foreign gene with an exogenous promoter up to 3.2 kb in size can be inserted into the adenoviral backbone (Hitt *et al.,* 1997). With additional deletions in the E2, E3, or E4 regions, genes up to 7.5 kb in size can be inserted. The maximal amount of DNA that can be encapsulated in the adenovirus is 38 kb (Berkner, 1992).

Adenovirus possesses several attractive characteristics for gene therapy. First, because adenovirus does not integrate into host genomic DNA, these viruses can target both replicating and quiescent cells alike. This characteristic is attractive for gene therapies directed at slow or nonreplicating cells, such as cardiac myocytes, hepatocytes, vascular smooth muscle cells (VSMC), and endothelial cells. In addition, genes transferred with this vector are not inherited, and transgene expression is limited to weeks. Second, adenoviral infection is efficient and is essentially complete within several minutes depending on the relative abundance of the CAR receptor. Third, the adenovirus has a particular affinity for human tissues, making it an ideal vector for human genetic therapies. Finally, the adenovirus can be produced in significantly higher titers than other vectors, often reaching 10^{13} plaque-forming units (pfu)/ml.

The adenoviral vector also has its shortcomings. Due to the nonintegrated nature of the adenoviral DNA, expression of the foreign gene is only transient, on the order of weeks. This characteristic may be considered both a potential benefit and a limitation. Transient gene expression may be ideal for disease processes that are acute, such as wound healing or intimal hyperplasia after arterial injury. However, when gene therapy is being directed at chronic and inherited diseases, transient gene delivery is unlikely to impact on disease outcome. Another important shortcoming of the adenoviral vector is the inflammatory response that it evokes. Current first-generation vectors still possess a number of viral genes that are transcribed, translated, and expressed. These viral proteins are recognized as foreign antigens by the host immune system and will evoke an immune response directed at eradicating the cells expressing the foreign proteins. Thus, the host immune response limits the duration of expression of the foreign gene. The immune response of the host will also diminish the success of subsequent attempts to deliver the same vector. The development of second-generation adenoviruses that express fewer viral products will hopefully reduce the inflammatory response and prolong transgene expression.

Adeno-Associated Virus

The adeno-associated virus (AAV) is another vector that has emerged onto the gene therapy scene. It is a member of the parvovirus family that has five different serotypes. AAV serotype 2 (AAV-2) is the most extensively characterized, and its genome has been completely sequenced (Srivastava *et al.,* 1983). AAV-2 virions contain a single-stranded linear DNA molecule that is approximately 4.7 kb long. Both plus and minus strands are packaged with equal frequency and are similarly infectious (Berns, 1990). The virion is encapsulated in a nonenveloped, icosahedral particle (Hoggan *et al.,* 1972). The genome of the AAV consists of two ITRs, a *rep* gene, and a *cap* gene. The *rep* gene encodes four proteins involved in DNA replication, and the *cap* gene encodes three proteins involved in the virion structure (Kotin, 1994). The ITRs are each 145 bases long; the first 125 bases of these repeats fold back on themselves in a T-shaped double strand which serves as the viral origin of DNA replication. Additionally, the ITRs are the only known *cis*-acting elements required for AAV packaging (Trempe, 1996).

Wild-type AAV has two distinct life cycles: latent and lytic. Latent infections occur when AAV infects a cell in the absence of a helper virus. AAV binds to cell surface heparan

sulfate proteoglycans (Summerford and Samulski, 1998). Viral DNA is released into the cytosol, migrates to the nucleus, and becomes integrated into the host genome. Viral integration does not occur in a completely random fashion; approximately 70% of integration events target chromosome 19 (Jolly, 1994). This targeting is believed to be related to the *rep* gene, because *rep* negative vectors do not integrate preferentially into chromosome 19 (Jolly, 1994). Once integrated, AAV exists as an integrated provirus without adversely affecting cell morphology or physiology. Cheung *et al.* (1980) demonstrated persistence of the AAV genome in tissue culture even after 100 passages. The lytic phase of the AAV life cycle is initiated by a helper virus or a cellular stress. When a cell possessing a latent AAV provirus is infected with a helper virus, such as the adenovirus or herpes simplex virus, the AAV genome is excised and enters into viral replication (Cheung *et al.,* 1980; Hoggan *et al.,* 1972). The helper virus provides the necessary gene products in altering host DNA synthesis to favor viral replication (Berns and Giraud, 1996). For example, the adenoviral early genes, namely, E1A, E1B, E2A, and E4, are involved in AAV viral replication.

The general strategy for generating a recombinant AAV vector (rAAV) is relatively straightforward owing to the simplicity of the AAV genome. The *rep* and *cap* genes can be deleted, and a foreign gene, along with an exogenous promoter, measuring up to 4.1–4.9 kb in size, can be inserted (Dong *et al.,* 1996). The ITRs are the only portion of the AAV genome that is required for its life cycle. A plasmid bearing the recombinant AAV and a plasmid bearing the *rep* and *cap* genes are cotransfected into a cell line expressing the necessary adenoviral genes. In the presence of adenoviral proteins, *rep* proteins rescue and amplify the replication of the recombinant AAV genome. The cap proteins package the single-stranded rAAV genome into an infectious AAV virion (Trempe, 1996). To isolate AAV virions, these cells are collected 48–72 hours after infection, heated to 56°C to inactivate the adenovirus (if an infectious adenovirus was used), and purified using a $CsCl_2$ equilibrium gradient. Viral titers produced using this technique have been shown to be as high as 10^{11} to 10^{12} total particles (Salvetti *et al.,* 1998). A problem with recombinant AAV vector production has been contamination of viral preparations with infectious helper virus. This has been greatly alleviated by using engineered packaging cells in the absence of infectious adenovirus (Grimm *et al.,* 1998; Salvetti *et al.,* 1998). Additionally, the use of a novel packaging cell line, generated from stably transfecting a *rep*/*cap*-containing plasmid into HeLa cells, has resulted in a 100-fold amplification and rescue of the AAV genome and in a high yield of recombinant AAV (Gao *et al.,* 1998; Salvetti *et al.,* 1998).

AAV has an unusually broad host range with diverse cell specificity. Lynch *et al.* (1997) infected different cell lines, including vascular cells, and showed a significant variation in the transfer efficiency between the different cells. Human smooth muscle cells (SMCs) infected with 100 DNase-resistant particles (DRP) per cell had a transfer efficiency of 5.2%, whereas rat SMCs had a transfer efficiency of only 0.032%. Human umbilical vein endothelial cells infected with 10 times as much virus revealed a transfer efficiency of 4.5%. Additionally, they demonstrated that rAAV had higher transfer efficiency *in vivo* in injured rat carotid arteries when viral infection was performed several days after creating the injury, indicating that rAAV preferentially infected actively proliferating cells in their model. Others have shown greater transduction efficiencies in vascular cells by infecting with even higher amounts of virus per cell. Maeda *et al.* (1997) were able to infect rat VSMC with a transfer efficiency of 50%. Another group examined the efficiency of gene transfer with the AAV in uninjured rat carotid artery (Arnold *et al.,* 1997) and found that 90% of intimal and medial cells contained the transgene 24 hours after infection, and that this expression could be detected at similar levels 1 month later. Although they did not specifically assay for inflammatory cells, microscopic evaluation did not show evidence of gross inflammation. It should be cautioned, however, that the titers used in this study were low, on the order of 10^5 IU/ml. Titers used with adenoviral-based gene delivery to the rat carotid artery have ranged from 10^6 to 10^{10} pfu/ml, which is significantly higher and more likely to provoke an immune response.

In summary, the AAV has many advantageous features that make this an ideal vector for gene transfer. Perhaps its most significant contribution will be toward gene therapy for chronic conditions, given that the AAV provirus is integrated into the host genome and has a relatively long duration of expression. This may prove to be the vector of choice when targeting genetic disorders or chronic disease processes such as transplant vasculopathy. Additionally, AAV produces virtually no viral antigenic proteins that can elicit a significant host immune response. Thus, the duration of transgene expression is even longer compared to some other viruses. AAV has a universal appeal secondary to its ability in successfully infecting a broad range of host cell types and species. The virus also appears to be quite safe for use with humans, with no evidence of pathogenicity in humans. Furthermore, the nonrandom integration event can reduce the theoretic mutagenic risk of random integration as seen with retroviruses. It is unknown, however, if this nonrandom insertion pattern will occur with recombinant adeno-associated viruses, since the *rep* gene, which is deleted with most rAAV, may be involved in this process.

One limitation with the use of AAV is that it can only accommodate the insertion of a small transgene ($<$4.9 kb), thus limiting the genes that can be carried by this vector. Another concern with its use for human gene therapy is that approximately 80% of the human population carry preformed antibodies secondary to prior exposure to the virus (Verma and Somia, 1997). These antibodies may make AAV gene transfer difficult by binding and clearing the vector prior to cellular infection. Currently, there are several ongoing clinical trials with rAAV bearing the cystic fibrosis transmembrane conductance regulator (CFTR) gene for treatment of patients with cystic fibrosis (Flotte *et al.,* 1996; Wagner *et*

al., 1995). Therefore, evaluation of these data may provide significant insight into this potential problem.

Cationic Liposomes

Prior to the advent of viral vectors, most gene transfer studies were performed using chemical methods of DNA delivery. Even today, however, safety concerns about administering infectious virus to humans have driven the continued development of nonviral modes of gene delivery. Cationic liposome complexes are positively charged synthetic lipid vesicles that encapsulate negatively charged DNA, RNA, and antisense molecules. These positively charged complexes fuse with the negatively charged cell membrane and release DNA into the cytoplasm. Typically, naked DNAs in cellular cytoplasm are rapidly degraded by DNases such that only a small percentage of the DNA actually reaches the nucleus. Methods of masking the DNA from cellular nucleases are being developed and should improve the gene transfer efficiency of these nonviral methods. Once in the nucleus, the DNA will remain unintegrated in an extrachromosomal fashion, resulting in a short duration of transgene expression. Various cationic liposome preparations are commercially available, and the efficiency of these preparations will vary with the preparation, the DNA/liposome concentration ratio, the cell type targeted, and the proliferation status of the cell (Feldman and Steg, 1997).

There are many advantageous features of using cationic liposomal-mediated gene transfer. The safety profile of cationic liposomes is superior to many other vectors because of the absence of viral products or viral progeny, the absence of an inflammatory response, and the low risk of insertional mutagenesis. Cationic complexes have been administered systemically via both intravenous and intra-arterial routes and locally, with minimal biochemical, hemodynamic, or cardiac toxicity (Nabel *et al.,* 1992, 1994; Stewart *et al.,* 1992). Construction of the lipid–DNA complex is very simple, and there is no limitation on the quantity of DNA that can be solubilized into the liposomes. These complexes can transfect both proliferating and nonproliferating cells. Disadvantages of cationic liposomes mainly involve the very poor gene transfer efficiency that is achieved, especially *in vivo.*

HVJ–Liposome Complexes

To increase the efficiency of liposomal gene transfer, a mediator of cell attachment and membrane fusion called hemagglutinating virus of Japan (HVJ) has been added to the DNA-containing liposome complexes. Synthesis of this modified fusion liposome is relatively straightforward. The DNA–liposome complex is fused with ultraviolet (UV)-inactivated HVJ virions, and the HVJ–liposomes are purified by sucrose-gradient ultracentrifugation. Subsequent fusion of the HVJ–liposome complex with the plasma membrane introduces the DNA directly into the cytoplasm. In several studies, use of HVJ–liposomes led to much greater levels of gene transfer than achieved with simple liposomes alone. Using these HVJ–liposomes to transfer genes into vascular smooth muscle *in vivo,* gene expression has been shown to be 10-fold higher than with lipofectin, and the length of liposome incubation with the tissue reduced from 24 hours to 30 min (Morishita *et al.,* 1993). There was no evidence of cellular toxicity, whereas lipofectin caused a significant decrease in cell viability.

Vasomotor Regulation

NO is a potent vasodilator and has been implicated in the regulation of blood pressure and flow in both animal models and human subjects (Dominiczak and Bohr, 1995; Huang *et al.,* 1995; Rees *et al.,* 1989; Vallance *et al.,* 1989). NO interacts with sGC, and the resultant increase in cGMP in the VSMCs leads to relaxation of the muscular layer and dilation of the vessel (Ignarro *et al.,* 1987; Palmer *et al.,* 1987). Endothelial NOS was found to play an even more integral role in determining resting blood pressure when mice genetically lacking the eNOS gene were found to be spontaneously hypertensive (Huang *et al.,* 1995). Additionally, patients with diabetes, hypertension, and atherosclerosis have been shown to have diminished levels of eNOS in their vasculature (Cohen, 1995; Moncada and Higgs, 1993). Therefore, the impact of reduced levels of vascular NO production on hypertension and other vascular pathophysiology has led to the development of gene therapy strategies to restore NO synthetic function to the blood vessel wall (Table III).

Carotid Artery

To determine if eNOS overexpression in the carotid artery could alter vasomotor function, Oobushi *et al.* (1997) collected rabbit carotid arteries and infected them with an adenoviral vector containing the eNOS (AdeNOS) gene using 3×10^9 pfu per arterial ring segment (3 mm in length). Twenty-four hours following infection, transgene expression was confirmed by immunohistochemistry and was localized to both endothelial and adventitial cells. Following precontraction with phenylephrine, vessels infected with AdeNOS demonstrated greater relaxation to acetylcholine than control vessels. A similar response was observed following treatment with the calcium ionophore A23187. To determine if this observed alteration in vascular tone was mediated predominately by the genetically engineered endothelial cells or adventitial fibroblasts, the experiments were repeated following endothelial denudation. Augmented relaxation was still observed following treatment with A23187. Therefore, overexpression of the eNOS gene in adventitial cells was sufficient to affect vasomotor function.

Since the above study was an *ex vivo* organ culture model, there was a need to determine if vasomotor function could be affected following *in vivo* gene transfer. Therefore, Kullo *et al.* infected rabbit carotid arteries intralumenally with AdeNOS or a control adenovirus for 20 min (Kullo *et al.,*

Table III Nitric Oxide Synthase Gene Transfer for the Regulation of Vasomotor Function

Author	Gene	Vector	Titer	Animal	Target tissue	Model	Effect
Janssens *et al.* (1996)	Human eNOS	Adeno[a]	3×10^9 pfu[b]	Rat	Lung (aerosol)	*In vivo*	Decrease in hypoxic-induced pulmondary HTN[c]
Lin *et al.* (1997)	Human eNOS	cDNA	1mg	SHR[d]	Systemic circulation	*In vivo*	Decrease in blood pressure
Chen *et al.* (1997a)	Bovine eNOS	Adeno	10^9–10^{11} pfu/ml	Dog	Basilar artery	*Ex vivo*	Increased vasomotor responsiveness
Chen *et al.* (1997b)	Bovine eNOS	Adeno	10^9 pfu/ml	Dog	CSF[e]/Cisterna magna	*In vivo*	Increased vasomotor responsiveness
Cable *et al.* (1997a)	Bovine eNOS	Adeno	5×10^9 pfu/ml	Pig	Coronary artery	*Ex vivo*	Increased vasomotor responsiveness
Ooboshi *et al.* (1997)	Bovine eNOS	Adeno	3×10^9 pfu	Rabbit	Carotid artery	*Ex vivo*	Increased vasomotor responsiveness
Kullo *et al.* (1997a)	Bovine eNOS	Adeno	1×10^9 pfu	Rabbit	Carotid artery	*In vivo*	Increased vasomotor responsiveness
Kullo *et al.* (1997b)	Bovine eNOS	Adeno	2×10^9 pfu	Rabbit	Carotid artery (adventitia)	*In vivo*	Increased vasomotor responsiveness
Tsutsui *et al.* (1998)	Bovine eNOS	Adeno	10^8–10^{10} pfu/ml	Dog	Basilar, coronary, femoral artery	*Ex vivo*	Increased vasomotor responsiveness in the basilar artery
Onoue *et al.* (1998)	Bovine eNOS	Adeno	10^{10} pfu/ml	Dog	Basilar artery	*Ex vivo*	Increased vasomotor responsiveness following SAH[f]
Ooboshi *et al.* (1998)	Bovine eNOS	Adeno	3×10^9 pfu/artery	Rabbit	Carotid artery	*Ex vivo*	Increased vasomotor responsiveness in normal and atherosclerotic vessels
Channon *et al.* (1998)	Rat nNOS	Adeno	6×10^8 pfu	Rabbit	Carotid artery	*In vivo*	Increased vasomotor responsiveness in normal and atherosclerotic vessels

[a] Adeno, adenovirus.
[b] pfu, plaque-forming units.
[c] HTN, hypertension.
[d] SHR, spontaneously hypertensive rat.
[e] CSF, cerebrospinal fluid.
[f] SAH, subarachnoid hemorrhage.

1997a). Four days following infection they determined that AdeNOS overexpression resulted in increased cGMP release as well as reduced contractions to phenylephrine, in an NO-dependent fashion. Additionally, acetylcholine-mediated vasorelaxation was enhanced in the AdeNOS-transduced vessels. The same investigators then showed that *in vivo* adventitial delivery of eNOS to the rabbit carotid artery could affect vasomotor function (Kullo *et al.,* 1997b). These experiments demonstrate that, by providing excess NO to the vasculature, vascular tone can be altered.

Atherosclerotic Carotid Artery

Delivering eNOS to normal vessels has been quite successful at modulating vasomotor function. Unfortunately, most dysfunctional blood vessels are diseased with atherosclerosis, and it is in this setting that endothelial-dependent relaxation is impaired (Forstermann *et al.,* 1988; Guerra *et al.,* 1989; Zeiher *et al.,* 1993). Although the mechanism of this impairment is poorly understood, possibilities include (1) impaired NO production through either reduced substrate or cofactor availability, or an effect of endogenous inhibitors; (2) enhanced NO scavenging, possibly through the increased production of reactive oxygen species; and/or (3) deleterious effects on downstream mediators of NO activity (Channon *et al.,* 1998). Guerra *et al.* measured NO release in normal and atherosclerotic vessels and found that atherosclerotic vessels released less NO than normal vessels. Intravenous administration of L-arginine was found to normalize endothelium-dependent relaxation in hypercholesterolemic rabbit thoracic aorta (Cooke *et al.,* 1991) and hind limb vessels (Girerd *et al.,* 1990). Similarly, administration of L-arginine via intracoronary infusion to hypercholesterolemic patients restored acetylcholine-induced increases in cardiac blood flow (Drexler *et al.,* 1991). However, endothelial cells at sites of atherosclerotic plaques are sources of superoxide anion (Ohara *et al.,* 1993), and this superoxide may interact with NO to generate peroxynitrite, which can result in local toxicity. This potential interaction makes it difficult to predict the outcome of eNOS overexpression in atherosclerotic vessels.

Ooboshi *et al.* (1998) extended their prior observation with eNOS gene transfer to normal carotid arteries to those of the carotid arteries of Watanabe heritable hyperlipidemic (WHHL) rabbits that display mild to moderate atherosclerotic lesions. Using an *ex vivo* organ culture model, the carotid arteries from WHHL rabbits and from New Zealand White (NZW) rabbits were incubated with either Adβgal or AdeNOS (3×10^9 pfu per artery) for 2 hours. Histochemistry for β-galactosidase and eNOS expression revealed similar patterns of staining among the vessels, with staining of the endothelial and adventitial cells but not the smooth muscle cells. However, expression of eNOS by Western blot analysis was greater in the WHHL than in the NZW carotid

arteries. The effect of eNOS transgene overexpression was analyzed by recording isometric tension in the vessel rings following infection. After precontraction with phenylephrine, acetylcholine produced less relaxation in the atherosclerotic (WHHL) compared to the control (NZW) vessels. However, both atherosclerotic (WHHL) and normal vessels (NZW) following eNOS gene transfer displayed greater relaxation to acetylcholine than control infected vessels. The enhanced relaxation was inhibited with N^{ω}-nitro-L-arginine. This was the first report utilizing adenovirus-mediated eNOS gene transfer to restore NO-mediated responses to acetylcholine in atherosclerotic rabbit carotid arteries. It will be important to examine the long-term consequences of such gene transfer to the arterial wall. Additionally, there may be other added benefits to this form of gene delivery, as some have speculated that long-term overexpression of eNOS in atherosclerotic vessels may lead to regression of existing plaques as a result of the known antiplatelet, antileukocyte, and antiproliferative effects of NO.

Coronary Artery

In 1997, Cable *et al.* investigated whether porcine coronary arteries could be transduced with an adenoviral vector encoding the eNOS gene (Cable *et al.,* 1997a). Using 5×10^9 pfu/ml of AdeNOS or the control adenoviral vector carrying the β-galactosidase gene (AdLacZ) the investigators infected porcine coronary arteries *ex vivo.* Localization studies revealed that endothelial and adventitial cells stained positive for β-galactosidase in AdLacZ-infected vessels, or eNOS in AdeNOS-infected vessels, but that medial smooth muscle cells were not infected. The investigators then performed nitrite measurements and isometric tension recordings. AdeNOS-infected segments produced significantly higher levels of nitrite and displayed greater relaxation to A23187, both of which effects were reversed with NG-monomethyl-L-arginine (L-NMMA). Since this was an *ex vivo* model, it will be important to determine the response of eNOS gene delivery to coronary arteries *in vivo,* perhaps by utilizing a percutaneous endovascular technique, as this will afford us knowledge more applicable to clinical scenarios.

Cerebral Arteries

Chen *et al.* were the first to demonstrate the feasibility of eNOS gene transfer to cerebral arteries using *ex vivo* gene transfer to rings of canine basilar artery (Chen *et al.,* 1997a). They delivered recombinant eNOS in an adenoviral vector using three different titers (10^9, 10^{10}, and 10^{11} pfu/ml). They found that arterial segments infected with AdeNOS demonstrated concentration-dependent reduction in the contractile responses to UTP and enhanced endothelium-dependent relaxation to the calcium ionophore A23187. They also demonstrated the feasibility of *in vivo* eNOS gene transfer to the cerebrovascular system via the cerebrospinal fluid (CSF) (Chen *et al.,* 1997b). Given that intraluminal gene transfer to the cerebral circulation is limited by risks of cerebral ischemia, the importance of demonstrating efficacy of adventitial vascular gene therapy to the cerebral circulation becomes evident. After injecting AdeNOS into the canine CSF via an intracisternal injection using 1×10^9 pfu/ml, they demonstrated increased expression of the eNOS protein in the adventitial fibroblasts of the vascular wall of all major cerebral arteries 24 hours following infection. Next, they demonstrated that AdeNOS-transduced arteries produced significantly higher levels of cGMP compared to AdLacZ-transduced arteries, and that AdeNOS-transduced arteries displayed increased bradykinin-induced relaxations. Both of these findings were reversed with L-NMMA, an inhibitor of eNOS, and the cGMP release was reversed in the absence of calcium, suggesting that these effects were not due to the upregulation of iNOS. Hence, these investigators showed that perivascular eNOS gene transfer is possible to the cerebrovascular circulation and does not require cessation of cerebral blood flow.

In 1998, Tsutsui *et al.* evaluated the efficacy of *ex vivo* gene transfer of eNOS to canine basilar, coronary, and femoral arteries, with and without endothelium, to determine if genetically modified adventitial fibroblasts could restore NO production and regulate vascular tone. Twenty-four hours following *ex vivo* gene transfer, staining for eNOS was predominately localized to adventitial fibroblasts, however, there was some staining of endothelial cells. Basilar artery staining was greater than that in both coronary and femoral artery. With the endothelium intact, bradykinin-induced relaxations were augmented in basilar arteries infected with AdeNOS compared to controls. However, coronary and femoral arteries infected with AdeNOS did not display augmented relaxation. Following removal of the endothelium in the basilar artery, AdeNOS infection still resulted in augmented relaxation to bradykinin. This study reveals two important findings. First, the efficiency and biological response from *ex vivo* adenoviral gene transfer was markedly greater in cerebral arteries than in peripheral arteries. Second, adventitial fibroblasts genetically modified with the eNOS gene were capable of modulating vasomotor tone in the cerebral circulation. This provides important insights concerning the role adventitial fibroblasts may play in maintaining or regulating vascular tone following gene transfer.

The etiology of vasospasm following subarachnoid hemorrhage (SAH) is poorly understood. However, impairment of the L-arginine–NO pathway as a result of increased oxyhemoglobin production has been implicated (Katusic *et al.,* 1993). Additionally, several investigators have shown that supplemental NO in the form of intravenous nitroglycerine (Frazee *et al.,* 1981), intracarotid NO infusion (Afshar *et al.,* 1995), or intracisternal injection of L-arginine or superoxide dismutase (Kajita *et al.,* 1994) can reverse cerebral vasospasm following SAH. Given that the cerebral vasospasm occurs 4 to 12 days after an incident of SAH, attempts to overexpress eNOS to the cerebral vasculature using an adenoviral vector would be ideal. This hypothesis was investigated by Onoue *et al.* (1998), who transferred eNOS using an adenoviral vector to canine basilar arteries *ex vivo* obtained from control dogs and dogs subjected to SAH. They

found that gene expression was twofold higher in arteries from animals suffering from SAH than in normal arteries and that the transgene localized primarily to the adventitia. Relaxation to bradykinin was augmented in arteries from both normal and SAH animals following AdeNOS infection. Additionally, the presence or absence of endothelium did not affect this augmentation, and NOS inhibitors reversed the beneficial effects of eNOS gene transfer, indicating NO-specific effects. Thus, *ex vivo* adventitial eNOS gene transfer was able to restore vasomotor tone to spastic arteries resulting from SAH in dogs. This may prove to be a feasible form of therapy for the alleviation of the devastating complications of cerebral vasospasm associated with subarachnoid hemorrhage.

Systemic Administration

Given the role NO plays in maintaining vasomotor tone, it is logical to hypothesize that systemic delivery of eNOS would attenuate hypertension in spontaneously hypertensive rats (SHR). Lin *et al.* (1997) delivered a single injection of naked eNOS cDNA through the tail vein of SHR and monitored blood pressure, urinary and serum levels of cGMP and nitrite/nitrate, heart rate, body weight, food and water intake, and urine output. The investigators found that systemic delivery of eNOS increased the production and excretion of cGMP and nitrite/nitrate, and was associated with a significant reduction in systolic blood pressure (181.5 $\pm$ 1.90 eNOS cDNA versus 195.5 $\pm$ 1.2 control) while the other parameters were unaffected. A single reinjection of eNOS cDNA prolonged the beneficial effects for up to 12 weeks. The authors did not evaluate where the transgene was being expressed or determine if certain vascular beds were more susceptible to genetic modulation than others. This may have significant implications for future studies. However, based on these results, the authors concluded that a single intravenous injection of eNOS cDNA may be efficacious in compensating for the dysfunction in the L-arginine–NO pathway in patients with essential hypertension.

Neuronal Nitric Oxide Synthase

Gene transfer of nNOS to the vasculature has also been shown to affect vasomotor tone. Channon *et al.* in 1996 delivered an adenoviral vector encoding the rat nNOS cDNA to human VSMCs and human umbilical vein endothelial cells (HUVECs). Cells infected with AdnNOS expressed increased levels of nNOS protein and exhibited marked NO production in response to A23187, acetylcholine, or bradykinin. Supplementation with the tetrahydrobiopterin precursor sepiapterin further enhanced NOS activity in all cells. To extend these observations to an *in vivo* model, the same investigators delivered a low titer of AdnNOS (6×10^8 pfu for 20 min) to carotid arteries of normal and cholesterol-fed rabbits (Channon *et al.,* 1998). In normal carotid arteries, AdnNOS enhanced endothelium-dependent vascular relaxation to acetylcholine (control 47 $\pm$ 6% versus AdnNOS 86 $\pm$ 4% reduction in precontracted tension). In the cholesterol-fed rabbits, baseline acetylcholine-induced relaxation was impaired. However, this abnormality was nearly completely corrected following delivery of nNOS to the carotid artery. Thus, both eNOS and nNOS gene transfer successfully restored vasomotor tone in normal and atherosclerotic arteries in different animal models. These studies taken together provide significant evidence that NOS overexpression is beneficial in this setting and that clinical trials should be performed to critically evaluate this form of gene therapy in both normal and atherosclerotic human vessels.

Pulmonary Hypertension

Nitric oxide has been shown to be an important regulatory molecule in the pulmonary vasculature. NO has a number of biological effects that are relevant in the lung: vasodilation, bronchodilation, and inhibition of platelet aggregation, leukocyte adhesion, and pulmonary smooth muscle cell proliferation (Assender *et al.,* 1992; Moncada and Higgs, 1993). NO is an important vasoactive substance that regulates pulmonary blood blow and maintains a low vascular resistance (Roberts *et al.,* 1995). Furthermore, several studies have suggested that NO plays a key role in the pulmonary vascular response to hypoxia. Hypoxia reduces eNOS gene transcription and NO production by transcriptional and posttranscriptional mechanisms (McQuillan *et al.,* 1994). Adnot *et al.* (1991) have shown that chronic hypoxia is associated with loss of NO-mediated relaxation in the pulmonary vasculature. Additionally, patients with pulmonary hypertension were shown to have less eNOS mRNA and protein, and the levels of eNOS mRNA and protein inversely correlated with the severity of pulmonary hypertension (Giaid and Saleh, 1995). Collectively, these results suggest that hypoxia-induced pulmonary hypertension may reflect a deficit in NO activity within the pulmonary vascular endothelium.

A therapeutic increase of NO activity within the lung may be a reasonable strategy in management of pulmonary hypertension. The additive benefits of the anti-inflammatory and bronchodilatory actions associated with NO may also be beneficial in difficult diseases such as obliterative bronchiolitis occurring post-lung transplantation. Gene transfer of NOS into lungs to restore deficient NO activity has the advantage of acting locally and may avoid hypotensive side effects that can occur with systemic NO delivery. Janssens *et al.* (1996) examined the efficacy of adenoviral-mediated eNOS gene transfer in attenuating hypoxic pulmonary vasoconstriction. Aerosol treatments with 3×10^9 pfu of a recombinant adenovirus containing the eNOS cDNA (AdCMV eNOS) to rats resulted in eNOS gene expression in alveoli and pulmonary vasculature as confirmed 4 days postinfection. Concomitant increases in NO activity and cGMP levels were detected. When subjected to hypoxia, the pulmonary artery pressures were significantly attenuated in the AdCMVeNOS-treated rats while systemic blood pressure remained unaffected. These results suggested that eNOS gene

transfer may have therapeutic utility in attenuating hypoxia-induced pulmonary hypertension.

Portal Hypertension

Portal hypertension is a common and significant complication in end-stage liver disease. The elevated portal pressure is a result of increase resistance in the portal venous system and hyperdynamic blood flow. Both fibrosis and active vasoconstriction are important in augmenting intrahepatic resistance in cirrhotic livers (Bhathal and Grossman, 1985). Hepatic stellate cells play an important effector role in maintaining vascular resistance, and endothelium-derived NO has shown to be a critical vasodilator in this setting (Rockey, 1997). Fevery and colleagues (1998) have investigated cirrhotic livers in rat and in humans and found decreased eNOS expression when compared with normal livers. To determine the impact of eNOS levels on portal hypertension, eNOS gene transfer was performed in cirrhotic rat livers using AdeNOS injected into the portal vein. Immunohistochemical analysis confirmed recombinant eNOS expression in sinusoidal-lining cells and this expression was associated with a reduction in mean portal pressures. These results indicate the importance of hepatic NO synthesis in regulating intrahepatic vascular resistance and the potential utility of NOS gene therapy in the palliation of portal hypertension associated with a variety of liver disorders.

Arterial Injury

Restenosis following percutaneous angioplasty remains a significant clinical dilemma. Approximately 30–50% of patients who have undergone angioplasty develop hemodynamically significant stenosis within 3–6 months as a result of intimal hyperplasia. Intraluminal stents have impacted on the patency rates, but restenosis and intimal hyperplasia remain problems. In addition, long-term patency of coronary artery bypass grafts and lower extremity vein grafts remains poor because of intimal thickening at sites of anastomoses. Current therapeutic modalities directed toward preventing this process, including antithrombotics, antioxidants, antiproliferative agents, and irradiation, have only offered limited benefit. Significant time and effort are being invested into the study of the arterial injury response and elucidating the mechanism of intimal hyperplasia. The rat carotid artery has served as the primary animal model in studying this problem because injury can be easily induced, and the cascade of cellular events has become fairly well understood. Since the 1970s, this simple model has advanced our knowledge of the vascular injury response considerably. The work of investigators such as Clowes, Reidy, Schwartz, Ross, and Nabel, has paved the way for the tremendous amount of research currently devoted to this area of study. Understanding the processes that occur following arterial injury will provide a better understanding of the logic behind any vascular gene therapy project.

The Vascular Injury Response

Arterial injury, either through balloon angioplasty or as a result of surgical intervention, causes endothelial denudation with possible rupture of the internal elastic lamina (IEL) and damage of the underlying medial SMCs (for reviews, see Clowes, 1997, Clowes *et al.,* 1983a,b, 1986, Reidy, 1992, Reidy *et al.,* 1992, and Ross, 1993). The exposed IEL and SMCs provide a nidus for platelet aggregation (Badimon *et al.,* 1998). Leukocyte adherence follows (Guzman *et al.,* 1995; Skantze *et al.,* 1998), and an array of cytokines and growth factors which have multiple effects are secreted. Basic fibroblast growth factor (bFGF) release is responsible for the first wave of smooth muscle cell proliferation following injury (Lindner *et al.,* 1991; Lindner and Reidy, 1991). Platelet-derived growth factor (PDGF) is mainly responsible for the migration of SMCs from the media to the intima, where the cells continue to proliferate (Fingerle *et al.,* 1989). Transforming growth factor β (TGF-β) potently stimulates interstitial collagen gene expression by human SMCs and results in extracellular matrix deposition (Liau and Chan, 1989). Although these growth factors have different mechanisms of action, there is tremendous redundancy in this regulatory process, as evidenced by studies in which just one growth factor was inhibited with either antibodies or ASOs, with intimal thickening being only partially inhibited.

One of the earliest transcriptional events associated with growth factor stimulation is the expression of nuclear protooncogenes. As early as 30 min postinjury, c-*myc,* c-*fos,* and c-*jun* have been shown to be upregulated in aortic SMCs (Miano *et al.,* 1990). This response persists for only a few hours (Hamon *et al.,* 1995). The products of these oncogenes appear to be transcriptional regulatory proteins that are involved in the G1 phase of the cell cycle and are necessary for progression to the S phase (Pardee, 1989). Therefore, it can be reasoned that gene therapy directed to counter the effects of these protooncogenes could alter the vascular injury response in a favorable manner.

Activation of SMCs by growth factors leads to a change in the SMC phenotype from a contractile to a synthetic phenotype. Medial SMC proliferation begins as early as 24 hours following injury and continues to occur for at least 2 weeks. For SMCs to migrate from the media to the intima, the extracellular matrix and IEL must undergo some degree of degradation. Plasminogen activators, which can lyse clots and activate matrix-degrading enzymes, are upregulated along with matrix metalloproteinases, which degrade collagen and elastin. SMCs then migrate to the intima between 1 and 3 days following injury where they continue to proliferate for several weeks leading to the development of intimal thickening. Concurrently, endothelial regeneration occurs through the stimulation of bFGF within 24 hours after injury and can continue for 6–10 weeks (Lindner *et al.,* 1990). Last, following injury there is a marked upregulation of the

genes that encode for extracellular matrix proteins such as collagen and elastin. Deposition of the extracellular matrix is a significant component of the vascular remodeling that occurs after injury. The end result of this complex process is the development of intimal hyperplasia at the site of arterial injury, and this encroachment of lumen may be alleviated by vascular remodeling with a compensatory increase in vessel diameter.

Vascular gene therapy projects have focused on methods to retard the arterial injury response. Inhibition of any one of the pathways described above may reduce intimal hyperplasia. However, the most successful approaches are ones that target central mechanisms. Gene transfer of NOS is attractive because NO can inhibit many different aspects of the injury response (Fig. 1). In addition to inhibiting platelet aggregation (Radomski *et al.,* 1987a,b) and leukocyte adherence (Kubes *et al.,* 1991) to the injured vasculature, NO inhibits VSMC proliferation and migration (Garg and Hassid, 1989). NO also promotes endothelial cell growth, protects them from lipopolysaccharide (LPS) and cytokine-induced apoptosis (Dimmeler *et al.,* 1997; Tzeng *et al.,* 1997), and has been shown to affect vascular remodeling (Rudic *et al.,* 1998).

Endothelial Nitric Oxide Synthase

One common event that occurs following arterial injury is endothelial disruption. This leads to the loss of the normal source of eNOS and with it the vasoprotective properties of NO. Gene therapy approaches using different NOS isoforms are being utilized to restore or increase NO synthesis at the site of injury in order to attenuate the arterial injury response (Table IV).

In 1995, von der Leyen *et al.* were the first to deliver eNOS cDNA using HVJ-modified liposomes to rat carotid arteries intralumenally following balloon induced injury. This eNOS gene transfer resulted in enhanced relaxation to calcium ionophore A23187 4 days following injury infection as well as a 70% reduction in the neointima/media ratio by 14 days postinjury. Other investigators have subsequently delivered the eNOS gene to the vasculature. Janssens *et al.* delivered an adenovirus encoding eNOS cDNA to rat carotid arteries following balloon-induced injury (Janssens *et al.,* 1998). AdeNOS-infected arteries experienced a 72% reduction in the intima/media ratio by 2 weeks postinjury, which was associated with a reduction in the 5-bromo-2'-deoxyuridine (BrdU) labeling index (29 $\pm$ 6% versus 45 $\pm$ 4%, $p < 0.05$). Chen *et al.* (1998) used a retrovirus to deliver eNOS to syngeneic rat arterial SMCs. These cells demonstrated increased cGMP release and decreased DNA synthesis and cell proliferation. These SMCs were then seeded onto the luminal surface of balloon-injured rat carotid arteries. Two weeks after SMC seeding, vessels treated with eNOS-engineered SMCs displayed a 37% reduction in neointimal thickness and a threefold increase in vessel diameter. Orally administered NOS inhibitor reversed these effects.

Another vascular bed that is subjected to injury as result of therapeutic maneuvers is the coronary vascular bed. To determine if coronary artery SMCs could support eNOS gene transfer, Kullo *et al.* infected cultured porcine coronary artery SMCs with AdeNOS (Kullo *et al.,* 1997c). AdeNOS-infected cells demonstrated increased nitrite production, increased cGMP release, and inhibited cellular proliferation. Recently, Varenne *et al.* (1998) performed *in vivo* eNOS gene transfer to porcine coronary arteries following angioplasty and demonstrated reduction in neointimal thickness and percent stenosis as compared with control vessels (0.75 $\pm$ 0.21 vs. 1.04 $\pm$ 0.25 mm, $p = 0.019$; 53 $\pm$ 15% vs. 75 $\pm$ 11%, $p = 0.006$, respectively). Therefore, the coronary bed appears to be receptive to eNOS gene transfer with a reduction in intimal hyerplasia and lumenal stenosis. Percutaneous delivery of an adenovirus encoding eNOS to coronary arteries following balloon angioplasty is feasible as well as efficacious.

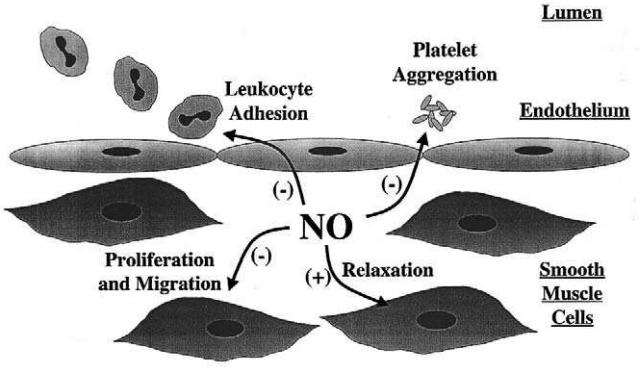


Figure 1 Beneficial effects of nitric oxide (NO) in the vasculature. NO exerts its protective effects in the vasculature by several mechanisms: (1) induction of vasorelaxation, (2) inhibition of smooth muscle cell proliferation and migration, (3) inhibition of platelet aggregation, and (4) inhibition of leukocyte adhesion.

Inducible Nitric Oxide Synthase

Inducible NOS is capable of producing much greater quantities of NO in a sustained, calcium-independent fashion as compared to eNOS. With this increased enzymatic specific activity, the use of iNOS for gene therapy may be advantageous over eNOS. Theoretically, lower levels of iNOS gene transfer would be necessary to achieve the same level of NO production generated by eNOS gene delivery. This is an important consideration when performing gene delivery using viral-based vectors to minimize patient exposure and to reduce the likelihood of an inflammatory reaction. In 1996, Tzeng *et al.* delivered retroviral-mediated human iNOS to isolated porcine arterial segments *ex vivo* (Tzeng *et al.,* 1996b). Vessels infected with iNOS produced more nitrite and cGMP compared to vector-infected vessels. Additionally, myointimal thickening following balloon catheter-induced injury was completely inhibited by 1% iNOS gene transfer. The investigators demonstrated a dependency of the iNOS enzyme on its cofactor tetrahydrobiopterin (BH_4). BH_4 is required for the NOS enzyme to dimerize into an active

Table IV Nitric Oxide Synthase Gene Transfer for Arterial Injury

Author	Gene	Vector	Titer	Model	Effect
von der Leyen *et al.* (1995)	Bovine eNOS	HVJ[a]	30 μg/ml	Rat carotid artery	70% reduction in neointimal area
Janssens *et al.* (1998)	Human eNOS	Adenovirus	0.9×10^{10} pfu[b]	Rat carotid artery	72% reduction in I/M[c] area ratio
Chen *et al.* (1998)	Human eNOS	Retrovirus	5×10^5 cfu[d]/ml	Transfected RASMCs[e] seeded onto the rat carotid artery	37% reduction in neointimal area
Varenne *et al.* (1998)	Human eNOS	Adenovirus	1.5×10^9 pfu	Pig coronary artery	28% reduction in maximal neointimal thickness
Shears *et al.* (1998)	Human iNOS	Adenovirus	2×10^6 pfu	Rat carotid artery	98% reduction in I/M area ratio
Shears *et al.* (1998)	Human iNOS	Adenovirus	5×10^8 pfu	Pig iliac artery	52% reduction in I/M area ratio

[a] HVJ, hemagglutinating virus of Japan.
[b] pfu, plaque-forming units.
[c] I/M, intimal/media.
[d] cfu, colony-forming units.
[e] RASMCs, rat aortic smooth muscle cells.

quaternary structure (Tzeng *et al.,* 1995). Hence, if the supply of BH_4 is low, iNOS function will not be optimized. By providing excess exogenous BH_4, Tzeng *et al.* demonstrated greater nitrite and cGMP production compared to cells transduced with the iNOS gene alone (Tzeng *et al.,* 1996c). Another method to increase the supply of BH_4 is to overexpress the rate-limiting enzyme involved in the synthesis of BH_4, namely, guanosine triphosphate cyclohydrolase I (GTPCH). Delivery of human GTPCH to rat aortic SMC and 3T3 cells infected with a retroviral iNOS vector resulted in increased NO production in these BH_4-deficient cell types (Tzeng *et al.,* 1996c). Thus, it is possible to maximize the efficiency of the iNOS enzyme through gene transfer of the GTPCH gene or by providing exogenous BH_4. Whether this will be a significant concern with *in vivo* studies remains to be determined.

Shears *et al.* (1998) demonstrated that *in vivo* adenoviral delivery of the human iNOS gene (AdiNOS) to injured rat and pig arteries was efficacious in preventing the development of intimal hyperplasia. First, AdiNOS transferred intraluminally to the rat carotid artery following balloon-induced injury was performed using very low titers of virus (2×10^6 pfu/artery) as compared to other gene therapy studies. Evaluation of the carotid arteries 2 weeks following injury and AdiNOS infection showed a 96.7% inhibition of intimal hyperplasia. This effect was NO dependent. Additionally, the inhibition of intimal hyperplasia persisted in animals sacrificed 6 weeks following injury and infection. The efficacy of iNOS gene transfer was also assessed in a model more relevant to humans, namely, the pig iliac artery injury model. Delivery of AdiNOS, again in low titers (5×10^8 pfu/artery), resulted in a 51.8% reduction in the intima/media area ratio in the vessels. This study demonstrated that infection with very low titers of AdiNOS was able to have profound effects in two different animal models of arterial injury. For clinical trials, one can see why it would be advantageous in many ways to be able to deliver such low titers. Demand versus supply in the production of adenoviral vectors encoding NOS enzymes is one very important issue that would be mitigated by using such low titers. Additionally, the concern of evoking the host immune response would be lessened using such low titers. Therefore, both eNOS and iNOS gene transfer to the vasculature have proved successful in different animal models of arterial injury. Because the arteries that will be targeted for these therapies will not be normal, these same experiments must be repeated in atherosclerotic vessels to fully evaluate the efficacy of NOS gene therapy in inhibiting intimal hyperplasia.

Vein Graft Intimal Hyperplasia

Late vein graft failure has been a daunting problem since bypass surgery was first developed. Approximately 25% of peripheral artery vein bypass grafts fail within the first 5 years (Taylor *et al.,* 1990), and 32% of coronary artery bypass grafts fail within 10 years (Lawrie *et al.,* 1991). The etiology of this late failure is due to the development of intimal hyperplasia or progression of the underlying atherosclerotic vascular disease (FitzGibbon *et al.,* 1986; Karayannacos *et al.,* 1978). The pathophysiology of vein graft intimal hyperplasia is poorly understood. However, vein grafts have impaired endothelium-dependent relaxation when implanted into the arterial circulation (Cross *et al.,* 1988; Miller *et al.,* 1987). Likewise, supplemental dietary L-arginine resulted in the reduction of atherosclerotic lesions and intimal hyperplasia with preservation of vasomotor function in animal models of vein grafts (Davies *et al.,* 1994, 1995).

Given the impairment of the NO pathway in vein grafts and the finding that NOS gene transfer successfully inhibits neointimal formation following balloon injury in arteries, it was hypothesized that overexpression of eNOS in vein grafts may attenuate the development of intimal hyperplasia and restore vasomotor function. Cable *et al.* exposed segments of human saphenous vein to AdeNOS in an *ex vivo* organ culture system (Cable *et al.,* 1997b). Immunohistochemical staining for eNOS localized enzyme expression in the endo-

thelium and the adventitia of the veins. Nitrite generation following stimulation with a calcium ionophore increased in segments infected with AdeNOS, but not in control segments. On examination of isometric tension recordings 48 hours following infection, vein segments infected with AdeNOS demonstrated augmented relaxation to calcium ionophore when compared to control vein segments. Hence, gene transfer of eNOS to vein segments using this *ex vivo* model was able to regulate vasomotor function.

More important will be the examination of vasomotor function following eNOS gene transfer *in vivo* over a longer period of investigation. Whether overexpression of eNOS in vein segments placed into the arterial circulation will result in persistent biological function has yet to be determined. This is important because, as noted, the endothelial cells and adventitial cells were transduced. A vein segment placed in the arterial circulation typically loses its endothelial cell layer through cell involution and denudation before becoming re-endothelialized (Ramos *et al.,* 1976). Hence, it is possible that only the adventitial cells initially infected with eNOS would remain to produce NO, and whether this is sufficient to effect a biological response needs to be investigated. Matsumoto *et al.* (1998) performed *in vivo* experiments with eNOS gene transfer to vein grafts. These investigators transferred bovine eNOS cDNA encapsulated in HVJ–liposomes to femoral veins of dogs using pressure-mediated distension (100 mm Hg) of the vein grafts for 10 min. These veins were then implanted as a reverse vein graft in an end-to-end fashion to the ipsilateral femoral artery. Under conditions of surgically created poor distal run-off, veins infected with the eNOS gene displayed a 55% reduction in neointimal area compared to sham-treated grafts evaluated 4 weeks following surgery. Using this model, the investigators found gene expression in the medial SMCs and adventitial cells in the vein wall. Thus, this study provides evidence that eNOS overexpression in vein grafts can affect long-term parameters, such as vein graft intimal hyperplasia. Evaluating these vein grafts at 1 year will provide even more important data, as the pathophysiology of the vein graft intimal hyperplasia is quite different from intimal hyperplasia following isolated arterial injury. Vein grafts are exposed to the continuous insult of the arterial circulation. The development of medial hypertrophy to a certain extent is desirable in the arterialization of the vein, and inhibition of this process may have detrimental sequela. Therefore, while this form of gene therapy has proved to be quite successful in these models, much remains to be learned about the long-term effects of NOS gene therapy in vein grafts.

Transplant Arteriosclerosis

Despite the success of modern cardiac transplantation, the development of accelerated coronary arteriosclerosis, also known as transplantation vasculopathy, has become a major obstacle to long-term allograft survival. It is the major cause of allograft dysfunction and late death in patients (Kaye, 1993; Weis and von Scheidt, 1997). Unfortunately, patients usually remain asymptomatic until the arteriosclerosis is advanced, manifested clinically by the development of ventricular arrhythmias, congestive heart failure, or sudden death. In a multi-institutional study by Costanzo and colleagues, the likelihood of angiographically visible allograft arteriosclerosis at 1, 2, and 4 years was 11, 22, and 45%, respectively (Costanzo *et al.,* 1998). This progressive disease is characterized by diffuse narrowing of the coronary arteries due to intimal hyperplasia as a result of abnormal medial SMC proliferation followed by migration to the intima (Tanaka *et al.,* 1995).

Although the exact pathogenesis of allograft arteriosclerosis has not been determined, several experimental studies indicate that it is an immune-mediated disease. Incompletely suppressed cellular and humoral immune responses directed toward disparate major histocompatibility antigens expressed on the endothelial surfaces of the allograft initiate an immune-mediated endothelial injury (Russell *et al.,* 1994a,b). This injury is then followed by the infiltration of activated macrophages and lymphocytes into the vessel wall and the initiation of the inflammatory cascade. Endothelial damage leads to exposure of the underlying SMCs to various mitogenic growth factors (Davis *et al.,* 1996). These activated SMCs are then converted from a normal, contractile nonproliferating phenotype to a secretory, proliferating phenotype (Suzuki *et al.,* 1995). The final pathologic lesion observed in the neointima of allograft arteriosclerosis consists of concentric intimal accumulation of SMC and late intimal fibrosis.

NO has been implicated as a potential mediator in each of the steps involved in the development of the transplant arteriosclerotic lesion. Russell *et al.* (1995) demonstrated in the Lewis to F344 rat cardiac transplantation model that iNOS transcription is significantly upregulated in cardiac allografts during both the acute and chronic stages of rejection extending from day 7 to day 75 posttransplantation. At all time points, iNOS was detected predominantly in the mononuclear inflammatory cells within the interstitium and perivascular spaces of the allograft. Akyurek *et al.* (1996) reported that iNOS expression was increased in the intima and adventitia of arteriosclerotic vessels in a rat aortic transplant model. Colocalization studies identified SMCs and macrophages as the cells expressing iNOS. Expression of iNOS in the media decreased over time, presumably due to the migration of medial SMCs to the intima. In a separate study by Lafond-Walker and colleagues (1997) examining coronary arteries of transplanted human hearts with accelerated allograft arteriosclerosis, iNOS mRNA and protein were found to be upregulated in macrophages. Despite these animal and human studies showing that iNOS is upregulated in transplant arteriosclerosis, the consequences of this upregulation remained unclear.

Adverse Effects of Nitric Oxide

Inducible NOS has been shown to contribute to the inflammatory process mediating transplant arteriosclerosis. Russell *et al.* (1995) modulated the inflammatory response

in rats with a diet deficient in essential fatty acids and reported significant reduction in the intimal thickening and percentage of diseased vessels in cardiac allografts. There was a correlated decrease in iNOS mRNA levels, suggesting that NO contributes to the inflammatory response mediating transplant arteriosclerosis. Indeed, high levels of NO has been shown to be cytotoxic to neighboring cells or even to the cells that are producing it (Moncada *et al.,* 1991). The targets of such cytotoxic actions have shown to be the iron-centered enzymes involved in mitochondrial respiration, aconitase activity, and DNA synthesis (Dusting and Macdonald, 1995). Another mechanism for NO induced cytotoxicity is through its interaction with superoxide anion (O_2^-). A product of the reaction between these two free radicals is the highly toxic peroxynitrite anion ($ONOO^-$), capable of initiating free radical-mediated lipid peroxidation and sulfhydryl oxidation (Beckman *et al.,* 1990). Peroxynitrite has been shown to induce permanent vascular dysfunction in isolated hearts (Villa *et al.,* 1994). Furthermore, addition of superoxide dismutase prevented peroxynitrite formation as well as vascular endothelial injury (Beckman *et al.,* 1990).

Cytoprotective Effects of Nitric Oxide

Despite the potential adverse effects of NO, there is a large body of literature indicating that NO is protective against intimal hyperplasia following arterial injury and in transplant arteriosclerosis. These protective effects are attributed to the ability of NO to inhibit SMC migration and proliferation (Garg and Hassid, 1989; Sarkar *et al.,* 1996) as well as to inhibit platelet (Mellion *et al.,* 1983) and neutrophil adhesion (Kubes *et al.,* 1991) (Fig. 1). Lou and coworkers demonstrated in a rabbit heterotopic heart transplantation model that dietary supplementation with L-arginine significantly attenuated intimal hyperplasia in allograft coronary arteries, presumably by suppressing SMC proliferative response (Lou *et al.,* 1996).

In transplant arteriosclerosis, immune-mediated injury to the vascular endothelium might induce the expression of adhesion molecules such as selectins and integrins on the endothelial cells. The adhesion molecules interact with ligands on platelets and leukocytes, and these cell–cell interactions can contribute to subsequent thrombus and lesion formation. NO may help maintain the integrity of the vascular endothelium by inhibiting adhesion molecule expression. NO has direct platelet actions by inhibiting ADP-induced human platelet aggregation, which is mediated by a cGMP-dependent mechanism (Mellion *et al.,* 1983). Furthermore, NO produced from platelets during platelet aggregation inhibits the extent of further platelet activation (Broekman *et al.,* 1991). These studies suggest that NO may protect against thrombus formation on the endothelium after transplantation. Conversely, impaired NO activity in damaged endothelium predisposes to formation of thrombi. NO has been shown *in vitro* and *in vivo* to attenuate leukocyte adhesion and chemotaxis, important steps in arteriosclerotic formation. Enhanced leukocyte adhesion to venular endothelium during infusion of NOS inhibitors has been reported, and this effect was reversed by administering L-arginine or an antibody to the leukocyte adhesion complex CD11b/CD18 (Kubes *et al.,* 1991). In another study, NO inhibited monocyte chemotaxis *in vitro* via a cGMP-dependent pathway (Bath, 1993). These observations, along with others, suggest that NO blocks inflammatory cell adhesion and migration into the intimal space, thereby limiting the deleterious effects of the inflammatory cascade and development of transplant arteriosclerosis.

Inducible Nitric Oxide Sythase Gene Therapy

It is possible that iNOS-mediated effects on the endothelial and vascular SMC function may differ during acute and chronic conditions (Liu *et al.,* 1998). Although acute iNOS-mediated effects may contribute to endothelial cell injury, NO may play a protective role under chronic conditions, possibly by suppressing intimal SMC accumulation. Indeed, cardiac transplantation into recipient iNOS −/− knockout mice resulted in acceleration of allograft arteriosclerosis (Koglin *et al.,* 1998). These findings suggest that during chronic rejection, iNOS activity protects allografts by suppressing intimal thickening.

Previous experiments performed in our laboratory corroborated these findings. Shears and co-workers (1997) showed in a model of ACI to Wistar Furth rat aortic allograft transplantation that iNOS mRNA and protein as well as intimal thickness were significantly increased by 28 days post-transplantation. Inhibiting NO production with an iNOS inhibitor, L-N^6-(1-iminoethyl)-lysine (L-NIL), increased the intimal width by 57%, indicating that NO participates in suppressing allograft arteriosclerosis. The effects of cyclosporine (CsA) on iNOS expression and allograft arteriosclerosis were also investigated. CsA inhibited iNOS expression in aortic allografts and was also associated with a 65% increase in intimal thickening. These results indicate that even low levels of rejection are associated with an increase in iNOS expression and that this iNOS expression may play a role in limiting the development of intimal thickening so often seen with transplant vasculopathy. Using an adenoviral vector carrying the human iNOS cDNA (AdiNOS) to deliver supraphysiological levels of iNOS and NO to the allograft, allograft arteriosclerosis was dramatically inhibited in recipient animals infected with AdiNOS compared to those infected with AdLacZ. The presence or absence of CsA in the animals infected with iNOS did not affect the beneficial results. This experiment supported that iNOS expression in allografts is important in protecting the allograft and that iNOS gene transfer strategies may have great therapeutic potential in preventing the development of allograft arteriosclerosis.

In contemplating iNOS gene therapy for allograft vasculopathy, the proper gene delivery vector is vitally important. Allograft arteriosclerosis is believed to reflect the outcome of chronic rejection. Chronic rejection, even in the face of appropriate pharmacologic immunosuppression, is an ongoing process for the life span of the allograft. Therefore, iNOS

gene therapy for the prevention of arteriosclerosis would be best served by the use of a vector that permits long-term transgene expression such as retrovirus or AAV. If, however, the pathogenesis of allograft vasculopathy is initiated by an early rejection event that is then propagated by the use of iNOS-inhibiting immunosuppression such as CsA, transient iNOS gene delivery may suffice and provide supplemental NO until vascular healing has occurred. These are all important considerations that must be further evaluated.

Lung Transplant Rejection

Jeppsson *et al.* (1998) investigated the efficiency, distribution, and toxicity of transbronchial adenovirus-mediated eNOS gene transfer to transplanted lungs with the future goal of potentially modulating acute or chronic rejection following lung transplantation. Syngeneic orthotopic single-lung transplantation was performed in rats with transbronchial administration of AdeNOS. Endothelial NOS transgene expression was confirmed by polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR). More than 75% of the pneumocytes stained for eNOS in the AdeNOS-treated group, and a significant increase in NOS activity was also detected. There was no significant difference in the amount of inflammation between control and AdeNOS-treated animals. Therefore, the feasibility of transbronchial eNOS gene transfer to transplanted lungs suggests an alternate and perhaps safer and easier route of AdeNOS administration than via a vascular route. Future studies examining the effects such gene transfer actually has on transplant rejection will further our knowledge on the role of eNOS in this pathophysiological process.

Erectile Dysfunction

Erectile dysfunction, a problem afflicting 10–15 million men in the United States, is mainly a function of inadequate relaxation of the penile corpora cavernosal smooth muscle during sexual stimulation (Murray *et al.,* 1995). NO has been shown to be the main mediator of penile erection (Ignarro *et al.,* 1990; Burnett *et al.,* 1992; Rajfer *et al.,* 1992). NO is released from penile nonadrenergic, noncholinergic nerve terminals and activates soluble guanylyl cyclase, leading to cGMP release in the penile smooth muscle. Cyclic GMP mediates a reduction in intracellular Ca^{2+} and results in relaxation of the penile corpora cavernosal smooth muscle. The engorgement and entrapment of blood in the corporal sinusoids result in penile tumescence. Unraveling the role of NO in penile physiology has led to the development of many pharmacologic agents directed at increasing penile NO activity to treat impotence. The widely publicized and utilized drug Viagra (sildenafil) is a potent phosphodiesterase inhibitor that prevents the degradation of cGMP in the corpus cavernosum, thus prolonging and augmenting the action of endogenously produced NO.

Another therapeutic approach has been directed at augmenting local NO production by stimulating NO synthase. Garban and colleagues (1997) investigated whether high levels of NO production by either cytokine stimulation of iNOS or following iNOS gene therapy can mitigate age-associated erectile dysfunction in the rat. Continuous infusion of iNOS inducers consisting of a mixture of bacterial lipopolysaccharide, rat γ-interferon, tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β) into the penile corpora cavernosa of old rats significantly corrected aging-associated erectile dysfunction. Western blot assays confirmed the upregulation of endogenous iNOS protein within the penile tissue. These investigators subsequently cloned the iNOS cDNA from rat corpora cavernosa and demonstrated that a single intracavernosal injection of an expression plasmid containing the rat penile iNOS cDNA also corrected age-associated erectile dysfunction in rats. Expression of recombinant iNOS was confirmed in cavernosal tissue by PCR. These results demonstrated that NOS gene transfer is a feasible strategy in the management of erectile dysfunction. However, there are concerns about prolonged NOS gene expression with resultant priapism. Mechanisms will have to be devised to control levels of NOS gene expression or NO production in order to prevent such an outcome.

Wound Healing

Attempts to elucidate the factors regulating wound healing have revealed a dependence on arginine metabolism and the NO synthase pathway (Albina *et al.,* 1990). End products of NO synthesis, nitrite and nitrate, were found to be transiently elevated in fluid collected from sponges implanted in subcutaneous wounds (Albina *et al.,* 1990). Systemic administration of arginine, the substrate for NO synthesis, enhanced wound healing (Barbul *et al.,* 1990; Kirk *et al.,* 1993). Topical application of an NO donor has been shown to accelerate closure of excisional wounds in rats (Shabani *et al.,* 1996), whereas NOS inhibitors given systemically (Schaffer *et al.,* 1996) or applied locally to wounds (Bulgrin *et al.,* 1995) have resulted in retardation of wound healing.

Studies have shown that NO plays a role in collagen matrix deposition, a process necessary in healing wounds. In models of impaired wound healing induced by malnutrition or diabetes, reductions in both wound collagen and NO levels were measured (Schaffer *et al.,* 1996, 1997). Moreover, systemic administration of the NOS inhibitor *S*-methylisothiouronium impaired wound collagen accumulation in mice (Schaffer *et al.,* 1996). Thornton and colleagues (1998) investigated the effect of iNOS gene delivery to cutaneous wounds using subcutaneously implanted polyvinyl alcohol sponges instilled with a mammalian expression plasmid (pMP6) containing the murine iNOS cDNA in rats that had received a dorsal midline incision. They observed that cells within the wound had efficient gene transfer with maximal

iNOS expression measured by 48 hours. By 7 days postimplantation, the iNOS expression plasmid-impregnated sponges had significantly greater collagen content than control sponges, implicating the role of NO in enhancing wound collagen deposition.

Previous experiments in our laboratory examined the role of iNOS in wound closure and explored the impact of topical iNOS gene transfer on the rate of wound healing (Yamasaki *et al.,* 1998). In iNOS −/− knockout mice, wound repair was delayed by 31% compared to wild-type mice. A similar delay in wound closure was seen in wild-type mice treated with a continuous infusion of L-NIL. Wound closure rates in iNOS −/− mice could be accelerated to a rate comparable to that in wild-type mice with a single topical application of AdiNOS at the time of wounding. Inducible NOS mRNA expression was confirmed in treated mice by PCR. This study demonstrated a critical role for iNOS in wound repair and the feasibility of topical iNOS gene transfer therapy. Future application of such gene therapy strategy may be possible in the setting of iNOS-deficient states such as in diabetes and during steroid treatment.

Cancer

Nitric oxide has been implicated in both tumor biology and tumor surveillance. It has been shown to be involved in virtually all components of solid tumor growth, such as tumor angiogenesis, infiltrating inflammatory cells, and tumor cells themselves (Vamvakas and Schmidt, 1997). High levels of NO produced from either iNOS expressed in activated macrophages and endothelial cells or exogenous NO donors can have cytotoxic effects on tumor cells. These cytotoxic effects can include inhibition of cell proliferation or the induction of apoptosis (Cifone *et al.,* 1995; Li *et al.,* 1991; Xie *et al.,* 1997a). Supraphysiological levels of NO may impair DNA synthesis, glycolysis, cellular respiration, or the citric acid cycle. NO-induced tumor cytotoxicity may also be mediated through its interaction with superoxide anion (O_2^-) and the generation of the highly toxic peroxynitrite anion ($ONOO^-$). Although peroxynitrite is very reactive, it is stable enough to diffuse to surrounding tumor cells and initiate free radical-mediated lipid peroxidation and sulfhydryl oxidation (Beckman *et al.,* 1990).

A direct correlation between expression of iNOS and regression of metastatic murine sarcoma has been reported (Xie *et al.,* 1995). Furthermore, the induction of iNOS expression by cytokines in the same model induced apoptosis in tumor cells *in vitro* and *in vivo.* Similar NO-mediated apoptosis was observed in metastatic murine K-1735 melanoma. After induction with different cytokines or LPS, primary nonmetastatic tumor cells demonstrate high levels of iNOS expression and NO synthesis, whereas tumor cells isolated from metastatic lesions have a much lower NO expression (Xie *et al.,* 1996). In dramatic contrast to its tumoricidal activities, NO has also been reported to facilitate angiogenesis within tumors and promote tumor growth. Endogenous NO activity has been shown to have a direct correlation with tumor grade in some human carcinomas (Jenkins *et al.,* 1995). Therefore, NO appears to have a dual action on tumor development. The precise effect NO has on a particular tumor may be related to local concentrations, the susceptibility of different tumors to NO, or the pleomorphic regulatory signals acting in these tumors (Vamvakas and Schmidt, 1997). NOS activity was two orders of magnitude lower during conditions when NO promoted tumor growth (Jenkins *et al.,* 1995). By activating differential signaling mechanisms, NO can initiate both tumorigenesis and tumor toxicity (Fig. 2). Low NO concentration might activate soluble guanylyl cyclases with a resulting increase in the second messenger molecule cGMP (Schmidt *et al.,* 1993). This could in turn stimulate the endothelial cell migration and proliferation (Ziche *et al.,* 1994) that are essential to tumor neovascularization and tumor development. Low concentrations of NO can also have a protective effect by preventing apoptosis (Kim *et al.,* 1997). Furthermore, NO may be tumorigenic itself. Nguyen *et al.* (1992) reported that NO can cause mutations by increasing DNA strand breaks and mediating the deamination of *S*-methylcytosine to thymine, and others have implicated increased iNOS activity in the genesis of certain human cancers (Bartsch *et al.,* 1992).

Equipped with the understanding of the dual actions of NO on cancer, one can attempt to achieve a therapeutic potential for NO by maximizing the local NO activity within tumors to inhibit tumor cell proliferation and to induce apoptosis. One way to do this is to supply NO with NO donor compounds. Indeed, Pipili-Synetos *et al.* (1995) showed that isosorbide mononitrate and dinitrate inhibited angiogenesis and tumor growth as well as prevented metastasis. Alternatively, endogenous NO production may be stimulated by induction of iNOS by various cytokines, such as TNF-α. Tumor necrosis factor α can activate both NO and O_2^- pro-

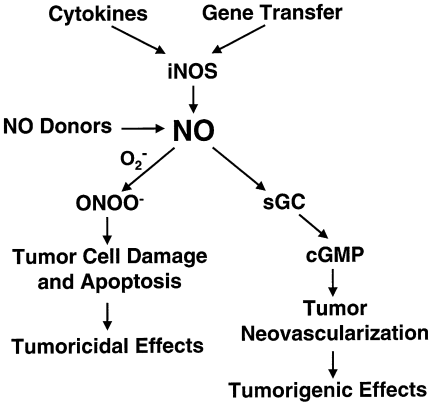


Figure 2 Dual tumoricidal and tumorigenic effects of nitric oxide (NO). NO donors, cytokine, and nitric oxide synthase (NOS) gene transfer therapies can all result in overproduction of NO. Interaction of NO with superoxide anion (O_2^-) yields highly toxic peroxynitrite ($ONOO^-$), ultimately leading to tumor cell killing. However, low levels of NO might activate soluble guanylyl cyclase (sGC), resulting in an increase in cyclic guanosine monophosphate (cGMP) and tumor neovascularization.

duction, providing an environment ideal for the generation of the highly toxic peroxynitrite (McCall and Vallance, 1992). Unfortunately, high-dose cytokine therapy is not a feasible mode of increasing local iNOS expression.

A more practical and direct method of locally increasing NO production is through the transfer of the iNOS cDNA to tumor cells. Transfer of iNOS into tumor cells has been successfully carried out by Xie *et al.* (1995) and by Ambs *et al.* (1998). Ambs *et al.* transfected human carcinoma cells expressing either a functional wild-type p53 or a nonfunctional mutated p53 gene with a retroviral vector carrying the human iNOS cDNA (DFGiNOS) (Tzeng *et al.,* 1995). Transfected cells carrying wild-type p53 and producing NO constitutively exhibited reduced tumor growth in athymic nude mice. Cells carrying mutated p53 and producing NO had accelerated tumor growth. This accelerated tumor growth was associated with increased vascular endothelial growth factor expression and neovascularization. These results suggest that, in some selective tumor cells with mutant p53, NO production might promote their growth. A role for p53 in determining the effect of NO on tumor cells may exist. Treatment of the tumors carrying a mutant p53 with inhibitors of iNOS may have therapeutic potential (Ambs *et al.,* 1998), whereas tumors with a normal p53 gene may be better treated with iNOS gene transfer to augment NO synthesis.

The role of NO and tumor metastasis has been examined. Highly metastatic murine K-1735 melanoma cells, which normally express low levels of iNOS, were transfected with a functional iNOS gene (Xie *et al.,* 1995). Cells overexpressing iNOS were then cultured, harvested, and injected intravenously into mice. The iNOS-transfected cells lost their metastatic potential and underwent apoptosis within 48 to 72 hours after tumor injection. Similarly, human renal carcinoma cells infected with retroviral vectors containing murine macrophage iNOS cDNA were injected into nude mice and produced few if any lung metastases, whereas injection of control cells resulted in large numbers of metastases (Juang *et al.,* 1998). Therefore, this form of gene therapy represents another way to manipulate tumor growth and development.

High levels of endogenous NO cause lysis of bystander cells in addition to autolysis of transfected cells (Xie *et al.,* 1997b). Murine K-1735 C4 parental melanoma cells transfected with an iNOS expression plasmid underwent autolysis that was NO mediated. When these cells were mixed with control cells, the control cells also underwent lysis, demonstrating a bystander effect as a result of the diffusable nature of NO. By engineering a few cells within a tumor to synthesize high levels of NO, tumor lysis may still be induced. These studies of iNOS gene transfer indicate that high local NO production can be achieved in tumor cells and may be used to treat primary tumors and prevent metastatic disease (Juang *et al.,* 1998).

Endothelial NOS gene transfer has also been applied to the treatment of tumors (Liu *et al.,* 1998). Liu *et al.* performed stable transfection of eNOS cDNA in human oral carcinoma cells. Similar to the results achieved with iNOS gene transfer, eNOS-transfected cells had a lower growth rate compared with control cells. When these eNOS-engineered tumor cells were injected into nude mice, tumor growth was retarded during the first 25 days, but then tumor growth exceeded that of control tumors. The ineffectiveness of eNOS gene transfer to limit tumor growth *in vivo* may be due to the lower levels of NO generated as compared with iNOS gene transfer. Alternatively, a different set of regulatory signals can be turned on by eNOS that may favor tumor growth *in vivo*. Further studies are necessary in elucidating these cellular signaling mechanisms.

References

Adnot, S., Raffestin, B., Eddahibi, S., Braquet, P., and Chabrier, P. E. (1991). Loss of endothelium-dependent relaxant activity in the pulmonary circulation of rats exposed to chronic hypoxia. *J. Clin. Invest.* **87,** 155–162.

Afshar, J. K., Pluta, R. M., Boock, R. J., Thompson, B. G., and Oldfield, E. H. (1995). Effect of intracarotid nitric oxide on primate cerebral vasospasm after subarachnoid hemorrhage. *J. Neurosurg.* **83,** 118–122.

Akyurek, L. M., Fellstrom, B. C., Yan, Z. Q., Hansson, G. K., Funa, K., and Larsson, E. (1996). Inducible and endothelial nitric oxide synthase expression during development of transplant arteriosclerosis in rat aortic grafts. *Am. J. Pathol.* **149,** 1981–1990.

Albina, J. E., Mills, C. D., Henry, W. L. J., and Caldwell, M. D. (1990). Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.* **144,** 3877–3880.

Ambs, S., Merriam, W. G., Ogunfusika, M. O., Bennett, W. P., Ishibe, N., Hussain, S. P., Tzeng, E. E., Geller, D. A., Billiar, T. R., and Harris, C. C. (1998). p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. *Nat. Med.* **4,** 1371–1376.

Arnold, T. E., Gnatenko, D., and Bahou, W. F. (1997). In vivo gene transfer into rat arterial walls with novel adeno-associated virus vectors. *J. Vasc. Surg.* **25,** 347–355.

Assender, J. W., Southgate, K. M., Hallett, M. B., and Newby, A. C. (1992). Inhibition of proliferation, but not of Ca^{2+} mobilization, by cyclic AMP and GMP in rabbit aortic smooth-muscle cells. *Biochem. J.* **288,** 527–532.

Babiss, L. E., Ginsberg, H. S., and Darnell, J. E. J. (1985). Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol. Cell. Biol.* **5,** 2552–2558.

Badimon, J. J., Ortiz, A. F., Meyer, B., Mailhac, A., Fallon, J. T., Falk, E., Badimon, L., Chesebro, J. H., and Fuster, V. (1998). Different response to balloon angioplasty of carotid and coronary arteries: Effects on acute platelet deposition and intimal thickening. *Atherosclerosis* **140,** 307–314.

Barbul, A., Lazarou, S. A., Efron, D. T., Wasserkrug, H. L., and Efron, G. (1990). Arginine enhances wound healing and lymphocyte immune responses in humans. *Surgery* **108,** 331–336.

Bartsch, H., Ohshima, H., Pignatelli, B., and Calmels, S. (1992). Endogenously formed *N*-nitroso compounds and nitrosating agents in human cancer etiology. *Pharmacogenetics* **2,** 272–277.

Bath, P. M. (1993). The effect of nitric oxide-donating vasodilators on monocyte chemotaxis and intracellular cGMP concentrations in vitro. *Eur. J. Clin. Pharmacol.* **45,** 53–58.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Berkner, K. L. (1992). Expression of heterologous sequences in adenoviral vectors. *Curr. Top. Microbiol. Immunol.* **158,** 39–66.

Berns, K. I. (1990). Parvovirus replication. *Microbiol. Rev.* **54,** 316–329.

Berns, K. I., and Giraud, C. (1996). Biology of adeno-associated virus. *Curr. Top. Microbiol. Immunol.* **218,** 1–23.

Bhathal, P. S., and Grossman, H. J. (1985). Reduction of the increased portal vascular resistance of the isolated perfused cirrhotic rat liver by vasodilators. *J. Hepatol.* **1,** 325–337.

Broekman, M. J., Eiroa, A. M., and Marcus, A. J. (1991). Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension: Blockade of aggregation and secretion by an aspirin-insensitive mechanism. *Blood* **78,** 1033–1040.

Bulgrin, J. P., Shabani, M., Chakravarthy, D., and Smith, D. J. (1995). Nitric oxide synthesis is suppressed in steroid-impaired and diabetic wounds. *Wounds* **7,** 48–57.

Burnett, A. L., Lowenstein, C. J., Bredt, D. S., Chang, T. S., and Snyder, S. H. (1992). Nitric oxide: A physiologic mediator of penile erection. *Science* **257,** 401–403.

Cable, D. G., O'Brien, T., Kullo, I. J., Schwartz, R. S., Schaff, H. V., and Pompili, V. J. (1997a). Expression and function of a recombinant endothelial nitric oxide synthase gene in porcine coronary arteries. *Cardiovasc. Res.* **35,** 553–559.

Cable, D. G., O'Brien, T., Schaff, H. V., and Pompili, V. J. (1997b). Recombinant endothelial nitric oxide synthase-transduced human saphenous veins: Gene therapy to augment nitric oxide production in bypass conduits. *Circulation* **96,** II-8.

Channon, K. M., Blazing, M. A., Shetty, G. A., Potts, K. E., and George, S. E. (1996). Adenoviral gene transfer of nitric oxide synthase: High level expression in human vascular cells. *Cardiovasc. Res.* **32,** 962–972.

Channon, K. M., Qian, H., Nepliouevа, V., Blazing, M. A., Olmez, E., Shetty, G. A., Youngblood, S. A., Pawloski, J., McMahon, T., Stamler, J. S., and George, S. E. (1998). In vivo gene transfer of nitric oxide synthase enhances vasomotor function in carotid arteries from normal and cholesterol-Fed rabbits. *Circulation* **98,** 1905–1911.

Chen, A. F., O'Brien, T., Tsutsui, M., Kinoshita, H., Pompili, V. J., Crotty, T. B., Spector, D. J., and Katusic, Z. S. (1997a). Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery. *Circ. Res.* **80,** 327–335.

Chen, A. Y., Jiang, S. W., Crotty, T. B., Tsutsui, M., Smith, L. A., O'Brien, T., and Katusic, Z. S. (1997b). Effects of in vivo adventitial expression of recombinant endothelial nitric oxide synthase gene in cerebral arteries. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 12568–12573.

Chen, L., Daum, G., Forough, R., Clowes, M., Walter, U., and Clowes, A. W. (1998). Overexpression of human endothelial nitric oxide synthase in rat vascular smooth muscle cells and in balloon-injured carotid artery. *Circ. Res.* **82,** 862–870.

Cheung, A. K., Hoggan, M. D., Hauswirth, W. W., and Berns, K. I. (1980). Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. *J. Virol.* **33,** 739–748.

Cifone, M. G., Cironi, L., Meccia, M. A., Roncaioli, P., Festuccia, C., De Nuntiis, G., D'Alo, S., and Santoni, A. (1995). Role of nitric oxide in cell-mediated tumor cytotoxicity. *Adv. Neuroimmunol.* **5,** 443–461.

Clowes, A. W. (1997). Vascular gene therapy in the 21st century. *Thromb. Haemostasis* **78,** 605–610.

Clowes, A. W., Reidy, M. A., and Clowes, M. M. (1983a). Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab. Invest.* **49,** 327–333.

Clowes, A. W., Reidy, M. A., and Clowes, M. M. (1983b). Mechanisms of stenosis after arterial injury. *Lab. Invest.* **49,** 208–215.

Clowes, A. W., Clowes, M. M., and Reidy, M. A. (1986). Kinetics of cellular proliferation after arterial injury. III. Endothelial and smooth muscle growth in chronically denuded vessels. *Lab. Invest.* **54,** 295–303.

Cohen, R. A. (1995). The role of nitric oxide and other endothelium-derived vasoactive substances in vascular disease. *Prog. Cardiovasc. Dis.* **38,** 105–128.

Cooke, J. P., Andon, N. A., Girerd, X. J., Hirsch, A. T., and Creager, M. A. (1991). Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta [see comments]. *Circulation* **83,** 1057–1062.

Costanzo, M. R., Naftel, D. C., Pritzker, M. R., Heilman, J. K., Boehmer, J. P., Brozena, S. C., Dec, G. W., Ventura, H. O., Kirklin, J. K., Bourge, R. C., and Miller, L. W. (1998). Heart transplant coronary artery disease detected by coronary angiography: A multiinstitutional study of preoperative donor and recipient risk factors. Cardiac Transplant Research Database. *J. Heart Lung Transplant.* **17,** 744–753.

Cross, K. S., el-Sanadiki, M. N., Murray, J. J., Mikat, E. M., McCann, R. L., and Hagen, P. O. (1988). Functional abnormalities of experimental autogenous vein graft neoendothelium. *Ann. Surg.* **208,** 631–638.

Davies, M. G., Kim, J. H., Dalen, H., Makhoul, R. G., Svendsen, E., and Hagen, P. O. (1994). Reduction of experimental vein graft intimal hyperplasia and preservation of nitric oxide-mediated relaxation by the nitric oxide precursor L-arginine. *Surgery* **116,** 557–568.

Davies, M. G., Dalen, H., Kim, J. H., Barber, L., Svendsen, E., and Hagen, P. O. (1995). Control of accelerated vein graft atheroma with the nitric oxide precursor: L-arginine. *J. Surg. Res.* **59,** 35–42.

Davis, S. F., Yeung, A. C., Meredith, I. T., Charbonneau, F., Ganz, P., Selwyn, A. P., and Anderson, T. J. (1996). Early endothelial dysfunction predicts the development of transplant coronary artery disease at 1 year posttransplant. *Circulation* **93,** 457–462.

Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A. M. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin- 1β-converting enzyme (ICE)-like and cysteine protease protein (CPP)- 32-like proteases. *J. Exp. Med.* **185,** 601–607.

Dominiczak, A. F., and Bohr, D. F. (1995). Nitric oxide and its putative role in hypertension. *Hypertension* **25,** 1202–1211.

Dong, J. Y., Fan, P. D., and Frizzell, R. A. (1996). Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum. Gene Ther.* **7,** 2101–2112.

Drexler, H., Zeiher, A. M., Meinzer, K., and Just, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* **338,** 1546–1550.

Dusting, G. J., and Macdonald, P. S. (1995). Endogenous nitric oxide in cardiovascular disease and transplantation. [Review] [123 refs.]. *Ann. Med.* **27,** 395–406.

Feldman, L. J., and Steg, G. (1997). Optimal techniques for arterial gene transfer. *Cardiovasc. Res* **35,** 391–404.

Fevery, J., Roskams, T., Van de Casteele, M., Omasta, A., Janssens, S., Desmet, V., and Nevens, F. (1998). NO synthase in the liver: Prospects of in vivo gene transfer. *Digestion* **59**(Suppl. 2), 58–59.

Fingerle, J., Johnson, R., Clowes, A. W., Majesky, M. W., and Reidy, M. A. (1989). Role of platelets in smooth muscle cell proliferation and migration after vascular injury in rat carotid artery. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8412–8416.

FitzGibbon, G. M., Leach, A. J., Keon, W. J., Burton, J. R., and Kafka, H. P. (1986). Coronary bypass graft fate. Angiographic study of 1,179 vein grafts early, one year, and five years after operation. *J. Thorac. Cardiovasc. Surg.* **91,** 773–778.

Flotte, T., Carter, B., Conrad, C., Guggino, W., Reynolds, T., Rosenstein, B., Taylor, G., Walden, S., and Wetzel, R. (1996). A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum. Gene Ther.* **7,** 1145–1159.

Flugelman, M. Y., Jaklitsch, M. T., Newman, K. D., Casscells, W., Brathauer, G. L., and Dichek, D. A. (1992). Low level in vivo gene transfer into the arterial wall through a perforated balloon catheter [see comments]. *Circulation* **85,** 1110–1117.

Forstermann, U., Mugge, A., Alheid, U., Haverich, A., and Frolich, J. C. (1988). Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. *Circ. Res.* **62,** 185–190.

Frazee, J. G., Giannotta, S. L., and Stern, W. E. (1981). Intravenous nitroglycerin for the treatment of chronic cerebral vasoconstriction in the primate. *J. Neurosurg.* **55,** 865–868.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Gao, G. P., Qu, G., Faust, L. Z., Engdahl, R. K., Xiao, W., Hughes, J. V., Zoltick, P. W., and Wilson, J. M. (1998). High-titer adeno-associated

viral vectors from a Rep/Cap cell line and hybrid shuttle virus. *Hum. Gene Ther.* **9,** 2353–2362.

Garban, H., Marquez, D., Magee, T., Moody, J., Rajavashisth, T., Rodriguez, J. A., Hung, A., Vernet, D., Rajfer, J., and Gonzalez-Cadavid, N. F. (1997). Cloning of rat and human inducible penile nitric oxide synthase. Application for gene therapy of erectile dysfunction. *Biol. Reprod.* **56,** 954–963.

Garg, U. C., and Hassid, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83,** 1774–1777.

Giaid, A., and Saleh, D. (1995). Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* **333,** 214–221.

Girerd, X. J., Hirsch, A. T., Cooke, J. P., Dzau, V. J., and Creager, M. A. (1990). L-arginine augments endothelium-dependent vasodilation in cholesterol- fed rabbits. *Circ. Res.* **67,** 1301–1308.

Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J. A. (1998). Novel tools for production and purification of recombinant adenoassociated virus vectors [In Process Citation]. *Hum. Gene Ther.* **9,** 2745–2760.

Guerra, R. J., Brotherton, A. F., Goodwin, P. J., Clark, C. R., Armstrong, M. L., and Harrison, D. G. (1989). Mechanisms of abnormal endothelium-dependent vascular relaxation in atherosclerosis: Implications for altered autocrine and paracrine functions of EDRF. *Blood Vessels* **26,** 300–314.

Guzman, L. A., Forudi, F., Villa, A. E., and Topol, E. J. (1995). Role of leukocytes in neointimal formation after balloon angioplasty in the rabbit atherosclerotic model. *Coron. Artery Dis.* **6,** 693–701.

Hamon, M., Bauters, C., McFadden, E. P., Wernert, N., Lablanche, J. M., Dupuis, B., and Bertrand, M. E. (1995). Restenosis after coronary angioplasty. *Eur. Heart J.* **16**(Suppl. I), 33–48.

Hierholzer, J. C., Wigand, R., Anderson, L. J., Adrian, T., and Gold, J. W. (1988). Adenoviruses from patients with AIDS: A plethora of serotypes and a description of five new serotypes of subgenus D (types 43–47). *J. Infect. Dis.* **158,** 804–813.

Hitt, M. M., Addison, C. L., and Graham, F. L. (1997). Human adenovirus vectors for gene transfer into mammalian cells. *Adv. Pharmacol.* **40,** 137–206.

Hoggan, M. D., Thomas, G. F., Thomas, F. B., and Johnson, T. B. (1972). Continuous carriage of adenovirus associated virus genome in cell culture in the absence of helper adenovirus. *In* ''Proceedings of the Fourth Lepetit Colloquium, Cocoyac, Mexico,'' pp. 243–249. North-Holland Publ., Amsterdam.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase [see comments]. *Nature* **377,** 239–242.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269.

Ignarro, L. J., Bush, P. A., Buga, G. M., Wood, K. S., Fukuto, J. M., and Rajfer, J. (1990). *Biochem. Biophys. Res. Commun.* **170,** 843–850.

Janssens, S. P., Bloch, K. D., Nong, Z., Gerard, R. D., Zoldhelyi, P., and Collen, D. (1996). Adenoviral-mediated transfer of the human endothelial nitric oxide synthase gene reduces acute hypoxic pulmonary vasoconstriction in rats. *J. Clin. Invest.* **98,** 317–324.

Janssens, S., Flaherty, D., Nong, Z., Varenne, O., Van Pelt, N., Haustermans, C., Zoldhelyi, P., Gerard, R., and Collen, D. (1998). Human endothelial nitric oxide synthase gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation after balloon injury in rats. *Circulation* **97,** 1274–1281.

Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Baylis, S. A., Rhodes, P., Westmore, K., Emson, P. C., and Moncada, S. (1995). Roles of nitric oxide in tumor growth. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4392–4396.

Jeppsson, A., Pellegrini, C., O'Brien, T., Miller, V. M., Tazelaar, H. D., and McGregor, C. G. (1998). Transbronchial gene transfer of endothelial nitric oxide synthase to transplanted lungs. *Ann. Thorac. Surg.* **66,** 318–324.

Jolly, D. (1994). Viral vector systems for gene therapy. *Cancer Gene Ther.* **1,** 51–64.

Juang, S. H., Xie, K., Xu, L., Shi, Q., Wang, Y., Yoneda, J., and Fidler, I. J. (1998). Suppression of tumorigenicity and metastasis of human renal carcinoma cells by infection with retroviral vectors harboring the murine inducible nitric oxide synthase gene. *Hum. Gene Ther.* **9,** 845–854.

Kajita, Y., Suzuki, Y., Oyama, H., Tanazawa, T., Takayasu, M., Shibuya, M., and Sugita, K. (1994). Combined effect of L-arginine and superoxide dismutase on the spastic basilar artery after subarachnoid hemorrhage in dogs. *J. Neurosurg.* **80,** 476–483.

Karayannacos, P. E., Hostetler, J. R., Bond, M. G., Kakos, G. S., Williams, R. A., Kilman, J. W., and Vasko, J. S. (1978). Late failure in vein grafts: Mediating factors in subendothelial fibromuscular hyperplasia. *Ann. Surg.* **187,** 183–188.

Katusic, Z. S., Milde, J. H., Cosentino, F., and Mitrovic, B. S. (1993). Subarachnoid hemorrhage and endothelial L-arginine pathway in small brain stem arteries in dogs. *Stroke* **24,** 392–399.

Kaye, M. P. (1993). The Registry of the International Society for Heart and Lung Transplantation: Tenth official report—1993. *J. Heart Lung Transplant.* **12,** 541–548.

Kim, Y. M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Kirk, S. J., Hurson, M., Regan, M. C., Holt, D. R., Wasserkrug, H. L., and Barbul, A. (1993). Arginine stimulates wound healing and immune function in elderly human beings. *Surgery* **114,** 155–159.

Koglin, J., Glysing-Jensen, T., Mudgett, J. S., and Russell, M. E. (1998). Exacerbated transplant arteriosclerosis in inducible nitric oxide-deficient mice. *Circulation* **97,** 2059–2065.

Kotin, R. M. (1994). Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum. Gene Ther.* **5,** 793–801.

Kubes, P., Suzuki, M., and Granger, D. N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4651–4655.

Kullo, I. J., Mozes, G., Schwartz, R. S., Gloviczki, P., Tsutsui, M., Katusic, Z. S., and O'Brien, T. (1997a). Enhanced endothelium-dependent relaxations after gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries. *Hypertension* **30,** 314–320.

Kullo, I. J., Mozes, G., Schwartz, R. S., Gloviczki, P., Crotty, T. B., Barber, D. A., Katusic, Z. S., and O'Brien, T. (1997b). Adventitial gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries alters vascular reactivity. *Circulation* **96,** 2254–2261.

Kullo, I. J., Schwartz, R. S., Pompili, V. J., Tsutsui, M., Milstien, S., Fitzpatrick, L. A., Katusic, Z. S., and O'Brien, T. (1997c). Expression and function of recombinant endothelial NO synthase in coronary artery smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **17,** 2405–2412.

Lafond-Walker, A., Chen, C. L., Augustine, S., Wu, T. C., Hruban, R. H., and Lowenstein, C. J. (1997). Inducible nitric oxide synthase expression in coronary arteries of transplanted human hearts with accelerated graft arteriosclerosis. *Am. J. Pathol.* **151,** 919–925.

Lawrie, G. M., Morris, G. C. J., and Earle, N. (1991). Long-term results of coronary bypass surgery. Analysis of 1698 patients followed 15 to 20 years. *Ann. Surg.* **213,** 377–385.

Li, L. M., Kilbourn, R. G., Adams, J., and Fidler, I. J. (1991). Role of nitric oxide in lysis of tumor cells by cytokine-activated endothelial cells. *Cancer Res.* **51,** 2531–2535.

Liau, G., and Chan, L. M. (1989). Regulation of extracellular matrix RNA levels in cultured smooth muscle cells. Relationship to cellular quiescence. *J. Biol. Chem.* **264,** 10315–10320.

Lin, K. F., Chao, L., and Chao, J. (1997). Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. *Hypertension* **30,** 307–313.

Lindner, V., and Reidy, M. A. (1991). Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 3739–3743.

Lindner, V., Majack, R. A., and Reidy, M. A. (1990). Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. *J. Clin. Invest.* **85,** 2004–2008.

Lindner, V., Lappi, D. A., Baird, A., Majack, R. A., and Reidy, M. A. (1991). Role of basic fibroblast growth factor in vascular lesion formation. *Circ. Res.* **68,** 106–113.

Linial, M. L., and Miller, A. D. (1990). Retroviral RNA packaging: Sequence requirements and implications. *Curr. Top. Microbiol. Immunol.* **157,** 125–152.

Liu, R., Oberley, T. D., and Oberley, L. W. (1998). Effects of endothelial nitric oxide synthase gene expression on the tumor biology of human oral carcinoma SCC-25 cells. *Cell Growth Differ.* **9,** 239–246.

Liu, Z., Wildhirt, S. M., Weismuller, S., Schulze, C., Conrad, N., and Reichart, B. (1998). Nitric oxide and endothelin in the development of cardiac allograft vasculopathy. Potential targets for therapeutic interventions. *Atherosclerosis* **140,** 1–14.

Lou, H., Kudama, T., Wang, Y. N., Katz, N., Ramwell, P., and Foegh, M. L. (1996). L-arginine prevents heart transplant arteriosclerosis by modulating the vascular cell proliferative response to insulin-like growth factor-I and interleukin-6. *J. Heart Lung Transpl.* **15,** 1248–1257.

Lynch, C. M., Hara, P. S., Leonard, J. C., Williams, J. K., Dean, R. H., and Geary, R. L. (1997). Adeno-associated virus vectors for vascular gene delivery. *Circ. Res.* **80,** 497–505.

McCall, T., and Vallance, P. (1992). Nitric oxide takes centre-stage with newly defined roles. *Trends Pharmacol. Sci* **13,** 1–6.

McQuillan, L. P., Leung, G. K., Marsden, P. A., Kostyk, S. K., and Kourembanas, S. (1994). Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms [published errata appear in *Am. J. Physiol.* (1995) Feb.; **268**(2 Pt. 2):Section H following table of contents and (1995) June; **268**(6 Pt. 3):Section H following table of contents]. *Am. J. Physiol.* **267,** H1921–H1927.

Maeda, Y., Ikeda, U., Ogasawara, Y., Urabe, M., Takizawa, T., Saito, T., Colosi, P., Kurtzman, G., Shimada, K., and Ozawa, K. (1997). Gene transfer into vascular cells using adeno-associated virus (AAV) vectors. *Cardiovasc. Res.* **35,** 514–521.

Matsumoto, T., Komori, K., Yonemitsu, Y., Morishita, R., Sueishi, K., Kaneda, Y., and Sugimachi, K. (1998). Hemagglutinating virus of Japan-liposome-mediated gene transfer of endothelial cell nitric oxide synthase inhibits intimal hyperplasia of canine vein grafts under conditions of poor runoff. *J. Vasc. Surg.* **27,** 135–144.

Mellion, B. T., Ignarro, L. J., Myers, C. B., Ohlstein, E. H., Ballot, B. A., Hyman, A. L., and Kadowitz, P. J. (1983). Inhibition of human platelet aggregation by *S*-nitrosothiols. Heme- dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation. *Mol. Pharmacol.* **23,** 653–664.

Miano, J. M., Tota, R. R., Vlasic, N., Danishefsky, K. J., and Stemerman, M. B. (1990). Early proto-oncogene expression in rat aortic smooth muscle cells following endothelial removal. *Am. J. Pathol.* **137,** 761–765.

Miller, A. D. (1992). Retroviral vectors. *Curr. Top. Microbiol. Immunol.* **158,** 1–24.

Miller, D. G., Adam, M. A., and Miller, A. D. (1990). Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection [published erratum appears in *Mol. Cell. Biol.* (1992) Jan.; **12**(1): 433]. *Mol. Cell. Biol.* **10,** 4239–4242.

Miller, V. M., Reigel, M. M., Hollier, L. H., and Vanhoutte, P. M. (1987). Endothelium-dependent responses in autogenous femoral veins grafted into the arterial circulation of the dog. *J. Clin. Invest.* **80,** 1350–1357.

Moncada, S., and Higgs, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329,** 2002–2012.

Moncada, S., Palmer, R. M., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Morishita, R., Gibbons, G. H., Kaneda, Y., Ogihara, T., and Dzau, V. J. (1993). Novel in vitro gene transfer method for study of local modulators in vascular smooth muscle cells. *Hypertension* **21,** 894–899.

Murray, F. T., Geisser, M., and Murphy, T. C. (1995). Evaluation and treatment of erectile dysfunction. *Am. J. Med. Sci.* **309,** 99–109.

Nabel, E. G., Plautz, G., and Nabel, G. J. (1990). Site-specific gene expression in vivo by direct gene transfer into the arterial wall. *Science* **249,** 1285–1288.

Nabel, E. G., Gordon, D., Yang, Z. Y., Xu, L., San, H., Plautz, G. E., Wu, B. Y., Gao, X., Huang, L., and Nabel, G. J. (1992). Gene transfer in vivo with DNA-liposome complexes: Lack of autoimmunity and gonadal localization. *Hum. Gene Ther.* **3,** 649–656.

Nabel, E. G., Pompili, V. J., Plautz, G. E., and Nabel, G. J. (1994). Gene transfer and vascular disease. *Cardiovasc. Res.* **28,** 445–455.

Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S., and Tannenbaum, S. R. (1992). DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 3030–3034.

Ohara, Y., Peterson, T. E., and Harrison, D. G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* **91,** 2546–2551.

Onoue, H., Tsutsui, M., Smith, L., Stelter, A., O'Brien, T., and Katusic, Z. S. (1998). Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery after experimental subarachnoid hemorrhage. *Stroke* **29,** 1959–1965.

Ooboshi, H., Chu, Y., Rios, C. D., Faraci, F. M., Davidson, B. L., and Heistad, D. D. (1997). Altered vascular function after adenovirus-mediated overexpression of endothelial nitric oxide synthase. *Am. J. Physiol.* **273,** H265–H270.

Ooboshi, H., Toyoda, K., Faraci, F. M., Lang, M. G., and Heistad, D. D. (1998). Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1752–1758.

Palmer, R. M., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. *Science* **246,** 603–608.

Perricaudet, M., and Stratford-Perricaudet, L. D. (1995). Adenovirus-mediated in vivo gene therapy. *In* ''Viruses in Human Gene Therapy'' (Jean-Michel H. Vos, 1st, ed.), pp. 1–32. Carolina Academic Press, Durham, North Carolina, Chapman & Hall, London and New York.

Pipili-Synetos, E., Papageorgiou, A., Sakkoula, E., Sotiropoulou, G., Fotsis, T., Karakiulakis, G., and Maragoudakis, M. E. (1995). Inhibition of angiogenesis, tumour growth and metastasis by the NO-releasing vasodilators, isosorbide mononitrate and dinitrate. *Br. J. Pharmacol.* **116,** 1829–1834.

Radomski, M. W., Palmer, R. M., and Moncada, S. (1987a). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2,** 1057–1058.

Radomski, M. W., Palmer, R. M., and Moncada, S. (1987b). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem. Biophys. Res. Commun.* **148,** 1482–1489.

Rajfer, J., Aronson, W. J., Bush, P. A., Dorey, F. J., and Ignarro, L. J. (1992). Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N. Engl. J. Med.* **326,** 90–94.

Ramos, J. R., Berger, K., Mansfield, P. B., and Sauvage, L. R. (1976). Histologic fate and endothelial changes of distended and nondistended vein grafts. *Ann. Surg.* **183,** 205–228.

Rees, D. D., Palmer, R. M., and Moncada, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3375–3378.

Reidy, M. A. (1992). Factors controlling smooth-muscle cell proliferation. *Arch. Pathol. Lab. Med.* **116,** 1276–1280.

Reidy, M. A., Fingerle, J., and Lindner, V. (1992). Factors controlling the development of arterial lesions after injury. *Circulation* **86,** III43–III46.

Roberts, J. D. J., Roberts, C. T., Jones, R. C., Zapol, W. M., and Bloch, K. D. (1995). Continuous nitric oxide inhalation reduces pulmonary arterial structural changes, right ventricular hypertrophy, and growth retardation in the hypoxic newborn rat. *Circ. Res.* **76,** 215–222.

Rockey, D. (1997). The cellular pathogenesis of portal hypertension: Stellate cell contractility, endothelin, and nitric oxide. *Hepatology* **25,** 2–5.

Roelvink, P. W., Lizonova, A., Lee, J. G., Li, Y., Bergelson, J. M., Finberg, R. W., Brough, D. E., Kovesdi, I., and Wickham, T. J. (1998). The coxsackievirus–adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J. Virol.* **72,** 7909–7915.

Ross, R. (1993). The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **362,** 801–809.

Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O., Segal, S. S., and Sessa, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J. Clin. Invest.* **101,** 731–736.

Russell, M. E., Wallace, A. F., Wyner, L. R., Newell, J. B., and Karnovsky, M. J. (1995). Upregulation and modulation of inducible nitric oxide synthase in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. *Circulation* **92,** 457–464.

Russell, P. S., Chase, C. M., Winn, H. J., and Colvin, R. B. (1994a). Coronary atherosclerosis in transplanted mouse hearts. I. Time course and immunogenetic and immunopathological considerations. *Am. J. Pathol.* **144,** 260–274.

Russell, P. S., Chase, C. M., Winn, H. J., and Colvin, R. B. (1994b). Coronary atherosclerosis in transplanted mouse hearts. II. Importance of humoral immunity. *J. Immunol.* **152,** 5135–5141.

Salvetti, A., Oreve, S., Chadeuf, G., Favre, D., Cherel, Y., Champion-Arnaud, P., David-Ameline, J., and Moullier, P. (1998). Factors influencing recombinant adeno-associated virus production. *Hum. Gene Ther.* **9,** 695–706.

Sarkar, R., Meinberg, E. G., Stanley, J. C., Gordon, D., and Webb, R. C. (1996). Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. *Circ. Res.* **78,** 225–230.

Schaffer, M. R., Tantry, U., Gross, S. S., Wasserburg, H. L., and Barbul, A. (1996). Nitric oxide regulates wound healing. *J. Surg. Res.* **63,** 237–240.

Schaffer, M. R., Tantry, U., Efron, P. A., Ahrendt, G. M., Thornton, F. J., and Barbul, A. (1997). Diabetes-impaired healing and reduced wound nitric oxide synthesis: A possible pathophysiologic correlation. *Surgery* **121,** 513–519.

Schmidt, H. H., Lohmann, S. M., and Walter, U. (1993). The nitric oxide and cGMP signal transduction system: Regulation and mechanism of action. *Biochim. Biophys. Acta* **1178,** 153–175.

Seth, P., Fitzgerald, D., Ginsberg, H., Willingham, M., and Pastan, I. (1984). Evidence that the penton base of adenovirus is involved in potentiation of toxicity of *Pseudomonas* exotoxin conjugated to epidermal growth factor. *Mol. Cell. Biol.* **4,** 1528–1533.

Shabani, M., Pulfer, S. K., Bulgrin, J. P., and Smith, D. J. (1996). Enhancement of wound repair with a topically applied nitric oxide-releasing polymer. *Wound Repair Regen.* **4,** 353–362.

Shears, L. L., Kawaharada, N., Tzeng, E., Billiar, T. R., Watkins, S. C., Kovesdi, I., Lizonova, A., and Pham, S. M. (1997). Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. *J. Clin. Invest.* **100,** 2035–2042.

Shears, L. L., Kibbe, M. R., Murdock, A. D., Billiar, T. R., Lizonova, A., Kovesdi, I., Watkins, S. C., and Tzeng, E. (1998). Efficient inhibition of intimal hyperplasia by adenovirus-mediated inducible nitric oxide synthase gene transfer to rats and pigs in vivo. *J. Am. Coll. Surg.* **187,** 295–306.

Skantze, H. B., Kaplan, J., Bondjers, G., Manuck, S., and Pettersson, K. (1998). Endothelial injury and leukocyte adherence in Hautchen preparations from coronary arteries and aorta of cynomolgus monkeys. *Atherosclerosis* **136,** 33–42.

Srivastava, A., Lusby, E. W., and Berns, K. I. (1983). Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.* **45,** 555–564.

Stewart, M. J., Plautz, G. E., Del Buono, L., Yang, Z. Y., Xu, L., Gao, X., Huang, L., Nabel, E. G., and Nabel, G. J. (1992). Gene transfer in vivo with DNA-liposome complexes: Safety and acute toxicity in mice. *Hum. Gene Ther.* **3,** 267–275.

Stratford-Perricaudet, L. D., and Perricaudet, M. (1994). Gene therapy: The advent of adenovirus. *In* ''Gene Therapeutics: Methods and Applications of Direct Gene Transfer,'' pp. 344–362, (J. A. Wolff, ed.), Birkhaeuser, Boston.

Straus, S. E. (1984). Adeonvirus infections in humans. *In* ''The Adenoviruses'' (H. S. Ginsberg, ed.), Plenum, New York and London.

Summerford, C., and Samulski, R. J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72,** 1438–1445.

Suzuki, J., Aikawa, M., Isobe, M., Sekiguchi, M., Yazaki, Y., and Nagai, R. (1995). Altered expression of smooth muscle and non-muscle myosin heavy chain isoforms in rejected hearts: A sensitive marker for acute rejection and graft coronary arteriosclerosis. *Transplant. Proc.* **27,** 578.

Tanaka, H., Swanson, S. J., Sukhova, G., Schoen, F. J., and Libby, P. (1995). Smooth muscle cells of the coronary arterial tunica media express tumor necrosis factor-α and proliferate during acute rejection of rabbit cardiac allografts. *Am. J. Pathol.* **147,** 617–626.

Taylor, L. M. J., Edwards, J. M., and Porter, J. M. (1990). Present status of reversed vein bypass grafting: Five-year results of a modern series. *J. Vasc. Surg.* **11,** 193–205.

Thornton, F. J., Schaffer, M. R., Witte, M. B., Moldawer, L. L., MacKay, S. L., Abouhamze, A., Tannahill, C. L., and Barbul, A. (1998). Enhanced collagen accumulation following direct transfection of the inducible nitric oxide synthase gene in cutaneous wounds. *Biochem. Biophys. Res. Commun.* **246,** 654–659.

Tomko, R. P., Xu, R., and Philipson, L. (1997). HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3352–3356.

Trempe, J. P. (1996). Packaging systems for adeno-associated virus vectors. *Curr. Top. Microbiol. Immunol.* **218,** 35–50, 35–50.

Tsutsui, M., Chen, A. F., O'Brien, T., Crotty, T. B., and Katusic, Z. S. (1998). Adventitial expression of recombinant eNOS gene restores NO production in arteries without endothelium. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1231–1241.

Tzeng, E., Billiar, T. R., Robbins, P. D., Loftus, M., and Stuehr, D. J. (1995). Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11771–11775.

Tzeng, E., Shears, L. L., Lotze, M. T., and Billiar, T. R. (1996a). Gene therapy. *Curr. Probl. Surg* **33,** 961–1041.

Tzeng, E., Shears, L. L., Robbins, P. D., Pitt, B. R., Geller, D. A., Watkins, S. C., Simmons, R. L., and Billiar, T. R. (1996b). Vascular gene transfer of the human inducible nitric oxide synthase: Characterization of activity and effects on myointimal hyperplasia. *Mol. Med.* **2,** 211–225.

Tzeng, E., Yoneyama, T., Hatakeyama, K., Shears, L. L., and Billiar, T. R. (1996c). Vascular inducible nitric oxide synthase gene therapy: Requirement for guanosine triphosphate cyclohydrolase I. *Surgery* **120,** 315–321.

Tzeng, E., Kim, Y. M., Pitt, B. R., Lizonova, A., Kovesdi, I., and Billiar, T. R. (1997). Adenoviral transfer of the inducible nitric oxide synthase gene blocks endothelial cell apoptosis. *Surgery* **122,** 255–263.

Vallance, P., Collier, J., and Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man [see comments]. *Lancet* **2,** 997–1000.

Vamvakas, S., and Schmidt, H. H. (1997). Just say NO to cancer? [editorial; comment]. *J. Natl. Cancer Inst.* **89,** 406–407.

Varenne, O., Pislaru, S., Gillijns, H., Van Pelt, N., Gerard, R. D., Zoldhelyi, P., Van de Werf, F., Collen, D., and Janssens, S. P. (1998). Local adenovirus-mediated transfer of human endothelial nitric oxide synthase reduces luminal narrowing after coronary angioplasty in pigs. *Circulation* **98,** 919–926.

Varmus, H. (1988). Retroviruses. *Science* **240,** 1427–1435.

Verma, I. M., and Somia, N. (1997). Gene therapy—promises, problems and prospects [news]. *Nature* **389,** 239–242.

Villa, L. M., Salas, E., Darley-Usmar, V. M., Radomski, M. W., and Moncada, S. (1994). Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12383–12387.

von der Leyen, Gibbons, G. H., Morishita, R., Lewis, N. P., Zhang, L., Nakajima, M., Kaneda, Y., Cooke, J. P., and Dzau, V. J. (1995). Gene therapy inhibiting neointimal vascular lesion: In vivo transfer of endothelial cell nitric oxide synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1137–1141.

Wagner, J. A., Chao, A. C., and Gardner, P. (1995). Molecular strategies for therapy of cystic fibrosis. *Annu. Rev. Pharmacol. Toxicol.* **35,** 257–276.

Weis, M., and von Scheidt, W. (1997). Cardiac allograft vasculopathy: A review. *Circulation* **96,** 2069–2077.

Wickham, T. J., Tzeng, E., Shears, L. L., Roelvink, P. W., Li, Y., Lee, G. M., Brough, D. E., Lizonova, A., and Kovesdi, I. (1997). Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J. Virol.* **71,** 8221–8229.

Xie, K., Huang, S., Dong, Z., Gutman, M., and Fidler, I. J. (1995). Direct correlation between expression of endogenous inducible nitric oxide synthase and regression of M5076 reticulum cell sarcoma hepatic metastases in mice treated with liposomes containing lipopeptide CGP 31362. *Cancer Res.* **55,** 3123–3131.

Xie, K., Dong, Z., and Fidler, I. J. (1996). Activation of nitric oxide synthase gene for inhibition of cancer metastasis. *J. Leukocyte Biol.* **59,** 797–803.

Xie, K., Wang, Y., Huang, S., Xu, L., Bielenberg, D., Salas, T., McConkey, D. J., Jiang, W., and Fidler, I. J. (1997a). Nitric oxide-mediated apoptosis of K-1735 melanoma cells is associated with downregulation of Bcl-2. *Oncogene* **15,** 771–779.

Xie, K., Huang, S., Dong, Z., Juang, S. H., Wang, Y., and Fidler, I. J. (1997b). Destruction of bystander cells by tumor cells transfected with inducible nitric oxide (NO) synthase gene [see comments]. *J. Natl. Cancer Inst.* **89,** 421–427.

Yamasaki, K., Edington, H. D., McClosky, C., Tzeng, E., Lizonova, A., Kovesdi, I., Steed, D. L., and Billiar, T. R. (1998). Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J. Clin. Invest.* **101,** 967–971.

Zeiher, A. M., Drexler, H., Saurbier, B., and Just, H. (1993). Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J. Clin. Invest.* **92,** 652–662.

Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., Geppetti, P., and Ledda, F. (1994). Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J. Clin. Invest.* **94,** 2036–2044.

CHAPTER 33

Functional Influence of Gene Transfer of Recombinant Nitric Oxide Synthase to Cardiovascular System

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NITRIC OXIDE (NO) EXERTS CRITICAL AND DIVERSE FUNCTIONS IN THE CARDIOVASCULAR SYSTEM. AN IMPAIRED NO PRODUCTION AND/OR FUNCTION CONTRIBUTES TO THE PATHOPHYSIOLOGY OF A VARIETY OF CARDIOVASCULAR DISEASES. ALTHOUGH NO THERAPY IS DESIRABLE, THE SHORT HALF-LIFE OF NO AND THE TOLERANCE AND SIDE EFFECTS OF NO DONORS LIMIT THE EFFECTIVENESS OF THEIR CLINICAL APPLICATIONS. SINCE 1996, RECOMBINANT NO SYNTHASE (NOS) GENE TRANSFER HAS BEEN SUCCESSFULLY CARRIED OUT BOTH *EX VIVO* AND *IN VIVO* IN VARIOUS ANIMAL MODELS OF CARDIOVASCULAR DISEASE AND HUMAN BLOOD VESSELS WITH ENCOURAGING RESULTS. THIS CHAPTER PROVIDES AN OVERVIEW OF THE CONCEPT AND PRINCIPLES OF NOS GENE THERAPY FOR A NUMBER OF CARDIOVASCULAR DISEASES INCLUDING ATHEROSCLEROSIS AND THROMBOSIS, RESTENOSIS, CEREBRAL VASOSPASM, TRANSPLANT VASCULOPATHY, HYPERTENSION, DIABETES MELLITUS, CORONARY HEART DISEASE, IMPOTENCY, AND DELAYED WOUND HEALING. IN ADDITION, THE LIMITATIONS OF THE CURRENTLY AVAILABLE VECTORS AND TECHNICAL MEANS OF NOS GENE DELIVERY TO THE CARDIOVASCULAR SYSTEM ARE DISCUSSED.

Introduction

Nitric oxide (NO) exerts critical and diverse functions in the cardiovascular system, as has been discussed in depth in other chapters in this book. Impairment of NO production and/or function plays a key role in a number of cardiovascular disease processes, including atherosclerosis and thrombosis, restenosis, cerebral vasospasm, transplant vasculopathy, hypertension, diabetes mellitus, coronary heart disease, impotency, and delayed wound healing (Cooke and Dzau, 1997). Although NO therapy under these circumstances is beneficial and highly desirable, the short half-life (several seconds) and high reactivity of NO and the tolerance and other unwanted effects caused by some NO donor agents (e.g., hypotension) often limit the effectiveness of their clinical applications. On the other hand, the excessive production of NO induced by immunological stimuli under certain diseased conditions (e.g., sepsis-induced hypotensive shock)

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contributes to the pathology of these diseases and thus to the logic for selectively inhibiting individual isoforms of nitric oxide synthase (NOS) (Higgs *et al.,* 1999).

Gene therapy refers to the transfer of functional genes to the host tissue to correct the malfunction of a specific gene or to replace a missing gene, with resultant alleviation of the symptoms of a particular disease. Cardiovascular gene transfer is not only a powerful technique for studying vascular biology, but also a novel and promising strategy for treating cardiovascular disease. Beginning in the late 1990s, a number of investigators including our group have begun to explore the functional influences of NOS gene transfer to the cardiovascular system, aiming initially at a variety of animal models of cardiovascular disease, with the long-term goal of applying this technology in future human therapy. Since 1996, NOS gene transfer studies have been successfully carried out both *ex vivo* and *in vivo* in different cardiovascular tissues with encouraging results (A. F. Y. Chen *et al.,* 1998a, 1999). This chapter provides an overview of the concept and principles of NOS gene therapy for cardiovascular disease and the currently available technical means of NOS gene delivery to the cardiovascular system.

Principles of Cardiovascular Gene Therapy

Concept of Gene Transfer and Therapy

Somatic gene therapy has a fundamental distinction from germ-line gene manipulation. In the former, gene expression is modified only in somatic cells, whereas in latter, permanent changes are made in the germ-line. In animal species, manipulation of germ-line genes is useful in that it provides models of human genetic disease, helps to elucidate the functions of novel genes, facilitates the large-scale production of recombinant therapeutic proteins, and even creates genetically modified animal tissues transferable to humans (i.e., xenotransplantation). In contrast, alterations in the human gene pool are clearly not the aim of somatic human gene therapy. In pharmacological and therapeutic terms, gene therapy can be considered to be the use of functional genes in treatment of diseases. In this regard, a prodrug gene (e.g., a NOS cDNA) is delivered to a host by an appropriate vector, via a chosen route of administration (e.g., intraluminal, perivascular), resulting in the release of the active drug (e.g., NO) after biotransformation processes of the genetic prodrug (Table I). Thus, an ideal system for gene delivery should consist of a safe formula (the vector) and a drug (the gene product) with maximal therapeutic effects and minimal side effects.

Vectors for Cardiovascular Gene Transfer

The success of gene therapy depends, to a large extent, on the effectiveness with which the vector system can deliver the functional gene to the host tissue. An ideal vector for cardiovascular gene delivery should have several characteristics. Such would be the capacity to achieve efficient transduction *in vivo* in both dividing and nondividing vascular cells; maintenance of stable transgene expression for a desired duration; inability to induce immune response and/or mutagenesis in the host cells. In general, two categories of vector system exist: viral and nonviral vectors (Verma and Somia, 1997). Nonviral vectors are either in the form of naked DNA or plasmid DNA in conjugates (e.g., liposomes, molecular conjugates). Although nonviral vectors possess a very good safety profile for human gene therapy, various attempts to use them for cardiovascular gene transfer have been unsuccessful, attributable to their inability of achieving efficacious *in vivo* transgene expression.

Viral vectors are produced from wild-type viruses by genetic engineering. The recombinant viruses are rendered replication incompetent by deletion of the particular portions of the viral genome responsible for initiating transcription. However, their ability to infect via interaction of their surface proteins with the receptors on the host cells is maintained. There are a number of viral vectors available for cardiovascular gene transfer. Retroviruses can integrate into the host cell genome and are thus capable of achieving prolonged transgene expression. However, only dividing cells can be transduced—a major drawback for cardiovascular gene transfer. Recombinant adenoviruses are perhaps the most efficient vascular gene transfer vector currently available, owing to their ability to transduce both dividing and nondividing vascular cells with high efficiency, and to grow to the high titers [i.e., up to 10^{12} plaque-forming units (pfu)/ml] required for *in vivo* gene transfer (Schneider and French, 1993; Wilson, 1996). But the use of these vectors for human gene therapy has currently been limited by the inflammatory response they provoke in host cells (see later). More recently, an ultraviolet light-inactivated hemagglutinating virus of

Table I Gene Therapy versus Traditional Drug Therapy

	Drug therapy	Gene therapy
Prodrug	A compound containing the active drug	A functional gene
Drug	The active compound	The gene product (RNA, enzyme, protein, etc.)
Biotransformation	Metabolic or enzymatic conversion of prodrug	Transcriptional and/or translational conversion of transgene
Formulation	Coated tablets or capsules, solutions, etc.	Viral or nonviral vector solutions, powders, etc.
Route of administration	Oral, intramuscular, intravenous, etc.	Intraluminal, perivascular, etc.

Japan (HVJ; Sendai virus), a member of the mouse paramyxovirus family, has been combined with multi- or unilamellar liposomes for cardiovascular gene transfer with 30 to 50% transduction efficiency rate and limited toxicity (Dzau *et al.,* 1996; Yonemitsu *et al.,* 1998). In addition, adeno-associated virus and lentivirus are currently being developed to address the limitations of other viral vectors (Verma and Somia, 1997) (compared in Table II).

Adenoviral Vectors and Receptors

BIOLOGY OF ADENOVIRUSES

There are 47 human adenovirus serotypes, divided into six groups (A–F), among the more than 100 serotypes identified in animal species since the 1960s, based on their individual type-specific antigens. The human serotypes 2 and 5 in subgroup C are the two best characterized and most frequently used types as gene transfer vectors. Adenoviruses are double-stranded, nonenveloped linear DNA viruses of approximately 36 kilobases in size that contain hexon, fiber protein, and penton base in their icosahedral capsid.

There are four early regions (E1–E4) of adenoviral genome expressed during the initial stages of host infection. The region essential for viral replication is E1 (E1A and E1B). This region encodes for a number of multifunctional regulatory proteins responsible for transactivation of other early genes involved in the replication cycle. The E2 region has two transcription units which encode both a DNA-binding protein (DBP) and a DNA polymerase (Ad pol) involved in viral replication. The E3-encoded proteins such as the 19-kDa glycoprotein play an important role in modulating the host immune responses, as they are associated with the major histocompatibility complex (MHC) class I molecules. The functions of the E4 region include facilitation of DNA replication and late viral gene expression and protein synthesis as well as inhibition of host protein synthesis. The first generation recombinant adenoviral vectors are rendered replication-incompetent after the deletion of the E1 region from the viral genome. Deletion of other early regions (e.g., E2, E3, and E4) has been used for construction of a second generation of adenoviral vectors. Foreign genes are then inserted as expression cassettes under the control of heterologous eukaryotic or viral promoters. (Wilson, 1996; Hitt *et al.,* 1997).

Several features of adenovirus biology make recombinant adenoviruses the choice for gene transfer studies in the cardiovascular system. Adenoviral vectors can readily be propagated and purified to yield high-titer stocks. A broad spectrum of vascular cells (both dividing and quiescent) and tissues of different species including human can be transduced by adenovirus *ex vivo* and *in vivo* at high efficiency. In addition, since adenoviruses remain episomal after entering the host cell nucleus, random insertional mutagenesis is not a concern.

PROBLEMS WITH FIRST AND SECOND GENERATION ADENOVIRAL VECTORS

Despite the obvious advantages, the currently available adenoviral vectors possess a number of problems which impede their successful use in human gene therapy today. Although the first and second generations of adenoviral vectors are replication incompetent, owing to deletion of the E1 or

Table II Cardiovascular Gene Transfer Vectors

	Advantages	Disadvantages
Viral vectors		
Retrovirus	Stable chromosomal integration High efficiency for *ex vivo* transduction Long-term transgene expression	Transduces only dividing cells Inefficient *in vivo* transduction Limited gene size (~7 kb)
Adenovirus	Transduces nondividing cells High efficiency High vector titers (~10^{12} pfu/ml)	Host immune responses Episomal (transient expression) Limited gene size (~7 kb)
Adeno-associated virus	Transduces nondividing cells Not associated with known human diseases Naturally replication deficient	Often low efficiency Requires helper viruses Limited gene size (~4.7 kb)
Lentivirus (e.g., HIV)	Transduces nondividing cells Stable *in vivo* transgene expression	Biosafety concerns Cytopathogenic?
Nonviral vectors		
Naked DNA	Easy to prepare No limit to gene size Safe	Episomal (transient expression) Low efficiency Nontropism
Anionic and cationic liposomes	Transduces nondividing cells No limit to gene size Safe	Episomal (transient expression) Low efficiency Nontropism
DNA–protein molecular conjugates (e.g., poly-L-lysine, HVJ-liposomes	No limit to gene size Safe	Episomal (transient expression) Transduces nondividing cells

E1 and other early regions, residual viral proteins can be expressed in the transduced host cells. As a result, both a cellular (i.e., $CD4^+$, $CD8^+$ T cells) and a humoral (i.e., hexon-specific neutralizing antibodies produced by B cells) immune response is evoked. The host immunity not only limits the duration of transgene expression but also prevents the repeated administration of the virus. In addition, adult humans have a high prevalence of preexisting immunity to adenovirus serotypes 2 and 5. In contrast, most experimental animal species lack prior exposure to human adenoviruses (Schulick *et al.,* 1997).

Newer Adenoviral Vectors for Cardiovascular Gene Transfer

In more recent years, a number of advances in the design and improvement of adenoviral vectors have been reported. These modifications aim to block either the expression of the immunogenic viral proteins or the host immune response. Most recently, the third generation adenoviral vectors, deleted of all viral coding sequences, have been constructed. These vectors offer the prospect of decreased host immune responses, decreased cellular toxicity, and increased capacity to accommodate large cDNA inserts. The use of these ''gutted'' vectors has resulted in persistent transgene expression, with a diminished immune response (Kochanek *et al.,* 1996; Haecker *et al.,* 1996; H.-H. Chen *et al.,* 1997; Schiedner *et al.,* 1998; Burcin *et al.,* 1999). In addition, cell type-specific and inducible promoters for cell targeting and transcriptionally targeted and regulatory vectors are being developed, further contributing to the optimization and refinement of the vectors for human gene therapy (Nabel, 1999). An adenoviral vector under the transcriptional control of a smooth muscle cell-specific promoter SM-22 has been shown to selectively transduce vascular smooth muscle cells *in vivo* (S. Kim *et al.,* 1997). Technological advances in vector development may thus in the not too distant future allow the results of animal gene transfer studies to be applied in clinical gene therapy trials.

Receptors for Adenoviruses

The terminal domain of the adenoviral fiber protein known as the ''knob'' is involved in viral uptake into the host cells, through a process involving viral receptor-mediated endocytosis. The primary receptor responsible for this process has been cloned, a receptor shared by coxsackie B virus (a RNA virus) and adenovirus serotypes 2 and 5 termed CAR (Bergelson *et al.,* 1997; Tomko *et al.,* 1997). The receptor is a 46-kDa transmembrane glycoprotein consisting of 365 amino acids. Sequence analysis reveals that it belongs to the immunoglobulin (Ig) gene superfamily with two extracellular immunoglobulin-like domains. The adenovirus-binding activity of CAR is localized in the amino-terminal IgV-related domain, which is also shared by adenovirus serotype 12 (Freimuth *et al.,* 1999). Moreover, the deduced amino acid sequences are highly homologous between human and mouse CAR (i.e., 91%) (Bergelson *et al.,* 1998). The localization of human serotype 2 adenoviral receptor has been mapped to chromosome 21 (Mayr and Freimuth, 1997). The functionality of the receptor has been established because transfection of CAR cDNA in cell lines without native CAR leads to CAR expression with marked transgene expression following adenoviral-mediated marked β-galactosidase gene transfer in these cells (Roelvink *et al.,* 1998).

In addition to the fiber knob protein, the penton base of the viral capsid plays an important role in the internalization of the virus after CAR-mediated endocytosis. It possesses a typical Arg-Gly-Asp (RGD) sequence that facilitates the viral entry through its interaction with $\alpha_v\beta_{3/5}$ integrin, this adhesion receptor which serves as the secondary receptor for the virus, is also involved in vitronectin/fibronectin receptor-mediated cell adhesion. Once the virus enters the host cell nucleus, the viral genome is not integrated into that of the host cells but remains episomal. Hence, adenoviruses are not known to be associated with human malignancy (Hitt *et al.,* 1997).

Tropism of CAR Distribution and Heterogeneity of Transgene Expression

The identification of CAR as a functional receptor for adenovirus and its tissue distribution is likely to have great significance in targeted gene delivery to specific cardiovascular tissues. Northern blot analysis reveals that the mRNA for CAR is strongly expressed in human heart, brain, and pancreas, while its expression is weak in the liver, lung, and lymphocytes, and absent in the kidney, placenta, skeletal muscle, and spleen (Bergelson *et al.,* 1997; Tomko *et al.,* 1997). The lack of CAR expression results in resistance to adenoviral infection and inefficient transgene expression in well-differentiated ciliated airway epithelial cells (Zabner *et al.,* 1997; Pickles *et al.,* 1998), this mechanism perhaps accounting for the failure of initial gene therapy in cystic fibrosis. Indeed, the adenovirus fiber protein largely determines viral tropism, through interaction with CAR, because the infectivity of fiberless adenoviruses deleted of the fiber gene (thus without CAR) is diminished (Legrand *et al.,* 1999; von Seggern *et al.,* 1999). In addition, CAR may also be involved in assembly or maturation of certain viral structural proteins, as the processing of these proteins in the fiber mutants is defective. As a result, although the binding to the cellular integrins was functional in the fiberless viruses, the later step in the cell entry process is impaired due to the defect of the structural proteins associated with the lack of fiber in the viral particle. On the other hand, the ectopic expression of CAR in human dermal fibroblasts that express a relative low level of CAR, or lymphocytes that lack native CAR, results in significant transduction following adenovirus-mediated gene transfer (Leon *et al.,* 1998; Hidaka *et al.,* 1999). In the vasculature, the mRNA for CAR can be detected in human cerebral arteries, and heterogeneity existing between cerebral and peripheral arteries for adenoviral mediated recombinant eNOS expression may reflect differential CAR expression in these vessels (A. F. Y. Chen, *et al.,* 1998b; Tsutsui *et al.,* 1998). Hence, the efficacy of transgene expression mediated by adenovirus may be related to the degree of

CAR expression in different vascular tissues and cells. Future studies on the regulation and manipulation of viral receptor expression will not only provide insights regarding adenoviral-mediated gene expression, but may also help to achieve efficient transduction both *ex vivo* and *in vivo* in different tissues and cells for human gene therapy. Alternatively, the adenoviral tropism can be expanded through genetic modification of the fiber knob domain (e.g., incorporation of a RGD sequence into the H1 loop), resulting in the ability of the virus to utilize an alternative receptor during the cell entry process in CAR-deficient cells (Dmitriev *et al.,* 1998; Hidaka *et al.,* 1999).

Rationale for Gene Transfer of Nitric Oxide Synthases

Nitric oxide synthases catalyze the formation of NO from L-arginine. Three isoforms of NOS enzymes have been isolated and purified to date, and the genes encoding the three isoforms have been cloned. Human endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) genes are located on human chromosomes 7, 12, and 17, respectively. Of the three NOS isozymes, eNOS and nNOS are constitutively active in response to increases in intracellular Ca^{2+} and subsequent Ca^{2+}-calmodulin binding. They usually produce NO in physiological amounts (i.e., in nanamolar range) on demand (in seconds). In contrast, the activity of iNOS is usually Ca^{2+} independent, and its expression following immunological challenges requires a longer time for activation (in hours). However, once iNOS is expressed, it is capable of generating large amounts of NO (i.e., in the micromolar range) hence probably contributing to pathological events (Mayer and Hemmens, 1997). All three NOS isoforms are expressed throughout the cardiovascular system (Ignarro, 1996). Targeted disruption or addition of the three NOS genes in transgenic mice has provided useful animal models for probing the functional importance of each NOS isoform (Huang and Fishman, 1996). In eNOS knockout mice, for instance, vascular phenotypic changes result not only in chronic systemic and pulmonary hypertension with impaired endothelium-dependent relaxation, but also smooth muscle and myocyte cell proliferation in response to arterial injury or hypoxia (Huang *et al.,* 1995; Shesely *et al.,* 1996; Faraci *et al.,* 1998; Rudic *et al.,* 1998; Steudel *et al.,* 1998). Furthermore, a deficiency in eNOS might indicate a genetic predisposition to intimal proliferation, possibly enhanced in males. In females, however, the genetic predisposition may be overridden by pregnancy (Moroi *et al.,* 1998). Conversely, hypotension and, interestingly, decreased NO-mediated vasodilations have been observed in transgenic mice overexpressing eNOS (Ohashi *et al.,* 1998). Potential cardiovascular protective functions for NO include augmentation of vasodilation, promotion of endothelial cell growth, inhibition of leukocyte adhesion, platelet adhesion and aggregation, leukocyte–platelet–endothelial cell interactions, endothelial cell apoptosis, smooth muscle cell proliferation, and synthesis of various matrix proteins (Ignarro, 1996; Cooke and Dzau, 1997). These biological properties of NO form the basis of potential NOS gene therapy for a number of cardiovascular disorders in which a dysfunctional L-arginine–NO–cGMP pathway exists (Table III).

Table III Cardiovascular Diseases Potentially Suitable for NOS Gene Therapy

Atherosclerosis and thrombosis	Diabetes mellitus
Restenosis	Ischemic, congestive, or hypertrophic heart disease
Cerebral vasospasm	Portal hypertension and cirrhosis
Transplant vasculopathy	Impotence
Pulmonary hypertension	Wound healing
Essential hypertension	

NOS Gene Transfer and Cardiovascular Disease

Proliferative Vascular Diseases

Cell proliferation contributes to the pathophysiology of many vascular disorders including atherosclerosis, thrombosis, restenosis, transplant vasculopathy, and wound healing. Several prominent features exist in the proliferative processes, including thrombus formation, inflammatory cell infiltration within the intima and adventitia, endothelial cell apoptosis, intimal hyperplasia, extracellular matrix synthesis, and impaired recoil of the vessel wall (Gibbons, 1998). Gene therapy represents an attractive approach and an active area of research through which functional genes can be delivered to a local site, in order to inhibit the proliferative response to injury (Nable, 1995). A number of candidate genes have been used for gene transfer to selectively inhibit one or more growth pathways involved in cell proliferation (Nabel, 1998; Braun-Dullaeus *et al.,* 1998). Overexpression of antiplatelet and antithrombotic proteins such as tissue plasminogen activator (t-PA) or hirudin can prevent platelet aggregation and thrombosis at the site of injury, resulting in decreased release of platelet-derived growth factors (PDGFs). The herpes simplex virus thymidine kinase (HSV-tk) gene can be expressed, and cells that express the transgene can phosphorylate the nucleoside analog ganciclovir, resulting in formation of an active toxic metabolite and leading to inhibition of DNA replication and cell death. Alternatively, since the transition from G_1 to S phase in the cell cycle requires cyclin/cyclin-dependent kinase (CDK) complex, natural inhibitors of cyclin-CDK (e.g., p21, p27) or antisense oligonucleotides against cyclin-CDK (e.g., c-*myb,* cdk 2 kinase, proliferating-cell nuclear antigen, or their transcription factor, E2F) have been delivered to disrupt cell replication.

However, it appears unlikely that inhibition of a limited number of growth pathways will completely abate the com-

Table IV NOS Gene Transfer in Cultured Cells

Cell type	cDNA	Vector	Functional effect	Reference
Endothelial cells				
Sheep, pulmonary artery	iNOS	Retrovirus	Inhibition of apoptosis	Tzeng *et al.,* 1996; Ceneviva *et al.,* 1998
Sheep, pulmonary artery	iNOS	Adenovirus	Inhibition of apoptosis	Tzeng *et al.,* 1997
Human, umbilical vein	nNOS	Adenovirus	Unknown	Channon *et al.,* 1996
Smooth muscle cells				
Pig, coronary artery	eNOS	Adenovirus	Inhibition of proliferation	Kullo *et al.,* 1997a
			Inhibition of cell cycle progression	Sato *et al.,* 1998
Guinea pig, coronary artery	eNOS	Adenovirus	Inhibition of paxillin and FAK phosphorylation	Fang *et al.,* 1997
Rat, pulmonary artery	iNOS	Retrovirus	Inhibition of proliferation	Tzeng *et al.,* 1996
Human, umbilical vein	nNOS	Adenovirus	Unknown	Channon *et al.,* 1996
Human, aorta	eNOS	Adenovirus	Inhibition of cell cycle progression	Tanner *et al.,* 1999
Rat and human, aorta	iNOS	Plasmid	Induction of apoptosis	Iwashina *et al.,* 1998
Other cells				
Rat, hepatocytes	iNOS	Adenovirus	Inhibition of apoptosis	Tzeng *et al.,* 1998

plex process of vascular proliferation in response to injury. NO is a pleiotropic molecule that influences a number of cellular processes central to vascular proliferative disease, including inhibition of leukocyte adhesion, platelet adhesion and aggregation, smooth muscle cell proliferation, and synthesis of matrix proteins. Consequently, gene transfer of NOS may provide a pleiotropic therapeutic strategy for suppression or modification of the response to injury. Specifically, enhanced NO production from recombinant NOS expression may provide continuous NO supply to inhibit platelet activation, leukocyte infiltration, platelet–leukocyte interaction, migration of smooth muscle cells and adventitial fibroblasts, endothelial cell apoptosis, and matrix synthesis, while stimulating beneficial responses such as re-endothelialization and increased blood flow. In pursuit of these concepts, *ex vivo* and *in vivo* NOS gene transfer approaches have more recently been carried out in cell cultures, isolated vessels, and normal and diseased animal models (Tables IV–VI).

CULTURED CELLS

Endothelial and smooth muscle cells of the vasculature constitute important targets for site-specific vascular wall therapy, with the intent of modifying the arterial response to

Table V *Ex Vivo* NOS Gene Transfer

Tissue	cDNA	Vector	Transgene expression	Functional effect	Reference
Cerebral artery	eNOS	Adenovirus	Adventitial fibroblasts and endothelial cells	Inhibition of contraction and augmentation of relaxation	A.F.Y. Chen *et al.,* 1997b; Tsutsui *et al.,* 1998
Dog, normal					
Dog, subarachnoid hemorrhage				Improvement of impaired relaxation	Onoue *et al.,* 1998
Carotid artery	eNOS	Adenovirus	Adventitia and endothelial cells	Augmentation of relaxation	Kullo *et al.,* 1997c; Ooboshi *et al.,* 1997; Toyoda *et al.,* 1998
Rabbit, normal				Improvement of impaired relaxation	
Rabbit, atherosclerotic					Mozes *et al.,* 1998; Ooboshi *et al.,* 1998
Coronary artery	eNOS	Adenovirus	Adventitia and endothelial cells	Augmentation of relaxation	Cable *et al.,* 1997a; Tsutsui *et al.,* 1998
Pig and dog, normal					
Femoral artery	iNOS	Retrovirus	Endothelial cells	Augmentation of relaxation	Tzeng *et al.,* 1996
pig and dog, normal	eNOS	Adenovirus	Adventitia and endothelium		Tsutsui *et al.,* 1998
Aorta	eNOS	Adenovirus	Adventitia and endothelium	Improvement of impaired relaxation	Zanetti *et al.,* 1999
rabbit, diabetic					
Saphenous vein	eNOS	Adenovirus	Adventitia and endothelium	Augmentation of relaxation	Cable *et al.,* 1997b
human, normal					
Radial artery	eNOS	Adenovirus	Adventitia and endothelium	Inhibition of contraction	Cable *et al.,* 1998
human, normal					
Transplanted lung	eNOS	Adenovirus	Pneumocytes	Inhibition of obliterative bronchiolitis?	Jeppsson *et al.,* 1998b
rat, normal					

Table VI *In Vivo* NOS Gene Transfer

	cDNA	Vector	Transgene expression	Functional effect	Reference
Cerebral artery dog, normal	eNOS	Adenovirus	Adventitial fibroblasts	Adventitia-dependent relaxation	A.F.Y. Chen *et al.,* 1997a
Carotid artery Rabbit, normal	eNOS	Adenovirus	Endothelium Adventitia	Inhibition of contraction and augmentation of relaxation	Kullo *et al.,* 1997a Kullo *et al.,* 1997b
Rabbit, atherosclerotic	nNOS	Adenovirus	Endothelium and adventitia	Improvement of relaxation	Channon *et al.,* 1998
Rat, balloon-injured	eNOS	NVJ–liposome	Smooth muscle	Inhibition of intimal hyperplasia	von der Leyen *et al.,* 1995
	eNOS	Adenovirus	Smooth muscle and adventitia	Inhibition of intimal hyperplasia	Janssens *et al.,* 1998
	eNOS	Retrovirus	Smooth muscle	Inhibition of intimal hyperplasia	L Chen *et al.,* 1998
	iNOS	Adenovirus		Inhibition of intimal hyperplasia	Shears *et al.,* 1998
Iliac artery pig, balloon-injured	iNOS	Adenovirus	Smooth muscle	Inhibition of intimal byperplasia	Shears *et al.,* 1998
Coronary artery pig, balloon-injured	eNOS	Adenovirus	Smooth muscle and adventitia	Inhibition of intimal hyperplasia	Varenne *et al.,* 1998
Femoral vein graft dog, normal	eNOS	HVJ–liposome	Adventitia, smooth muscle, and endothelium	Inhibition of intimal hyperplasia	Matsumoto *et al.,* 1998
Aorta, other tissues? rat, SHR	eNOS	Plasmid	Unknown	Reduction of systemic pressure	Lin *et al.,* 1997
Aortic allograft ACI rat to Wistar rat	iNOS	Adenovirus	Endothelium	Reduced allograft arteriosclerosis	Shears *et al.,* 1997
Pulmonary artery rat, hypoxic lung	eNOS	Adenovirus	Adventitia and endothelium	Reduction of pulmonary pressure	Janssens *et al.,* 1996
Heart rat, normal	eNOS	HVJ–liposome	Cardic myocytes	Degradation of myoplasm, induction of apoptosis?	Shin *et al.,* 1996; Kawaguchi *et al.,* 1997
Skin Mouse, iNOS −/−	iNOS	Adenovirus	Fibroblasts, melanocytes, keratinocytes, endothelium Langerhans cells, epithelium?	Accelerated wound healing	Yamasaki *et al.,* 1998
Rat, normal	iNOS	Liposome	—	Increase of collagen synthesis	Thornton *et al.,* 1998
Liver rat, cirrhotic	eNOS	Adenovirus	Hepatocytes, stellate cells, sinusoidal endothelial cells	Reduction of portal pressure	Fevery *et al.,* 1998
Penile tissue rat, aged male	iNOS	Liposome	Corporal tissues	Improved erection response?	Garban *et al.,* 1997

injury. Endothelial dysfunction and attenuated endothelium-dependent relaxations are manifested in vascular diseases such as atherosclerosis, hypertension, and diabetes, thus providing the logic for endothelial cells as targets. On the other hand, smooth muscle cell response to arterial injury is involved in vascular proliferative diseases such as atherosclerosis, hypertension, and intimal hyperplasia and restenosis after coronary angioplasty. Expression of recombinant NOS in endothelial and/or smooth muscle cells may prove useful in site-specific therapy of these diseases.

NO is a potent inhibitor of both smooth muscle cell and cardiac myocyte growth in response to remodeling. Loss of NO effect results in hyperplasia or ventricular hypertrophy in eNOS knockout mice subjected to arterial ligation or hypoxia, respectively (Rudic *et al.,* 1998; Steudel *et al.,* 1998). NO suppresses smooth muscle mitogenesis by inhibiting protein kinase C (PKC) activity (Guh *et al.,* 1998), blocking cell cycle progression at multiple phases (e.g., G_0/G_1, S) via activation of cyclin-dependent kinase inhibitors such as p21 (Ishida *et al.,* 1997; Sciorati *et al.,* 1997; Sarkar *et al.,* 1997a,b), or through induction of apoptosis (Iwashina *et al.,* 1998). By contrast, compelling evidence indicates that NO is a strong inhibitor of endothelial cell apoptosis (Dimmeler and Zeiher, 1997; Y.-M. Kim *et al.,* 1999). NO abrogates angiotensin II, endotoxin, and tumor necrosis factor alpha (TNF-α) induced apoptosis in endothelial cells by interfering with the activation of the protease family of caspase cascade and Bcl-2 cleavage (Dimmeler *et al.,* 1997a,b), and NF-κB DNA binding activity (DeMeester *et al.,* 1998), respectively. The possible cellular mechanisms through which NO exerts its antiapoptotic effect include induction of cytoprotective stress proteins [e.g., heme oxygenase, heat-shock protein 70 (Hsp70)], cGMP-dependent activation of protein kinase G (PKG) and suppression of caspase activation and activity, and inhibition of cytochrome *c* release from mitochondria (Y.-M. Kim *et al.,* 1999).

The effects of recombinant NOS gene expression have been investigated in cultured endothelial and smooth muscle cells from human and animal species. In porcine coronary arterial smooth muscle cells, adenoviral mediated eNOS gene transfer increases eNOS enzymatic activity, and NO_2 and cGMP formation (Kullo *et al.,* 1997a). Serum-induced cell proliferation is inhibited 3 or 6 days after transduction, attributable to decreased DNA synthesis, when measured by

thymidine uptake. In rat pulmonary smooth muscle cells and sheep pulmonary endothelial cells, retroviral vector-mediated iNOS gene transfer increases iNOS mRNA and protein levels with increased NO_2 production (Tzeng *et al.,* 1996). Interestingly, in smooth muscle cells tetrahydrobiopterin (BH_4), an essential cofactor of NOS, augments eNOS-stimulated cGMP production and iNOS-induced NO production. Likewise, the transduction of cultured guinea pig coronary smooth muscle cells with the eNOS gene also results in recombinant eNOS expression in more than 90% of the cells after 48 hours (Fang *et al.,* 1997). Recombinant eNOS expression inhibits platelet-derived growth factor (PDGF)-stimulated tyrosine phosphorylation of two key molecules implicated in the cell adhesion process, paxillin and focal adhesion kinase (FAK), suggesting the FAK–paxillin pathway as a target for NO-mediated inhibition of cell migration and proliferation.

Similar to eNOS gene transfer, adenoviral mediated nNOS gene transfer resulted in recombinant nNOS expression in cultured human smooth muscle cells and umbilical vein endothelial cells (Channon *et al.,* 1996). Both cell types showed increased NO production in response to Ca^{2+} ionophore A23187, acetylcholine (ACh), or bradykinin stimulation 3 days after gene transfer. Supplement of the BH_4 precursor sepiapterin significantly augmented NO production in nNOS-transduced cells.

In sheep pulmonary endothelial cells, iNOS transduction by either adenovirus or retrovirus vectors, increased NO production in a viral titer-dependent fashion (Tzeng *et al.,* 1997; Ceneviva *et al.,* 1998). Overexpression of iNOS did not inhibit endothelial cell proliferation and viability, but inhibited endotoxin-induced apoptosis in these cells by reducing caspase-3-like protease activity, and possibly via induction of hsp70, heme oxygenase-1, and metallothionein. In contrast, recombinant iNOS transduction by plasmid lipofectin in rat and human smooth muscle cells can lead to a marked inhibition of DNA synthesis, as well as induction of apoptosis, both being abrogated by the NOS inhibitor *N*-monomethyl-L-arginine (L-NMMA) (Iwashina *et al.,* 1998). In both porcine coronary and human aortic smooth muscle cells, adenoviral mediated eNOS gene transfer also resulted in G_0/G_1 arrest of the regular cell cycle in a cGMP-dependent manner (Sato *et al.,* 1998). These studies suggest that NO produced from NOS gene expression can protect endothelial cells against apoptosis while inhibiting smooth muscle cell proliferation, both being desirable effects in the treatment of proliferative vascular diseases.

Atherosclerosis and Thrombosis

Atherosclerosis in coronary arteries, carotid arteries, iliac-femoral arteries, aortas, and renal arteries results in conditions such as unstable angina and myocardial infarction, brain ischemia and stroke, and claudication and leg ulcers. Hypercholesterolemia and hyperhomocysteinemia are associated with impaired NO-mediated endothelium-dependent relaxation, most likely due to a combination of increased superoxide generation, decreased antioxidant capacity, and reduced synthesis and/or increased degradation of NO (Harrison, 1997; Refsum *et al.,* 1998). Endothelial dysfunction occurs at an early stage of the disease process, which often leads to changes in endothelial redox state, activation of oxidant-sensitive transcriptional genes [e.g., monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule (VCAM)], adhesion of circulating platelets and monocytes, increased accumulation of lipids in the intima, and increased contraction, migration, and proliferation of smooth muscle cells (Maxwell *et al.,* 1998). Progressive development of atherosclerotic lesions results in the formation of lipid-laden plaques that are prone to fissure, ulceration, and rupture. Thrombosis resulting from the plaque rupture in the coronary arteries plays a pivotal role in the progression from atherosclerosis to myocardial infarction. Hence, NOS gene therapy might be expected to improve endothelium-dependent vasodilation, reverse the altered endothelial redox state (thus decreasing adhesion molecule secretion and endothelial adhesiveness), suppress smooth muscle cell proliferation, and stabilize vulnerable plaques.

Both *ex vivo* and *in vivo* NOS gene transfer to atherosclerotic arteries have been studied. In thoracic aortas of cholesterol-fed rabbits and carotid arteries of Watanabe heritable hyperlipidemic rabbits (WHHL) subjected to adenoviral mediated eNOS gene transfer, recombinant eNOS expression was evident in both endothelial cells and adventitia 24 hours after transduction (Mozes *et al.,* 1998; Ooboshi *et al.,* 1998). In atherosclerotic arteries, ACh-induced endothelium-dependent relaxation was significantly improved. Consistent with these results, *in vivo* transfer of an nNOS gene to the atherosclerotic carotid artery of cholesterol-fed rabbits ameliorated the impaired endothelium-dependent vasodilation in response to ACh (Channon *et al.,* 1998). Hence, gene transfer of the constitutive NOS enhances vascular NOS activity and improves vascular relaxation in vessels with dysfunctional endothelium. Furthermore, constitutive NOS may also be better suited than iNOS in this setting because their activities are subject to regulation by Ca^{2+} and endogenous agonists (discussed later).

The feasibility of adenoviral mediated gene transfer to atherosclerotic arteries of primates and humans has also been explored (Rios *et al.,* 1995; Ooboshi *et al.,* 1998; Rekhter *et al.,* 1998; Laitinen and Yla-Herituala, 1998). Adenovirus vectors encoding a β-galactosidase and a placental alkaline phosphatase have been transferred, to atherosclerotic monkey aortas and human coronary and carotid arteries, respectively. Transgene expression was significantly higher in atherosclerotic compared to normal monkey vessels. Human coronary and carotid arteries with advanced atherosclerosis showed efficient recombinant gene expression in nonendothelial intimal cells and in macrophages and smooth muscle cells. The areas of plaque rupture and thrombus could be specific sites targeted for expression of recombinant genes. In addition, collagenase and elastase treatment increased transgene expression sevenfold in the human vessels, suggesting that the pattern of gene expression can be affected by the amount of surrounding extracellular matrix. Taken together, these studies

indicate that it is likely feasible to achieve efficient transgene expression in vessels with atherosclerosis and thrombosis.

RESTENOSIS

Restenosis refers to the late loss of lumen size relative to the original acute gain in lumen dimension induced by angioplasty. Percutaneous transluminal coronary angioplasty (PTCA) has become a mainstay in the treatment of ischemic heart disease since its introduction in 1979, with an estimated 300,000 percutaneous procedures being performed annually in the United States alone, exceeding the number of surgical revascularization procedures. In spite of significant advances in reducing the acute complications of revascularization, and application of better techniques such as the use of intracoronary stents, chronic restenosis remains a serious problem, occurring in 30 to 50% of patients (Frishman *et al.,* 1998). Restenosis reaches its peak 1 to 4 months after PTCA, perhaps reflecting an exaggerated healing response to the original balloon injury. Chemokines and mitogens secreted from the platelets, leukocytes, and other cells, induce the migration of smooth muscle cells and adventitial fibroblasts from the media and adventitia to the intima, resulting in reocclusion of the lumen. Restenosis shares some prominent features with atherosclerosis and thrombosis, including thrombus formation, intimal hyperplasia, matrix expansion and modification, and inflammatory cell infiltration within the intima and adventitia. On the basis of these concepts, the feasibility of NOS gene transfer as an intervention strategy has been investigated in a variety of animal models.

Initial *ex vivo* studies using normal pig coronary and rabbit carotid arteries have demonstrated that adenoviral mediated eNOS gene transfer results in enhanced arterial relaxation in response to acetylcholine (receptor-mediated) or calcium ionophore A23187 (non-receptor-mediated) 1 to 4 days posttransduction (Cable *et al.,* 1997a; Ooboshi *et al.,* 1997; Toyoda *et al.,* 1998). *In vivo* transduction of recombinant eNOS in a normal rabbit carotid artery model has led to similar functional effects (Kullo *et al.,* 1997b). Observations include an enhancement in ACh-induced, endothelium-dependent relaxation, a reduction in phenylephrine-induced maximal contraction, and an increase in basal cGMP formation, at 4 days posttransduction.

In agreement with data obtained from normal vessel studies, *in vivo* NOS gene delivery to a number of injured animal models has also yielded encouraging results. The pioneering study of eNOS gene transfer in a rat carotid model of neointimal hyperplasia performed by Dzau and co-workers, employed a fusigenic HVJ virus–liposome conjugate encoding a bovine eNOS cDNA (von der Leyen *et al.,* 1995). After intraluminal delivery via a catheter to the balloon-injured common carotid arteries of Sprague-Dawley rats, endothelial NO production in injured arteries was restored to normal levels 4 days after transduction, resulting in increased relaxation in response to Ca^{2+} ionophore A23187. Neointimal formation was inhibited by 70% 2 weeks after balloon injury, attributable to suppressed DNA synthesis as measured by bromodeoxyuridine (BrdU) incorporation after eNOS gene transduction.

In a more recent study in the same rat model of carotid artery injury, reduced expression of the structural protein paxillin was observed in the proliferating smooth muscle cells (Fang *et al.,* 1999). Adenoviral mediated eNOS gene transfer *in vivo* to the injured site reduced neointimal formation for up to 4 weeks, with concomitant enhancement of paxillin recovery, suggesting that NO inhibition of intimal hyperplasia may be mediated in part by the enhanced recovery of injury-induced downregulation of paxillin. In a similar model of carotid artery injury in Fischer 344 rats, a retroviral vector encoding an eNOS gene was used to transduce aortic smooth muscle cells in primary culture, followed by the seeding of transduced cells onto the luminal surface of balloon-injured arteries (L. Chen *et al.,* 1998). *Ex vivo* gene transfer had to be conducted first in cultured smooth muscle cells, due to the requirement of cell division for retroviral-mediated gene transfer. Thus retroviral vectors may have limited use for *in vivo* arterial gene transfer. Nonetheless, the seeding of cells transduced with eNOS gene at the site of arterial injury resulted in inhibition of neointimal formation of up to 37% at 2 weeks postinjury. More importantly, the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a proline-rich protein substrate of both PKG and protein kinase A (PKA) and a participant in actin filament formation in platelets, endothelial cells, and smooth muscle cells, was significantly enhanced in both eNOS transduced cells and the seeded vessels. Since carotid artery injury results in a decrease in VASP phosphorylation *in vivo* (L. Chen *et al.,* 1998), increased VASP phosphorylation following recombinant eNOS expression may play an important role in the suppression of smooth muscle proliferation.

More recently, adenoviral mediated eNOS gene transfer to rat carotid artery and to pig coronary artery after balloon injury has been studied *in vivo* (Janssens *et al.,* 1998; Varenne *et al.,* 1998). Catheter-mediated instillation of vector resulted in efficient eNOS transgene expression in both models, resulting in significant reductions of intima-to-media ratios, approximately 70% in rat carotid artery at 2 weeks and 25% in pig coronary artery at 4 weeks postinjury. The discrepancy in the degree of reduction of intimal hyperplasia in these studies may be due to the viral titers used, as the viral dose applied to the rat carotid artery (3×10^{10} pfu/ml) was 20 times more than that used in the pig coronary artery (1.5×10^{9} pfu/ml).

An alternative method to increase localized vessel wall generation of NO is use of adenoviral-mediated iNOS gene transfer. This has also been investigated in rat carotid artery and pig iliac artery models following balloon angioplasty (Shears *et al.,* 1998). A very low dose of adenoviral vector encoding iNOS (2×10^{6} pfu) delivered to the rat carotid artery resulted in a near complete reduction (>95%) in neointimal formation up to 6 weeks postinjury. In the pig iliac artery, a higher viral dose (5×10^{8} pfu) led to a >50% decrease of intimal hyperplasia. This remarkable degree of suppression of restenosis was observed in both models when gene transfer was initiated shortly after balloon injury. However, iNOS gene transfer did not result in regression of

preformed neointimal lesions when gene transfer was carried out several days after balloon injury. Thus, expression of recombinant NOS may function to inhibit leukocyte and platelet adherence to the injured vessel while simultaneously promoting endothelial regrowth and inhibiting smooth muscle cell proliferation. It is conceivable that the initiation of cellular proliferative events can be effectively suppressed with recombinant iNOS expression, and gene therapy during the early stage of vessel injury may thus become a determining factor for a successful outcome. Transfer of an iNOS gene in this setting may be advantageous over eNOS transduction since a substantially lower amount of virus encoding iNOS may be needed to produce a therapeutic effect. This would decrease the likelihood of vector-induced cytotoxicity.

In addition to luminal delivery, successful *in vivo* eNOS gene transduction has also been achieved using adventitial delivery (Kullo *et al.,* 1997c). Here, the vector was instilled into the periarterial sheath at a final concentration of 10^{10} pfu/ml after surgical isolation of the carotid artery. Ca^{2+}-dependent eNOS enzymatic activity and cGMP levels were increased 4 days after gene transfer, indicating the expression of functionally active recombinant eNOS. Maximal contraction to phenylephrine was reduced and relaxation to Ca^{2+} ionophore A23187 was augmented. The adventitial response to arterial injury is also a determinant of intimal hyperplasia and restenosis. Balloon injury results in increased adventitial proliferation in which adventitial fibroblasts differentiate into myofibroblasts, migrate into the intima, and contribute to chronic arterial narrowing and restenosis (Zalewski and Shi, 1997). Targeting recombinant eNOS to adventitial fibroblasts with resulting increase in adventitial NO production in this setting may also prevent restenosis by inhibiting cellular proliferation and vasoconstriction.

Cerebral Vasospasm

Chronic cerebral vasospasm is a major cause of morbidity and mortality in patients with subarachnoid hemorrhage. It is a state of delayed onset of focal or diffuse narrowing of the major cerebral arteries 4–12 days after subarachnoid hemorrhage, resulting in cerebral ischemia and neurological deficits (Macdonald, 1997; Brown and Wiebers, 1998). The spastic cerebral arteries do not respond well to the currently available vasodilators and calcium channel blockers. Impairment of NO production and/or vasodilator function is an important mechanism associated with the pathogenesis of cerebral vasospasm (Sobey and Faraci, 1998; Faraci and Heistad, 1998), accompanied by decreased eNOS mRNA level, loss of nNOS immunoreactivity, and diminished cGMP formation. Consequently, experimental vasospasm can be alleviated by intravenous administration of nitroglycerin (a nitrovasodilator which releases NO within cells), intracarotid infusion of NO, intrathecal injection of NO donors, or restoration of endogenous NO production in arterial wall by administration of L-arginine and superoxide dismutase. However, the short half-life of NO and the unwanted effects of NO donor agents (e.g., hypotension) limit their clinical usefulness. In contrast, the administration of an adenoviral vector with functional expression of recombinant eNOS in cerebral arteries raises the possibility of providing continuous NO supply to the underlying smooth muscle cells. Cerebral vasospasm usually occurs between 4 and 12 days after subarachnoid hemorrhage and since it is a transient phenomenon, adenoviral vector-mediated transfer of recombinant eNOS gene may be useful in this setting for its transient nature.

In contrast to gene transfer to the peripheral vascular bed, *in vivo* gene delivery to the cerebral vasculature has unique problems. A segment of cerebral artery cannot be occluded in order to allow localized gene delivery due to problems with brain ischemia. One approach is to deliver genes to the perivascular site via the cerebrospinal fluid (CSF) (Heistad and Faraci, 1996). Such an approach is attractive since cerebral blood flow need not be interrupted as in luminal delivery of recombinant DNA. Adenovirus-mediated gene transfer to cerebral arteries can be achieved by vector injection into the CSF via the cisterna magna, either under normal conditions or in the presence of cisternal blood (Ooboshi *et al.,* 1995; Muhonen *et al.,* 1997). Consistent with these concepts, adenoviral vectors encoding eNOS gene have more recently been delivered *ex vivo* and *in vivo* into canine cerebral blood vessels (A. F. Y. Chen *et al.,* 1997a,b). Recombinant eNOS protein is known to be localized in the adventitial fibroblasts of major cerebral arteries 24 or 72 hours after *in vivo* gene delivery, demonstrated by eNOS immunohistochemical staining and electron microscopy immunogold labeling. Bradykinin-induced relaxation was significantly augmented in eNOS- but not β-galactosidase reporter gene-transduced cerebral arteries, with a concomitant increase in cGMP production. The augmented relaxations after gene transfer were adventitia dependent, as the transgene was not expressed in smooth muscle cells, and removal of endothelial cells did not affect relaxation (A. F. Y. Chen *et al.,* 1997a, Tsutsui *et al.,* 1998). In addition, *ex vivo* eNOS gene transfer to spastic canine cerebral arteries following subarachnoid hemorrhage resulted in enhanced expression of recombinant eNOS protein, with partial restoration of impaired NO-mediated relaxations via adventitial NO production (Onoue *et al.,* 1998). Thus, functional eNOS transgene expression in adventitial fibroblasts results in the alteration of cerebral vasoreactivity in normal and spastic cerebral arteries.

Although cerebrovascular gene transfer has been studied *ex vivo* and *in vivo* in various animal species, only relatively recently has information regarding the feasibility of transgene expression in human cerebral blood vessels become available (A. F. Y. Chen *et al.,* 1998b,c). Human pial arteries, obtained from surgically resected temporal lobectomy specimens for intractable epilepsy, were susceptible to adenoviral vector-mediated *ex vivo* gene transfer. The level of recombinant protein expression in human pial arteries is comparable to those in porcine cerebral vessels, but significantly less than those in canine or rabbit arteries. Expression of adenoviral receptor mRNA can also be detected in fresh human pial arteries as well as basilar and middle cerebral arteries

obtained post mortem. Hence, adenoviral vector-mediated gene transfer to human cerebral arteries is feasible, and adenoviral receptor CAR is present in these vessels. Furthermore, heterogeneity exists in transgene expression between human and animal cerebral arteries.

Graft Vasculopathy

VEIN GRAFT STENOSIS

The internal mammary artery (IMA) is the conduit of choice for myocardial revascularization. However the greater saphenous vein is most commonly used in this role due to its availability and versatility in reaching distal coronary branches that cannot be bypassed with the IMA. However, 12 to 20% of saphenous vein coronary artery bypass grafts occlude during the first postoperative year and subsequent risk of graft occlusion is approximately 4% per year (FitzGibbon *et al.,* 1986). Early graft attrition, exclusive of technical failure, is associated with luminal thrombosis, whereas subsequent failure of saphenous vein grafts is due to fibrointimal hyperplasia and atherosclerosis.

Preservation or augmentation of NO production in bypass vein conduits may serve as a potential molecular approach to reduce early and long-term vein graft occlusion. As NO is known to inhibit many of the putative processes involved in graft failure such as thrombus formation and smooth muscle cell proliferation, NOS overexpression may decrease the rate of occurrence of this problem.

The first successful eNOS gene transfer to human saphenous veins using an adenoviral vector was reported (Cable *et al.,* 1997b). Recombinant eNOS was detected in the adventitia and endothelium 48 hours after *ex vivo* gene transfer, resulting in augmented relaxation to Ca^{2+} ionophore A23187 and concomitant increase in NO_2 production. This approach is particularly attractive because gene transfer can be readily achieved between the time frame of tissue harvest and the coronary bypass procedure. Eventually, it may be used to improve the patency rate in the clinical setting.

To assess possible therapeutic effects of eNOS gene transfer on late graft failure, *in vivo* eNOS gene transfer has been carried out in autologous femoral vein grafts prior to implantation to ipsilateral femoral arteries in a dog model (Matsumoto *et al.,* 1998). Increased local NO production, as a result of recombinant eNOS expression mediated by HVJ virus-liposome conjugate, inhibited more than 50% of intimal hyperplasia in the vein grafts with poor runoff 4 weeks after the operation. Augmentation of local NO via NOS gene transfer may become a feasible means to prevent late graft failure.

ARTERIAL GRAFT VASOSPASM

Radial arteries represent another choice for coronary artery bypass because they are relatively resistant to atherosclerosis compared with autogenous vein grafts. However, radial artery grafts are prone to developing arterial spasm, partially due to a locally active renin–angiotensin system in these vessels (Chester *et al.,* 1998). Although improved drug therapy for arterial spasm is now available, vasospasm still occurs in 5 to 10% of radial artery grafts. Vasospasm of radial artery bypass grafts can cause hypoperfusion, stasis, graft thrombosis, and subsequent myocardial ischemia in early and late postoperative periods. Augmentation of NO production in radial artery conduits via NOS gene transfer can provide an effective means to antagonize the thrombotic and proliferative events, hence improving early and long-term patency results in the postoperative period.

Efficacious inhibition of radial artery contraction after adenoviral mediated gene transfer of eNOS was reported more recently (Cable *et al.,* 1998). Human radial arteries expressed recombinant eNOS protein 40 hours after transduction. Both KCl- and prostaglandin $F_{2\alpha}$-induced contractions were reduced by more than 50% in transduced arteries compared to normal control vessels. These results suggest that adenoviral-mediated transfer of eNOS may exert an important role in prevention of radial artery graft vasospasm in future clinical applications.

ALLOGRAFT ATHEROSCLEROSIS

The development of atherosclerosis in allograft transplants is a principal cause of allograft dysfunction and late death in cardiac transplant recipients (Ardehali, 1995). In more recent years, allograft vasculopathy has replaced acute rejection as the most common cause of graft loss following cardiac transplantation (Ventura *et al.,* 1995; Russell *et al.,* 1995). The early immune-mediated upregulation of endogenous iNOS expression in arterial grafts may partially protect allografts from the development of atherosclerosis (Akyurek *et al.,* 1996). However, immunosuppressive agents commonly used for reducing allograft rejection, such as cyclosporine A, often block endogenous iNOS gene expression and accelerate the atherosclerotic process.

Gene transfer of recombinant iNOS was tested in a rat aortic allograft transplant model (Shears *et al.,* 1997). Transduction of aortic allografts in donor rats with iNOS gene at a low viral titer (10^7 pfu/ml) inhibited the development of atherosclerosis 4 weeks after allograft transplantation. Unlike endogenously expressed iNOS, transgene expression was not inhibited by cyclosporine A since the CMV-driven promoter of the adenoviral vector was not subjected to transcriptional inhibition. Thus, iNOS overexpression by a constitutively driven potent promoter may avoid immunosuppressive-related downregulation and may have a beneficial therapeutic effect in this otherwise untreatable complication.

Heart and Lung Transplantation

Heart or lung transplantation has become a viable option for the treatment of end-stage cardiac or pulmonary disease in selected patients. Despite advances in surgical techniques and organ preservation, graft and recipient survival are suboptimal and retransplantation is seldom an option. Allograft rejection and transplant vasculopathy are major causes of morbidity and mortality after clinical heart or lung transplantation. The major complications influencing the long-term

survival rate are allograft arteriosclerosis in the transplant heart and obliterative bronchiolitis in the transplant lung, respectively. In both cases, cell proliferation and fibrosis are the central components contributing to narrowing and occlusion of coronary arteries and luminal obliteration of the terminal airways. NO produces potent vasodilation and bronchodilation and reduces platelet aggregation and leukocyte adhesion, all of which are advantageous in combating the process of vasculopathy in the transplanted organs. In keeping with this notion, inhaled NO has been used in transplant heart or lung animal models and humans with beneficial effects (Auler *et al.,* 1996; Meyer *et al.,* 1998). NOS gene transfer to the allograft heart or lung may be an ideal alternative, especially because there is a delay of as much as 4 hours from the time of organ retrieval to transplantation, a time when the donor organ could be subjected to NOS gene transfer.

Studies have demonstrated the feasibility of gene transfer to the transplanted heart and lung with adenoviral vectors. The intracoronary administration of the virus has resulted in widespread transgene expression in cardiac myocytes, coronary endothelial cells, and interstitial fibroblasts in a model of abdominal heterotopic heart transplantation in syngeneic rats with or without immunosuppression (Yap *et al.,* 1997; Pellegrini *et al.,* 1998). In a syngeneic rat model of orthotopic single-lung transplantation, bronchial infusion of adenoviral vectors caused transgene expression in pneumocytes and alveolar macrophages (Jeppsson *et al.,* 1998a). More recently, transbronchial gene delivery of eNOS in the same animal model of lung transplant resulted in recombinant eNOS protein expression in more than 75% pneumocytes 4 days following transduction (Jeppsson *et al.,* 1998b). Moreover, Ca^{2+} dependent eNOS enzymatic activity was increased dramatically in eNOS transduced lungs. Gene transfer of recombinant eNOS may thus be employed to inhibit allograft arteriosclerosis in transplanted hearts and obliterative bronchiolitis in transplanted lungs.

Hypertension

PULMONARY HYPERTENSION

Pulmonary hypertension is a disease with high morbidity and mortality which currently has limited therapeutic options. Specific diseases in this category include primary pulmonary hypertension and hypoxic chronic pulmonary hypertension. Inhalation of low concentrations (i.e., $<$80 ppm) of gaseous NO has been used to dilate pulmonary vasculature and improve the perfusion of ventilated lung, resulting in alleviation of the adult respiratory distress syndrome (Rossaint *et al.,* 1993) and reduced hypoxic vasoconstriction in newborns (Roberts *et al.,* 1997). Since reduced expression of eNOS and diminished endothelium-dependent relaxation are involved in pulmonary hypertension (Giaid and Saleh, 1995), transfer of the eNOS gene may also reduce vascular resistance of the pulmonary circulation and provide a local therapeutic approach to pulmonary hypertension.

Adenoviral-mediated human eNOS gene delivery using aerosolization has been reported in an acute hypoxic rat model of pulmonary hypertension (Janssens *et al.,* 1996). Recombinant eNOS was expressed in alveoli and small and medium-sized pulmonary vessels with increased eNOS activity and cGMP levels. Pulmonary arterial pressure was reduced to the control level without affecting systemic blood pressure. Thus, NOS gene transfer to the airways via aerosolization may be a potential therapeutic approach for pulmonary hypertension.

ESSENTIAL HYPERTENSION

Essential hypertension affects approximately 20% of the adult population and has a multifactorial origin arising from an interaction between susceptible genes and environmental factors. A dysfunctional L-arginine–NO pathway including a defect in eNOS gene has been implicated in the pathogenesis of hypertension (Arnal *et al.,* 1995; Huang *et al.,* 1995). A more recent study has investigated the effect of eNOS gene transfer to spontaneously hypertensive rats (SHR), using a human eNOS plasmid DNA driven by a CMV promoter (Lin *et al.,* 1997). A single injection of the eNOS plasmid DNA through the tail vein of SHR rats caused a significant reduction of systemic blood pressure for 6 weeks, and a second injection maintained the effect up to 12 weeks. In this experiment, increased NO production was determined as increased urinary and serum NO_2^-/NO_3^- content as well as increased cGMP levels in the urine and aorta. Somatic delivery of the eNOS gene using such a nonviral vector may be both safe and useful as a means for the treatment of essential hypertension.

Diabetes Mellitus

Accelerated atherosclerosis is a major cause of morbidity and mortality in patients with diabetes mellitus. Sustained elevation of serum glucose causes nonenzymatic glycation and oxidation of proteins and lipids, resulting in the accumulation of irreversibly formed advanced glycation end products (AGEs). The AGEs in diabetic vascular tissues are directly toxic to endothelial cells, leading to vascular dysfunction including impaired NO-mediated endothelium-dependent relaxation, vascular matrix expansion, and atheroand glomerulosclerosis. In addition, hyperglycemia or hyperinsulinemia can induce superoxide anion generation in endothelial cells, which may be mediated by specific isoforms of protein kinase C (Maxwell *et al.,* 1998).

Currently, studies are being conducted in animal models of diabetes mellitus to assess the effect of recombinant eNOS expression on the impairment of endothelium-dependent relaxation. In a rabbit model of alloxan-induced diabetes, stable hyperglycemia ($>$400 mg/dl) can be achieved, resulting in decreased ACh-induced relaxation in the aorta. Adenoviral-mediated eNOS gene transfer to the vessels *ex vivo* can result in eNOS transgene expression in both endothelial cells and the adventitial layer 24 hours following transduction. In this experiment, gene transfer of eNOS to the diabetic rabbit aortic rings augmented ACh-mediated relaxation, but did not reverse abnormal contraction in response to higher concentrations of ACh in diabetic vessels (Zanetti *et al.,* 1999).

Ischemic, Congestive, or Hypertrophic Heart Disease

NO is an important modulator of cardiac function, coronary vascular function, and myocardial metabolism (Drexler, 1998; Ingwall and Kelly, 1998). Although NO is thought to participate in events following reperfusion of the heart, as either a beneficial or a detrimental factor (Curtis and Pabla, 1997), compelling evidence suggests that diminished NO bioactivity causes constriction of coronary arteries, contributes to provocation of myocardial ischemia, and facilitates vascular inflammation with oxidation of lipoproteins. In both human and animal models of congestive heart failure, impaired endothelial NO release is observed in large coronary arteries and microvessels, which is attributable to reduced eNOS gene expression (Smith *et al.,* 1996). Reduced NO production can result in diastolic dysfunction (i.e., impaired relaxation and increased ventricular preload) and altered myocardial energy metabolism (Recchia *et al.,* 1998). Consequently, the therapeutic properties of NO donor agents (e.g., organic nitrates) in heart failure are based, to some extent, on the reduction of ventricular preload (Elkayam, 1996). Under these circumstances, eNOS gene transfer might be useful as an approach to assess the possibility of reversing myocardial endothelial dysfunction by enhancing the production and release of NO.

The direct *in vivo* transfer of the eNOS gene to the heart has been reported (Shin *et al.,* 1996; Kawaguchi *et al.,* 1997). HVJ (Sendai virus)-coated liposomes containing human eNOS cDNA were percutaneously injected into myocytes of the left ventricular wall in normal Wistar rats. Morphological analysis of transduced myocytes revealed cell shrinkage, loss of myofilaments, and mitochondrial swelling and accumulations only when the high dose of HVJ-liposomes encoding eNOS cDNA was given (i.e., 50 μg), 1 week after transduction. This report provides the first observations of cytotoxicity directly related to recombinant eNOS-induced NO production, as β-galactosidase reporter gene expression in these cells did not cause cell death. Given the high chemical reactivity of NO, it is not unlikely that when NO is produced in large amounts following transduction, it may contribute to increased susceptibility to cell injury. In addition, the possibility of peroxynitrite involvement as a result of interaction between NO and superoxide anion could not be ruled out in this setting. Future studies of gene transfer using various NOS isoforms in different doses in the setting of heart failure may provide information on the potential therapeutic effects of NO in the failing heart.

Portal Hypertension and Cirrhosis

Portal hypertension is a major complication of cirrhosis, portal venous thrombosis, and pre- and postsinusoidal liver disorders such as schistosomiasis and veno-occlusive disease. Augmentation of intrahepatic resistance in cirrhotic liver results from fibrosis and scarring, a process involving stellate cells (i.e., a cell type with smooth muscle feature). Activated stellate cells transform into myofibroblasts, a cell type common to wound healing, which contributes to endothelin-induced contraction in fibrous bands (Rockey, 1997). On the other hand, NO released from sinusoidal endothelial cell modulates portal pressure in response to hepatic flow, and its dysfunction may contribute to portal hypertension and facilitate the development of the hepatorenal syndrome (Shah *et al.,* 1997, 1998).

In cirrhotic liver of human and animal species, eNOS expression is reduced in sinusoidal and hepatic venular endothelial cells (Shah *et al.,* 1998). Perfusion of L-arginine reduces portal pressure in response to norepinephrine stimulation (Rockey, 1997), suggesting a protective role of endogenous NO in this state. In addition, endogenous iNOS expression by hepatocytes plays an important role in hepatocyte function and protects the liver during sepsis and ischemia reperfusion (Taylor *et al.,* 1998). NO prevents intravascular thrombosis by inhibiting platelet adhesion and neutralizing toxic oxygen radicals. It also blocks TNF-α-induced apoptosis and hepatotoxicity, perhaps in part by a thiol-dependent inhibition of caspase-3-like protease activity (Y.-M. Kim *et al.,* 1997).

Although NO has beneficial effects in the hepatic vasculature, the means to deliver NO to hepatocytes in a sustained manner are currently limited. More recently, the feasibility of NOS gene transfer was tested as an approach to deliver an intracellular source of NO in order that apoptosis in hepatocytes may be inhibited and that stellate cell contractility in cirrhosis may be decreased (Kim *et al.,* 1998; Tzeng *et al.,* 1998; Fevery *et al.,* 1998). Adenoviral-mediated iNOS gene transfer resulted in sustained NO production and protected hepatocytes from spontaneous and TNF-α-induced apoptosis in these cells (Tzeng *et al.,* 1998). The caspase-3-like protease activity associated with apoptosis was suppressed by iNOS gene transfer. In CCl_4-induced cirrhotic rats, an injection of adenoviral vector encoding eNOS into the portal vein resulted in recombinant eNOS expression in sinusoidal-lining endothelial cells (Fevery *et al.,* 1998). Portal pressure of the cirrhotic rats was significantly reduced 4 days after *in vivo* eNOS gene delivery. Targeted delivery of the NOS gene to selected cell types of the liver may hold promise as a means to treat portal hypertension and cirrhosis.

Impotence

NO plays a pivotal role in the regulation of human reproductive function and is a physiological mediator of penile erection (Rosselli *et al.,* 1998). NO mediates normal erection by relaxing the cavernosal smooth muscle of the penis. Although neuronal NO produced by nNOS of the cavernosal nerve terminals plays a major role in the process (Rajfer *et al.,* 1992; Burnett *et al.,* 1992), eNOS and iNOS are also present in smooth muscle and endothelial cells of corpus cavernosum and penile blood vessels, providing additional sources for endogenous NO production (Rajasekaran *et al.,* 1998). Erectile dysfunction due to the inability of the cavernosal smooth muscle to undergo complete relaxation is manifested in aging and in a number of diseases such as diabetes mellitus (Vernet *et al.,* 1995). Augmentation of NO levels by various methods has been tested in animal models and in

clinical trials of human impotence (Wegner *et al.,* 1995; Moody *et al.,* 1997). The use of NO donor agents and inhibitors of cGMP breakdown, while beneficial in ameliorating impotence, can cause side effects. Localized and targeted augmentation of NO release via NOS gene transfer to cavernosal smooth muscle and endothelial cells of penile vessels may represent an alternative future means for such treatment.

To this end, the possibility of whether the stimulation of penile NOS expression by local induction or gene transfer may mitigate erectile dysfunction in an aged rat model has been investigated (Garban *et al.,* 1997). Following the injection of a mix of iNOS inducers or iNOS cDNA to the rat penis, erectile dysfunction in the aged rats was ameliorated, as measured by the erectile response to electrical field stimulation of the cavernosal nerve. Future studies with targeted local gene transfer of different NOS isoforms to the aged animal and human penile tissue will help to shed more light on the feasibility of gene therapy for erectile dysfunction.

Wound Healing

NO plays a critical role in human skin physiology and pathophysiology. It serves as an autocrine and paracrine mediator in maintaining normal skin homeostasis and in promoting the wound healing process (Weller, 1997; Bruch-Gerharz *et al.,* 1998a,b). NO is produced in a variety of specialized human skin cells including keratinocytes (Baudoin and Tachon, 1996; Shimizu *et al.,* 1997; Roméro-Graillet *et al.,* 1997), melanocytes (Roméro-Graillet *et al.,* 1996, 1997; Jackson *et al.,* 1998), Langerhans cells (Qureshi *et al.,* 1996; Morhenn, 1997), fibroblasts (Wang *et al.,* 1996; Schaffer *et al.,* 1997a), and endothelial cells (Bull *et al.,* 1996).

Wound healing is a well-orchestrated and highly coordinated process involving angiogenesis, collagen synthesis, and re-epithelialization (Clark, 1996). Compelling evidence indicates that NO plays a key role in wound repair. Production of nitrite and nitrate, the stable NO metabolites, is elevated early and transiently in fluid obtained from sponges implanted in subcutaneous wounds (Albina *et al.,* 1990), while urinary nitrate excretion increases in a sustained manner after excisional wounding (Smith *et al.,* 1991) and burn injury (Becker *et al.,* 1993; Carter *et al.,* 1994). The presence of nitrite and nitrate, as an indicator of NOS activity, is directly correlated with collagen deposition within the wound and by dermal fibroblasts in culture (Schaffer *et al.,* 1996, 1997a–c). Furthermore, iNOS and GTP-cyclohydrolase I, the rate-limiting enzyme for the synthesis of NOS essential cofactor BH_4, are expressed in coordination and colocalized in keratinocytes and epithelium during the process of cutaneous wound repair in mice (Frank *et al.,* 1998a,b). Finally, a deficiency of the L-arginine–NO pathway under various diseased conditions such as diabetes and steroid treatment contributes to impaired wound healing (Bulgrin *et al.,* 1995; Schaffer *et al.,* 1997c; Veves *et al.,* 1998).

Conversely, NO promotes processes central to wound healing such as angiogenesis (Ziche *et al.,* 1994, 1997), migration, and proliferation of fibroblasts (Schaffer *et al.,* 1997a), epithelial cells (Noiri *et al.,* 1996), and endothelial cells (Papapetropoulos *et al.,* 1997; Parenti *et al.,* 1998). Indeed, systemic L-arginine administration improves wound healing in normal individuals (Barbul *et al.,* 1990) and in aged individuals with impaired wound healing (Kirk *et al.,* 1993), and topical application of a NO donor agent accelerates closure of excisional wounds in rats (Shabani *et al.,* 1996). In addition, NO formation as a direct result of cutaneous wounding induces expression of vascular endothelial growth factor (VEGF) in mice (Frank *et al.,* 1998a), and the angiogenesis effect of VEGF is mediated in part by NO (Ziche *et al.,* 1994, 1997; Papapetropoulos *et al.,* 1997). By contrast, nonspecific NOS inhibitors applied to the surface of the wound (Bulgrin *et al.,* 1995) or given systemically (Schaffer *et al.,* 1996) retard wound healing.

The possibility that recombinant NOS expression may promote endothelial regeneration after injury (hence accelerating vascular healing) has been investigated in iNOS knockout mice subjected to excisional wounding (Yamasaki *et al.,* 1998). Wound closure was delayed by 31% in iNOS knockout mice compared with wild-type animals, and the delay in wound healing was also observed in wild-type mice given N^6-(iminoethyl)-L-lysine (NIL), a relatively selective iNOS inhibitor. Delayed wound healing in iNOS-deficient mice was completely reversed by adenoviral-mediated iNOS gene transfer at the time of wounding. In male Sprague-Dawley rats that received a longitudinal dorsal midline incision, plasmid-mediated iNOS gene transfer with or without cationic liposomes resulted in recombinant iNOS expression and augmented NO production, which preceded an increase in collagen synthesis and accumulation (Thornton *et al.,* 1998). The results from these studies suggest that iNOS gene transfer may improve wound-healing processes in iNOS-deficient states such as diabetes (Schaffer *et al.,* 1997b) and during steroid treatment (Bulgrin *et al.,* 1995).

Interactions between Recombinant NOS Protein and Host Environment

Individual isoforms of endogenous NOS protein are regulated at transcriptional, translational, and posttranslational levels through multiple and complex cellular processes (Sessa, 1994; Sase and Michel, 1997). The Ca^{2+}-binding protein calmodulin interacts with all three NOS isoforms as its binding is required for their enzymatic activity. A variety of endogenous proteins can interact with nNOS, including 1-syntrophin, postsynaptic density proteins (PSD) 93 and 95, and the mammalian homolog of dynein light chain protein inhibitor of nNOS (PIN) (Christopherson and Bredt, 1997). The proteins can each bind with nNOS and localize it to specific subcellular region of myocytes or neurons. The heat shock protein 90 (hsp90) is involved in the regulation of both nNOS and eNOS activities, in addition to its role in protein folding and maturation processes (Garćia-Cardeña *et al.,* 1998; Bender *et al.,* 1999). Hsp90 has been shown to associate with eNOS *in vitro* and nNOS *in vivo* on activation by different agonists. Consequently, the binding of hsp90 to

eNOS or nNOS augments the activation process of the two constitutive isozymes. The inducible NOS was previously thought to be regulated primarily at the level of transcription, but most recent evidence indicates that iNOS can be modulated negatively at the posttranscriptional level by a cytosolic protein, kalirin (Ratovitski *et al.,* 1999). Likewise, different isoforms of recombinant NOS proteins may also be subjected to modulation via their interaction with various endogenous proteins and agonists. The tissue-specific regulation of recombinant NOS proteins may exert significant influences on the functionality and effects in different cardiovascular beds following NOS gene transfer.

Recombinant NOS and Ca^{2+} and Caveolin

Constitutive NOS functions are highly regulated by plasmalemmal caveolae, the small invaginations of the plasma membrane involved in the regulation of multiple signal transduction pathways (Michel and Feron, 1997; Anderson, 1998; Shaul and Anderson, 1998). Endothelial NOS is localized in caveolae of endothelial cells and cardiac myocytes through association with the caveolae integral-membrane structural proteins caveolins-1 and -3, respectively. Caveolin proteins play a key role in eNOS sequestration after the enzyme is palmitoylated and exert a direct inhibitory effect on eNOS activity (Michel and Feron, 1997), through a 20-amino acid region within the caveolin sequence named caveolin scaffolding domain (Li *et al.,* 1996). This inhibition can be completely reversed via a reciprocal regulatory cycle through which caveolin proteins interact with Ca^{2+}–calmodulin complex (Michel and Feron, 1997). In resting endothelial cells, the association between eNOS and caveolin suppresses eNOS enzyme activity. After agonist activation, the increase in intracellular Ca^{2+} promotes calmodulin binding to eNOS and the dissociation of caveolin from eNOS. The activated eNOS–calmodulin complex synthesizes NO until intracellular Ca^{2+} decreases to the point that calmodulin dissociates and the inhibitory eNOS–caveolin complex reforms. Moreover, the localization of eNOS in caveolae may also optimize NO production via a caveolar complex in which eNOS and an arginine transporter protein are colocalized and from which the direct transfer of extracellular arginine to membrane-bound eNOS may be facilitated (McDonald *et al.,* 1997). In addition, a number of G protein-coupled receptors such as muscarinic M_2, bradykinin B_2, and endothelin receptors are targeted to caveolae on agonist binding (Anderson, 1998; Shaul and Anderson, 1998). Activation of these receptors in caveolae promotes eNOS depalmitoylation that may lead to the release and translocation of functional eNOS from caveolae to the cytosol.

Adenoviral-mediated eNOS gene transfer to cerebral arteries not only results in recombinant eNOS expression in caveolae of adventitial fibroblasts, but also causes augmented arterial relaxation induced by bradykinin in the absence of endothelial cells (A. F. Y. Chen *et al.,* 1997a; Tsutsui *et al.,* 1998). Bradykinin receptors are present on fibroblasts, and activation of these receptors leads to both extracellular calcium influx and intracellular calcium release (Byron *et al.,* 1992; Baumgraten *et al.,* 1995). Hence, the adventitial fibroblasts of arterial walls are well equipped with the machinery for receptor-mediated, caveolae-associated activation of recombinant eNOS protein.

Neuronal NOS is similar to eNOS structurally (i.e., sharing >60% amino acid sequence identity) and in the requirement of Ca^{2+} for activity. It is expressed not only in neurons, but also in skeletal muscle where it is involved in the regulation of contractility (Christopherson and Bredt, 1997). Neuronal NOS in the skeletal muscle dystrophin complex interacts not only with 1-syntrophin but also with caveolin-3, and the latter interaction serves to inhibit nNOS catalytic activity (Venema *et al.,* 1997). Thus, the capacity to interact with caveolin proteins seems to be a general property of the Ca^{2+}-dependent NOS enzymes. Recombinant nNOS may also be targeted to and similarly modulated by caveolin-3 after nNOS gene transfer. In contrast, iNOS is unlikely to be targeted to caveolae or regulated by interactions with caveolin, since it binds calmodulin avidly, and the binding of caveolin and calmodulin are mutually exclusive.

The interactions between caveolin and constitutive NOS, but not iNOS, may thus play a critical role in the regulation of recombinant NOS function, and should be considered when choosing an isoform of NOS for gene transfer. It is hoped that future comparative studies of isoform-specific NOS gene expression in different vascular beds and in different animal models will shed more light on the interaction of recombinant NOS with caveolae-associated proteins.

Recombinant NOS and Oxygen Free Radicals

NO synthesis requires optimal supply of both L-arginine, the precursor, and tetrahydrobiopterin (BH_4), an essential cofactor of NOS (Katusic, 1996; Mayer and Hemmens, 1997). L-arginine facilitates NO production by competing as a substrate, either with a potent endogenous NOS inhibitor N^G, N^G-dimethyl-L-arginine (ADMA) produced in brain and other tissue, or with molecular oxygen to reduce the formation of superoxide anion. On the other hand, BH_4 participates in nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxygen activation during NO synthesis and its depletion leads to uncoupling of oxygen reduction and arginine oxidation, resulting in superoxide production via the heme moiety of NOS (Kinoshita *et al.,* 1997; Shimizu *et al.,* 1998; Werner *et al.,* 1998). Thus, in the absence of adequate L-arginine or BH_4, NOS can act as a superoxide-generating enzyme.

The degradation of NO *in vivo* largely results from its interaction with superoxide anion which inactivates NO rapidly to form peroxynitrite, a potent oxidant implicated in vascular injury involving protein nitrosylation and DNA damage (Wever *et al.,* 1998). In addition to its formation by NOS in the absence of L-arginine or BH_4, superoxide can also be produced from two other important sources: NADH/NADPH oxidase or arachidonic acid in the cyclooxygenase pathway (Harrison, 1997).

The consequence of the NO–superoxide reaction depends in part on the ratio of NO to superoxide. Under most

physiological conditions, NO plays a protective role in the cardiovascular system through its interactions with superoxide and NADPH oxidase (Harrison, 1997). NO inactivates NADPH oxidase by inhibiting its assembling process in neutrophils (Fujii *et al.,* 1997). Peroxynitrite formed by superoxide and NO mimics the effects of NO by inhibiting platelet aggregation and leukocyte adhesion to endothelial cells, producing vasodilation, and even acting as a NO donor agent (Wever *et al.,* 1998). On the other hand, NO may exacerbate the injuries by inducing peroxynitrite formation in diseases such as atherosclerosis and myocardial ischemia–reperfusion in which superoxide production may be enhanced (Curtis and Pabla, 1997). Excessive superoxide formation with perturbed NO synthesis in such cardiovascular diseases may result in accumulation of larger amounts of peroxynitrite and subsequent formation of highly toxic hydroxyl radicals (Katusic, 1996). Under these circumstances, overexpression of recombinant NOS may not necessarily be beneficial.

Future Prospects

The critical and detailed roles that NO plays in the cardiovascular system in health and disease have been reported extensively since the 1980s. As detailed knowledge regarding the function and regulation of individual NOS isoforms in disease is gained, therapeutic potentials of NO have gradually been explored. To this end, the feasibility of recombinant NOS gene transfer to various cardiovascular beds has now been established *ex vivo* and *in vivo* in a number of animal models and human tissues. Localized vascular NOS gene expression is advantageous over NO supplementation by either NO or NO donor agents. However, several obstacles must be overcome before NOS gene therapy can enter the clinical arena. In addition to choosing an appropriate isoform of NOS for gene transfer to specific vascular cells, a major issue is the limitations of currently available vectors. Another clinically formidable obstacle is finding a means to effectively deliver the vectors to the vessel wall. Improvements in both vector design and *in vivo* gene delivery methods are therefore still required prior to clinical application of recombinant NOS genes in therapy. Finally, caution is necessary regarding NOS overexpression in situations where the adverse consequences of NO–free radical interactions may outweigh the direct benefits of NO alone.

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References

Akyurek, L. M., Fellstrom, B. C., Yan, Z. Q., Hansson, G. K., Funa, K., and Larsson, E. (1996). Inducible and endothelial nitric oxide synthase expression during development of transplant arteriosclerosis in rat aortic allografts. *Am. J. Pathol.* **149,** 1981–1990.

Albina, J. E., Mills, C. D., Henry, W. L., Jr., and Caldwell, M. D. (1990). Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.* **144,** 3877–3880.

Anderson, R. G. W. (1998). The caveolae membrane system. *Annu. Rev. Biochem.* **67,** 199–225.

Ardehali, A. (1995). Heart transplantation: Accelerated graft atherosclerosis. *Adv. Cardiac Surg.* **6,** 195–205.

Arnal, J. F., Michel, J. B., and Harrison, D. G. (1995). Nitric oxide in the pathogenesis of hypertension. *Curr. Opin. Nephrol. Hypertens.* **4,** 182–188.

Auler, J. O., Jr., Carmona, M. J., Bocchi, E. A., Bacal, F., Fiorelli, A. I., Stolf, N. A., and Jatene, A. D. (1996). Low doses of inhaled nitric oxide in heart transplant recipients. *J. Heart Lung Transplant.* **15,** 443–450.

Barbul, A., Lazarou, S. A., Efron, D. T., Wasserkrug, H. L., and Efron, G. (1990). Arginine enhances wound healing and lymphocyte immune responses in humans. *Surgery* **108,** 331–337.

Baudoin, J. E., and Tachon, P. (1996). Constitutive nitric oxide synthase is present in normal human keratinocytes. *J. Invest. Dermatol.* **106,** 428–431.

Baumgraten, L. B., Lee, H. C., and Villereal, M. L. (1995). Multiple intracellular Ca^{2+} pools exists in human foreskin fibroblast cell: The effect of bradykinin on release and filling of the noncytosolic Ca^{2+} pools. *Cell Calcium* **17,** 41–52.

Becker, W. K., Shippee, R. L., McManus, A. T., Mason, A. D., Jr., and Pruitt, B. A. Jr. (1993). Kinetics of nitrogen oxide production following experimental thermal injury in rats. *J. Trauma* **34,** 855–862.

Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B., and Osawa, Y. (1999). Neuronal nitric-oxide synthase is regulated by the hsp90-based chaperone system in vivo. *J. Biol. Chem.* **274,** 1472–1478.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275,** 1320–1323.

Bergelson, J. M., Krithivas, A., Celi, L., Droguett, G., Horwitz, M. S., Wickham, T., Crowell, R. T., and Finberg, R. W. (1998). The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J. Virol.* **72,** 415–419.

Braun-Dullaeus, R. C., Mann, M. J., and Dzau, V. J. (1998). Cell cycle progression—new therapeutic target for vascular proliferative disease. *Circulation* **98,** 82–89.

Brown, R. D., and Wiebers, D. O. (1998). Subarachnoid hemorrhage and unruptured intracranial aneurysms. *In* ''Cerebrovascular Disease: Pathophysiology, Diagnosis, and Management'' (M. D. Ginsberg and J. Bogousslavsky, eds.), pp. 1502–1531. Blackwell, Malden, Massachusetts.

Bruch-Gerharz, D., Ruzicka, T., and Kolb-Bachofen (1998a). Nitric oxide in human skin—current status and future prospects. *J. Invest. Dermatol.* **110,** 1–7.

Bruch-Gerharz, D., Ruzicka, T., and Kolb-Bachofen (1998b). Nitric oxide and its implication in skin homeostasis and disease—a review. *Arch. Dermatol. Res.* **290,** 643–651.

Bulgrin, J. P., Shabani, M., Chakravarthy, D., and Smith, D. J. (1995). Nitric oxide synthesis is suppressed in steroid-impaired and diabetic wounds. *Wounds* **7,** 48–57.

Bull, H. A., Hothersall, J., Chowdhurry, N., Cohen, J., and Dowd, P. M. (1996). Neuropeptides induce release of nitric oxide from human dermal microvascular endothelial cells. *J. Invest. Dermatol.* **106,** 655–660.

Burcin, M. M., Schiedner, G., Kochanek, S., Tsai, S. Y., and O'Malley, B. W. (1999). Adenovirus-mediated regulable target gene expression in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 355–360.

Burnett, A. L., Lowenstein, C. J., Bredt, D. S., Chang, T. S., and Snyder, S. H. (1992). Nitric oxide: A physiologic mediator of penile erection. *Science* **257,** 401–403.

Byron, K. L., Babnigg, G., and Villereal, M. L. (1992). Bradykinin-induced Ca^{2+} entry, release, and refilling of intracellular Ca^{2+} stores: Relationships revealed by image analysis of individual human fibroblasts. *J. Biol. Chem.* **267,** 108–118.

Cable, D. G., O'Brien, T., Kullo, I. J., Schwartz, R. S., Schaff, H. V., and Pompili, V. J. (1997a). Expression and function of a recombinant endothelial nitric oxide synthase gene in porcine coronary arteries. *Cardiovasc. Res.* **35,** 553–559.

Cable, D. G., O'Brien, T., Schaff, H. V., and Pompili, V. J. (1997b). Recombinant endothelial nitric oxide synthase-transduced human saphenous veins: Gene therapy to augment nitric oxide production in bypass conduits. *Circulation* **96** (Suppl. II), II-173–II-178.

Cable, D. G., Caccitolo, J. A., Pearson, P. J., O'Brien, T., Mullany, C. J., Daly, R. C., Orszulak, T. A., and Schaff, H. V. (1998). New approaches to prevention and treatment of radial artery graft vasospasm. *Circulation* **98**(19 Suppl. S), II-15–II-21.

Carter, E. A., Derojas-Walker, T., Tamir, S., Tannenbaum, S. R., Yu, Y. M., and Tompkins, R. G. (1994). Nitric oxide production is intensely and persistently increased in tissue by thermal injury. *Biochem. J.* **304,** 201–204.

Ceneviva, G. D., Tzeng, E., Hoyt, D. G., Yee, E., Gallagher, A., Engelhardt, J. F., Kim, Y. M., Billiar, T. R., Watkins, S. A., and Pitt, B. R. (1998). Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells. *Am. J. Physiol.* **19,** L717–L728.

Channon, K. M., Blazing, M. A., Shetty, G. A., Potts, K. E., and George, S. E. (1996). Adenoviral gene transfer of nitric oxide synthase: High level expression in human vascular cells. *Cardiovasc. Res.* **32,** 962–972.

Channon, K. M., Qian, H., Neplioueva, V., Blazing, M. A., Olmez, E., Shetty, G. A., Youngblood, S. A., Pawloski, J., McMahon, T., Stamler, J. S., and George, S. E. (1998). In vivo gene transfer of nitric oxide synthase enhances vasomotor function in carotid arteries from normal and cholesterol-fed rabbits. *Circulation* **98,** 1905–1911.

Chen, A. F. Y., Jiang, S., Crotty, T. B., Tsutsui, M., Smith, L. A., O'Brien, T., and Katusic, Z. S. (1997a). Effects of *in vivo* adventitial expression of recombinant endothelial nitric oxide synthase gene in cerebral arteries. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 12568–12573.

Chen, A. F. Y., O'Brien, T., Tsutsui, M., Kinoshita, H., Pompili, V. J., Crotty, T. B., Spector, D. J., and Katusic, Z. S. (1997b). Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery. *Circ. Res.* **80,** 327–335.

Chen, A. F. Y., O'Brien, T., and Katusic, Z. S. (1998a). Transfer and expression of recombinant nitric oxide synthase genes in cardiovascular system. *Trends Pharmacol. Sci.* **19,** 276–286.

Chen, A. F. Y., O'Brien, T., and Katusic, Z. S. (1998b). Transfer of recombinant endothelial nitric oxide synthase gene to cerebral arteries. *J. Isr. Heart Soc.* **8,** 11–13.

Chen, A. F. Y., Mertens, L. L., Parisi, J. E., O'Brien, T., and Katusic, Z. S. (1998c). Adenoviral receptor-mediated gene transfer to human cerebral artery. *N.-S. Arch. Pharmacol.* **358** (Suppl. 1), R165.

Chen, A. F. Y., O'Brien, T., and Katusic, Z. S. (1999). Therapeutic implications of recombinant endothelial nitric oxide synthase gene expression in cerebral and peripheral arteries. *In* ''The Pathophysiology and Clinical Applications of Nitric Oxide'' (G. M. Rubanyi, ed.), pp. 555–567. Harwood, Reading, Pennsylvania.

Chen, H.-H., Mack, L. M., Kelly, R., Ontell, M., Kochanek, S., and Clemens, P. R. (1997). Persistence in muscle of an adenoviral vector that lacks all viral genes. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 1645–1650.

Chen, L., Daum, G., Forough, R., Clowes, M., Walter, U., and Clowes, A. W. (1998). Overexpression of human endothelial nitric oxide synthase in rat vascular smooth muscle cells and in balloon-injured carotid artery. *Circ. Res.* **82,** 862–870.

Chester, A. H., Amrani, M., and Borland, J. A. A. (1998). Vascular biology of the radial artery. *Curr. Opin. Cardiol.* **13,** 447–452.

Christopherson, K. S., and Bredt, D. S. (1997). Nitric oxide in excitable tissues: Physiological role and disease. *J. Clin. Invest.* **100,** 2424–2429.

Clark, R. A. F. (1996). Wound repair: Overview and general consideration. *In* ''The Molecular and Cellular Biology of Wound Repair'' (R. A. F. Clack, ed.), pp. 3–50. Plenum, New York.

Cooke, J. P., and Dzau, V. J. (1997). Nitric oxide synthase: Role in the genesis of vascular disease. *Annu. Rev. Med.* **48,** 489–509.

Curtis, M. J., and Pabla, R. (1997). Nitric oxide supplementation or synthesis block—which is the better approach to treatment of heart disease? *Trends Pharmacol. Sci.* **18,** 239–244.

DeMeester, S. L., Qiu, Y., Buchman, T. G., Hotchkiss, R. S., Dunnigan, K., Karl, I. E., and Cobb, J. P. (1998). Nitric oxide inhibits stress-induced endothelial cell apoptosis. *Crit. Care Med.* **26,** 1500–1509.

Dimmeler, S., and Zeiher, A. M. (1997). Nitric oxide and apoptosis: Another paradigm for the double-edged role of nitric oxide. *Nitric Oxide* **1,** 275–281.

Dimmeler, S., Rippmann, V., Weiland, U., Haendeler, J., and Zeiher, A. M. (1997a). Angiotensin II induces apoptosis of human endothelial cells: Protective effect of nitric oxide. *Circ. Res.* **81,** 970–976.

Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A. M. (1997b). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.* **185,** 601–608.

Dmitriev, I., Krasnykh, V., Miller, C. R., Wang, M., Kashentseva, E., Mikheeva, G., Belousova, N., and Curiel, D. T. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J. Virol.* **72,** 9706–9713.

Drexler, H. (1998). Endothelium as a therapeutic target in heart failure. *Circulation* **98,** 2652–2655.

Dzau, V. J., Mann, M. J., Morishita, R., and Kaneda, Y. (1996). Fusigenic viral liposome for gene therapy in cardiovascular diseases. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11421–11425.

Elkayam, U. (1996). Nitrates in the treatment of congestive heart failure. *Am. J. Cardiol.* **77,** 41C–51C.

Fang, S., Sharma, R. V., and Bhalla, R. C. (1997). Endothelial nitric oxide synthase gene transfer inhibits platelet-derived growth factor-BB stimulated focal adhesion kinase and paxillin phosphorylation in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **236,** 706–711.

Fang, S., Sharma, R. V., and Bhalla, R. C. (1999). Enhanced recovery of injury-caused downregulation of paxillin protein by eNOS gene expression in rat carotid artery: Mechanism of NO inhibition of intimal hyperplasia? *Arterioscler. Thromb. Vasc. Biol.* **19,** 147–152.

Faraci, F. M., and Heistad, D. D. (1998). Regulation of the cerebral circulation-role of endothelium and potassium channels. *Physiol. Rev.* **78,** 53–97.

Faraci, F. M., Sigmund, C. D., Shesely, E. G., Maeda, N., and Heistad, D. D. (1998). Responses of carotid artery in mice deficient in expression of the gene for endothelial NO synthase. *Am. J. Physiol.* **274,** H564–H570.

Fevery, J., Roskams, T., Vandecasteele, M., Omasta, A., Janssens, S., Desmet, V., and Nevens, F. (1998). NO synthase in the liver—prospects of in vivo gene transfer. *Digestion* **59**(Suppl. 2), 58–59.

FitzGibbon, G. M., Leach, A. J., Keon, W. J., Burton, J. R., and Kafka, H. P. (1986). Coronary bypass graft fate: Angiographic study of 1,179 vein grafts early, one year, and five years after operation. *J. Thorac. Cardiovasc. Surg.* **91,** 773–778.

Frank, S., Madlener, M., Pfeilschifter, J., and Werner, S. (1998a). Induction of inducible nitric oxide synthase and its corresponding tetrahydrobiopterin-cofactor-synthesizing enzyme GTP-cyclohydrolase I during cutaneous wound repair. *J. Invest. Dermatol.* **111,** 1058–1064.

Frank, S., Kolb, N., Werner, E. R., and Pfeilschifter, J. (1998b). Coordinated induction of inducible nitric oxide synthase and GTP-cyclohydrolase I is dependent on inflammatory cytokines and interferon-gamma in HaCaT keratinocytes: Implications for the model of cutaneous wound repair. *J. Invest. Dermatol.* **111,** 1065–1071.

Freimuth, P., Springer, K., Berard, C., Hainfeld, J., Bewley, M., and Flanagan, J. (1999). Coxsackievirus and adenovirus receptor amino-terminal

immunoglobulin V-related domain binds adenovirus type 2 and fiber knob from adenovirus type 12. *J. Virol.* **73,** 1392–1398.

Frishman, W. H., Chiu, R., Landzberg, R., and Weiss, M. (1998). Medical therapies for the prevention of restenosis after percutaneous coronary interventions. *Curr. Prob. Cardiol.* **23,** 535–635.

Fujii, H., Ichimori, K., Hoshiai, K., and Nakazawa, H. (1997). Nitric oxide inactivates NADPH oxidase in pig neutrophils by inhibiting its assembling process. *J. Biol. Chem.* **272,** 32773–32778.

Garban, H., Marquez, D., Magee, T., Moody, J., Rajavashisth, T., Rodriguez, J. A., Hung, A., Vernet, D., Rajfer, J., and Gonzalez-Cadavid, N. F. (1997). Cloning of rat and human inducible penile nitric oxide synthase. Application for gene therapy of erectile dysfunction. *Biol. Reprod.* **56,** 954–963.

García-Cardeña, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392,** 821–824.

Giaid, A., and Saleh, D. (1995). Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* **333,** 214–216.

Gibbons, G. H. (1998). The vascular response to injury. *In* ''Thrombosis and Hemorrhage'' (J. Loscalzo and A. I. Schafer, eds.), pp. 307–320. Williams & Wilkins, Baltimore, Maryland.

Guh, J. H., Hwang, T. L., Ko, F. N., Chueh, S. C., Lai, M. K., and Teng, C. M. (1998). Antiproliferative effect in human prostatic smooth muscle cells by nitric oxide donor. *Mol. Pharmacol.* **53,** 467–474.

Haecker, S. E., Stedman, H. H., Balice-Gordon, R. J., Smith, D. B., Greelish, J. P., Mitchell, M. A., Wells, A., Sweeney, H. L., and Wilson, J. M. (1996). In vivo expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. *Hum. Gene Ther.* **7,** 1907–1914.

Harrison, D. G. (1997). Cellular and molecular mechanisms of endothelial cell dysfunction. *J. Clin. Invest.* **100,** 2153–2157.

Heistad, D. D., and Faraci, F. M. (1996). Gene therapy for cerebral vascular disease. *Stroke* **27,** 1688–1693.

Hidaka, C., Milano, E., Leopold, P. L., Bergelson, J. M., Hackett, N. R., Finberg, R. W., Wickham, T. J., Kovesdi, I., Roelvink, P., and Crystal, R. G. (1999). CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. *J. Clin. Invest.* **103,** 579–587.

Higgs, A., Hobbs, A., and Moncada, S. (1999). Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu. Rev. Pharmacol. Toxicol.* **39,** 191–220.

Hitt, M. M., Addison, C. L., and Graham, F. L. (1997). Human adenovirus vectors for gene transfer into mammalian cells. *Adv. Pharmacol.* **40,** 137–206.

Huang, P. L., and Fishman, M. C. (1996). Genetic analysis of nitric oxide synthase isoforms: Targeted mutation in mice. *J. Mol. Med.* **74,** 415–421.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **377,** 196–197.

Ignarro, L. J. (1996). Physiology and pathophysiology of nitric oxide. *Kidney Int.* **55,** S2–S5.

Ingwall, J. S., and Kelly, R. A. (1998). Nitric oxide, myocardial oxygen consumption, and ATP synthesis. *Circ. Res.* **83,** 1067–1068.

Ishida, A., Sasaguri, T., Kosaka, C., Nojima, H., and Ogata, J. (1997). Induction of the cyclin-dependent kinase inhibitor p21Sdi1/Cip1/Waf1 by nitric oxide-generating vasodilator in vascular smooth muscle cells. *J. Biol. Chem.* **272,** 10050–10057.

Iwashina, M., Shichiri, M., Marumo, F., and Hirata, Y. (1998). Transfection of inducible nitric oxide synthase gene causes apoptosis in vascular smooth muscle cells. *Circulation* **98,** 1212–1218.

Jackson, M., Frame, F., Weller, R., and McKenzie, R. C. (1998). Expression of nitric oxide synthase III (eNOS) mRNA by human skin cells: Melanocytes but not keratinocytes express eNOS mRNA. *Arch. Dermatol. Res.* **290,** 350–352.

Janssens, S. P., Bloch, K. D., Nong, Z., Gerard, R. D., Zoldhelyi, P., and Collen, D. (1996). Adenoviral-mediated transfer of the human endothelial nitric oxide synthase gene reduces acute hypoxic pulmonary vasoconstriction in rats. *J. Clin. Invest.* **98,** 317–324.

Janssens, S. P., Flaherty, D., Nong, Z., Varenne, O., van Pelt, N., Haustermans, C., Zoldhelyi, P., Gerard, R. D., and Collen, D. (1998). Human endothelial nitric oxide synthase gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation after balloon injury in rats. *Circulation* **97,** 1274–1281.

Jeppsson, A., Lee, R., Pellegrini, C., O'Brien, T., Miller, V. M., Tazelaar, H. D., and McGregor, C. G. A. (1998a). Gene therapy in lung transplantation: Effective gene transfer via the airways. *J. Thorac. Cardiovasc. Surg.* **115,** 638–643.

Jeppsson, A., Pellegrini, C., O'Brien, T., Miller, V. M., Tazelaar, H. D., and McGregor, C. G. A. (1998b). Transbronchial gene transfer of endothelial nitric oxide synthase to transplanted lungs. *Annu. Thorac. Surg.* **66,** 318–324.

Katusic, Z. S. (1996). Superoxide anion and endothelial regulation of arterial tone. *Free Radicals Biol. Med.* **20,** 443–448.

Kawaguchi, H., Shin, W. S., Wang, Y., Inukai, M., Kato, M., Matsuo-Okai, Y., Sakamoto, A., Uehara, Y., Kaneda, Y., and Toyo-oka, T. (1997). In vivo gene transfection of human endothelial cell nitric oxide synthase in cardiomyocytes causes apoptosis-like cell death: Identification using Sendai virus-coated liposomes. *Circulation* **95,** 2441–2447.

Kim, S., Lin, H., Barr, E., Chu, L., Leiden, J. M., and Parmacek, M. S. (1997). Transcriptional targeting of replication-defective adenovirus transgene expression to smooth muscle cells in vivo. *J. Clin. Invest.* **100,** 1006–1014.

Kim, Y.-M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Kim, Y.-M., Kim, T. H., Seol, D. W., Talanian, R. V., and Billiar, T. R. (1998). Nitric oxide suppression of apoptosis occurs in association with an inhibition of BCL-2 cleavage and cytochrome C release. *J. Biol. Chem.* **273,** 31437–31441.

Kim, Y.-M., Bombeck, C. A., and Billiar, T. R. (1999). Nitric oxide as a bifunctional regulator of apoptosis. *Circ. Res.* **84,** 253–256.

Kinoshita, H., Tsutsui, M., Milstien, S., and Katusic, Z. S. (1997). Tetrahydrobiopterin, nitric oxide and regulation of cerebral vascular tone. *Prog. Neurobiol.* **52,** 295–302.

Kirk, S. J., Hurson, M., Regan, M. C., Holt, D. R., Wasserkrug, H. L., and Barbul, A. (1993). Arginine stimulates wound healing and immune function in elderly human beings. *Surgery* **114,** 155–160.

Kochanek, S., Clemens, P. R., Mitani, K., Chen, H.-H., Chan, S., and Caskey, C. T. (1996). A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galactosidase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5731–5736.

Kullo, I. J., Schwartz, R. S., Pompili, V. J., Tsutsui, M., Milstein, S., Fitzpatrick, L. A., Katusic, Z. S., and O'Brien, T. (1997a). Expression and function of recombinant endothelial nitric oxide synthase in coronary artery smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **17,** 2405–2412.

Kullo, I. J., Mozes, G., Schwartz, R. S., Gloviczki, P., Tsutsui, M., Katusic, Z. S., and O'Brien, T. (1997b). Enhanced endothelium-dependent relaxations after gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries. *Hypertension* **30,** 314–320.

Kullo, I. J., Mozes, G., Schwartz, R. S., Gloviczki, P., Crotty, T. B., Barber, D. A., Katusic, Z. S., and O'Brien, T. (1997c). Adventitial gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries alters vascular reactivity. *Circulation* **96,** 2254–2261.

Leon, R. P., Hedlund, T., Meech, S. J., Li, S., Schaack, J., Hunger, S. P., Duke, R. C., and DeGregori, J. (1998). Adenoviral-mediated gene transfer in lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 13159–13164.

Laitinen, M., and Yla-Herttuala, S. (1998). Vascular gene transfer for the treatment of restenosis and atherosclerosis. *Curr. Opin. Lipidol.* **9,** 465–469.

Legrand, V., Spehner, D., Schlesinger, Y., Settelen, N., Pavirani, A., and Mehtali, M. (1999). Fiberless recombinant adenoviruses: Virus maturation and infectivity in the absence of fiber. *J. Virol.* **73,** 907–919.

Li, S., Couet, J., and Lisanti, M. P. (1996). Src tyrosine kinases, G subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. *J. Biol. Chem.* **271,** 29182–29190.

Lin, K.-F., Chao, L., and Chao, J. (1997). Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. *Hypertension* **30,** 307–313.

McDonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric oxide synthase may explain the ''arginine paradox.'' *J. Biol. Chem.* **272,** 31213–31216.

Macdonald, R. L. (1997). Subarachnoid hemorrhage. *In* ''Primer on Cerebrovascular Diseases'' (K. M. A. Welch, L. R. Caplan, D. J. Reis, B. O. Siesjo, and B. Weir, eds.), pp. 490–497. Academic Press, San Diego.

Matsumoto, T., Komori, K., Yonemitsu, Y., Morishita, R., Sueishi, K., Kaneda, Y., and Sugimachi, K. (1998). Hemagglutinating virus of Japan-liposome-mediated gene transfer of endothelial cell nitric oxide synthase inhibits intimal hyperplasia of canine vein grafts under conditions of poor runoff. *J. Vasc. Surg.* **27,** 135–144.

Maxwell, A. J., Tsao, P. S., and Cooke, J. P. (1998). Modulation of the nitric oxide synthase pathway in atherosclerosis. *Exp. Physiol.* **83,** 573–584.

Mayer, B., and Hemmens, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem. Sci.* **22,** 477–481.

Meyer, K. C., Love, R. B., and Zimmerman, J. J. (1998). The therapeutic potential of nitric oxide in lung transplantation. *Chest* **113,** 1360–1371.

Mayr, G. A., and Freimuth, P. (1997). A single locus on human chromosome 21 directs the expression of a receptor for adenovirus type 2 in mouse A9 cells. *J. Virol.* **71,** 412–418.

Michel, T., and Feron, O. (1997). Nitric oxide synthases: Which, where, how, and why? *J. Clin. Invest.* **100,** 2146–2152.

Moody, J. A., Vernet, D., Laidlaw, S., Rajfer, J., and Gonzalez-Cadavid, N. F. (1997). Effects of long-term oral administration of L-arginine on the rat erectile response. *J. Urol.* **158,** 942–947.

Morhenn, V. B. (1997). Langerhans cells may trigger the psoriatic disease process via production of nitric oxide. *Immunol. Today* **18,** 433–436.

Moroi, M., Zhang, L., Yasuda, T., Virmani, R., Gold, H. K., Fishman, M. C., and Huang, P. L. (1998). Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J. Clin. Invest.* **101,** 1225–1232.

Mozes, G., Kullo, I. J., Mohacsi, T. G., Cable, D. G., Spector, D. J., Crotty, T. B., Gloviczki, P., Katusic, Z. S., and O'Brien, T. (1998). Ex vivo gene transfer of endothelial nitric oxide synthase to atherosclerotic rabbit aortic rings improves endothelium dependent relaxations. *Atherosclerosis* **141,** 265–271.

Muhonen, M. G., Ooboshi, H., Welsh, M. J., Davidson, B. L., and Heistad, D. D. (1997). Gene transfer to cerebral blood vessels after subarachnoid hemorrhage. *Stroke* **28,** 822–829.

Nabel, E. G. (1995). Gene therapy for cardiovascular disease. *Circulation* **91,** 541–548.

Nabel, E. G. (1998). Gene therapy of thrombotic and hemorrhagic disorders. *In* ''Thrombosis and Hemorrhage'' (J. Loscalzo and A. I. Schafer, eds.), pp. 1377–1389. Williams & Wilkins, Baltimore, Maryland.

Nabel, G. J. (1999). Development of optimized vectors for gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 324–326.

Noiri, E., Peresleni, T., Srivastava, N., Weber, P., Bahou, W. F., Peunova, N., and Goligorsky, M. S. (1996). Nitric oxide is necessary for a switch from stationary to locomoting phenotype in epithelial cells. *Am. J. Physiol.* **170,** C794–C802.

Ohashi, Y., Kawashima, S., Hirata, K., Yamashita, T., Ishida, T., Inoue, N., Sakoda, T., Kurihara, H., Yazaki, Y., and Yokoyama, M. (1998). Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J. Clin. Invest.* **102,** 2061–2071.

Onoue, H., Tsutsui, M., Smith, L., Stelter, A., O'Brien, T., and Katusic, Z. S. (1998). Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery after experimental subarachnoid hemorrhage. *Stroke* **29,** 1959–1965.

Ooboshi, H., Welsh, M. J., Rios, C. D., Davidson, B. L., and Heistad, D. D. (1995). Adenovirus-mediated gene transfer *in vivo* to cerebral blood vessels and perivascular tissue. *Circ. Res.* **77,** 7–13.

Ooboshi, H., Chu, Y., Rios, C. D., Faraci, F. M., Davidson, B. L., and Heistad, D. D. (1997). Altered vascular function after adenovirus-mediated overexpression of endothelial nitric oxide synthase. *Am. J. Physiol.* **273,** H265–H270.

Ooboshi, H., Toyoda, K., Faraci, F. M., Lang, M. G., and Heistad, D. D. (1998). Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1752–1758.

Papapetropoulos, A., Garcia-Cardena, G., Madri, J. A., and Sessa, W. C. (1997). Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100,** 3131–3139.

Parenti, A., Morbidelli, L., Cui, X. L., Douglas, J. G., Hood, J. D., Granger, H. J., Ledda, F., and Ziche, M. (1998). Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase ½ activation in postcapillary endothelium. *J. Biol. Chem.* **273,** 4220–4226.

Pellegrini, C., O'Brien, T., Yap, J., Jeppsson, A., Tazelaar, H. D., and McGregor, C. G. A. (1998). Systematic evaluation of distribution of transgene expression after adenovirus-mediated gene transfer to the transplanted heart. *Transplant. Int.* **11,** 373–377.

Pickles, R. J., McCarty, D., Matsui, H., Hart, P. J., Randell, S. H., and Boucher, R. C. (1998). Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. *J. Virol.* **72,** 6014–6023.

Qureshi, A. A., Hosoi, J., Xu, S., Takashima, A., Granstein, R. D., and Lerner, E. A. (1996). Langerhans cells express inducible nitric oxide synthase and produce nitric oxide. *J. Invest. Dermatol.* **107,** 815–821.

Rajasekaran, M., Mondal, D., Agrawal, K., Chen, I. L., Hellstrom, W., and Sikka, S. (1998). Ex vivo expression of nitric oxide synthase isoforms (eNOS/iNOS) and calmodulin in human penile cavernosal cells. *J. Urol.* **160,** 2210–2215.

Rajfer, J., Aronson, W. J., Bush, P. A., Dorey, F. J., and Ignarro, L. J. (1992). Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N. Engl. J. Med.* **326,** 90–94.

Ratovitski, E. A., Alam, M. R., Quick, R. A., McMillan, A., Bao, C., Kozlovsky, C., Hand, T. A., Johnson, R. C., Mains, R. E., Eipper, B. A., and Lowenstein, C. J. (1999). Kalirin inhibition of inducible nitric-oxide synthase. *J. Biol. Chem.* **274,** 993–999.

Recchia, F. A., McConnell, P. I., Bernstein, R. D., Vogel, T. R., Xu, X., and Hintze, T. H. (1998). Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. *Circ. Res.* **83,** 969–979.

Refsum, H., Ueland, P. M., Nygård, O., and Vollset, S. E. (1998). Homocysteine and cardiovascular disease. *Annu. Rev. Med.* **49,** 31–62.

Rekhter, M. D., Simari, R. D., Work, C. W., Nabel, G. J., Nabel, E. G., and Gordon, D. (1998). Gene transfer into normal and atherosclerotic human blood vessels. *Circ. Res.* **82,** 1243–1252.

Rios, C. D., Ooboshi, H., Piegors, D., Davidson, B. L., and Heistad, D. D. (1995). Adenovirus-mediated gene transfer to normal and atherosclerotic arteries—a novel approach. *Arterioscler. Thromb. Vas. Biol.* **15,** 2241–2245.

Roberts, J. D., Jr., Fineman, J. R., Morin III, F. C., Shaul, P. W., Rimar, S., Schreiber, M. D., Polin, R. A., Zwass, M. S., Zayek, M. M., Gross, I., Heymann, M. A., and Zapol, W. M. (1997). Inhaled nitric oxide and persistent pulmonary hypertension of the newborn. *N. Engl. J. Med.* **336,** 605–610.

Rockey, D. (1997). The cellular pathogenesis of portal hypertension: Stellate cell contractility, endothelin, and nitric oxide. *Hepatology* **25,** 2–5.

Roelvink, P. W., Lizonova, A., Lee, J. G. M., Li, Y., Bergelson, J. M., Finberg, R. W., Brough, D. E., Kovesdi, I., and Wickham, T. J. (1998). The coxsackievirus–adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J. Virol.* **72,** 7909–7915.

Roméro-Graillet, C., Aberdam, E., Biagoli, N., Massabni, W., Ortonne, J. P., and Ballotti, R. (1996). Ultraviolet B radiation acts through the nitric oxide and cGMP signal transduction pathway to stimulate melanogenesis in human melanocytes. *J. Biol. Chem.* **271,** 28052–28056.

Roméro-Graillet, C., Aberdam, E., Clément, M., Ortonne, J. P., and Ballotti, R. (1997). Nitric oxide produced by ultraviolet-irradiated keratinocytes stimulates melanogenesis. *J. Clin. Invest.* **99,** 635–642.

Rossaint, R., Falke, K. J., Lopez, F., Slama, K., Pison, U., and Zapol, W. M. (1993). Inhaled nitric oxide for the adult respiratory distress syndrome. *N. Engl. J. Med.* **328,** 399–405.

Rosselli, M., Keller, P. J., and Dubey, R. K. (1998). Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum. Reprod. Update* **4,** 3–24.

Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O., Segal, S. S., and Sessa, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J. Clin. Invest.* **101,** 731–736.

Russell, M. E., Wallace, A. F., Wyner, L. R., Newell, J. B., and Karnovsky, M. J. (1995). Upregulation and modification of inducible nitric oxide synthase in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. *Circulation* **92,** 457–464.

Sarkar, R., Gordon, D., Stanley, J. C., and Webb, R. C. (1997a). Dual cell cycle-specific mechanisms mediate the antimitogenic effects of nitric oxide in vascular smooth muscle cells. *J. Hypertens.* **15,** 275–283.

Sarkar, R., Gordon, D., Stanley, J. C., and Webb, R. C. (1997b). Cell cycle effects of nitric oxide on vascular smooth muscle cells. *Am. J. Physiol.* **272,** H1810–H1818.

Sase, K., and Michel, T. (1997). Expression and regulation of endothelial nitric oxide synthase. *Trends Cardiovasc. Med.* **7,** 28–37.

Sato, J., Fitzpatrick, L. A., Katusic, Z. S., and O'Brien, T. (1998). Transfer of the endothelial nitric oxide synthase gene to coronary smooth muscle cells blocks cell cycle progression in G1. *Circulation* **98**(Suppl. 1), 536.

Schaffer, M. R., Tantry, U., Gross, S. S., Wasserkrug, H. L., and Barbul, A. (1996). Nitric oxide regulates wound healing. *J. Surg. Res.* **63,** 237–240.

Schaffer, M. R., Efron, P. A., Thornton, F. J., Klingel, K., Gross, S. S., and Barbul, A. (1997a). Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. *J. Immunol.* **158,** 2382–2389.

Schaffer, M. R., Tantry, U., Ahrendt, G. M., Wasserburg, H. L., and Barbul, A. (1997b). Acute protein–calorie malnutrition impairs wound healing: A possible role of decreased wound nitric oxide synthesis. *J. Am. Coll. Surg.* **184,** 37–43.

Schaffer, M. R., Tantry, U., Efron, P. A., Ahrendt, G. M., Thornton, F. J., and Barbul, A. (1997c). Diabetes-impaired healing and reduced wound nitric oxide synthesis: A possible pathophysiologic correlation. *Surgery* **121,** 513–519.

Schiedner, G., Morral, N., Parks, R. J., Wu, Y., Koopmans, S. C., Langston, C., Graham, F. L., Beaudet, A. L., and Kochanek, S. (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* **18,** 180–183.

Schneider, M. D., and French, B. A. (1993). The advent of adenovirus: Gene therapy for cardiovascular disease. *Circulation* **88,** 1937–1942.

Schulick, A. H., Vassalli, G., Dunn, P. F., Dong, G., Rade, J. J., Zamarron, C., and Dichek, D. A. (1997). Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. Potential for immunosuppression and vector engineering to overcome barriers of immunity. *J. Clin. Invest.* **99,** 209–219.

Sciorati, C., Nistico, G., Meldolesi, J., and Clementi, E. (1997). Nitric oxide effects on cell growth: GMP-dependent stimulation of the AP-1 transcription complex and cyclic GMP-independent slowing of cell cycling. *Br. J. Pharmacol.* **122,** 687–697.

Sessa, W. C. (1994). The nitric oxide synthase family of proteins. *J. Vasc. Res.* **31,** 131–143.

Shabani, M., Pulfer, S. K., Bulgrin, J. P., and Smith, D. J. (1996). Enhancement of wound repair with a topically applied nitric oxide-releasing polymer. *Wound Repair Regen.* **4,** 353–362.

Shah, V., Haddad, F. G., Garcia-Cardena, G., Frangos, J. A., Mennone, A., Groszmann, R. J., and Sessa, W. C. (1997). Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of resistance in the hepatic sinusoids. *J. Clin. Invest.* **100,** 2923–2930.

Shah, V., Garcia-Cardena, G., Sessa, W. C., and Groszmann, R. J. (1998). The hepatic circulation in health and disease: Report of a single-topic symposium. *Hepatology* **27,** 279–288.

Shaul, P. W., and Anderson, R. G. W. (1998). Role of plasmalemmal caveolae in signal transduction. *Am. J. Physiol.* **275,** L843–L851.

Shears, L. L., Kawaharada, N., Tzeng, E., Billiar, T. R., Watkins, S. C., Kovesdi, I., Lizonova, A., and Pham, S. M. (1997). Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. *J. Clin. Invest.* **100,** 2035–2042.

Shears, L. L., Kibbe, M. R., Murdock, A. D., Billiar, T. R., Lizonova, A., Kovesdi, I., Watkins, S. C., and Tzeng, E. (1998). *J. Am. Coll. Surg.* **187,** 295–306.

Shesely, E. G., Maeda, N., Kim, H.-S., Desai, K. M., Krege, J. H., Laumback, V. E., Sherman, P. A., Sessa, W. C., and Smithies, O. (1996). Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 13176–13181.

Shimizu, S., Ishii, M., Momose, K., and Yamamoto, T. (1998). Role of tetrahydrobiopterin in the function of nitric oxide synthase, and its cytoprotective effect. *Int. J. Mol. Med.* **2,** 533–540.

Shimizu, Y., Sakai, M., Umemura, Y., and Ueda, H. (1997). Immunohistochemical localization of nitric oxide synthase in normal human skin: Expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes. *J. Dermatol.* **24,** 80–87.

Shin, W. S., Kawaguchi, H., Liao, J. K., Wang, Y., Inukai, M., and Toyooka, T. (1996). Toxic action of nitric oxide on myocardial cells: Direct evidence from gene transfer in vivo. *J. Cardiac Failure* **2**(4 Suppl.), S149–S153.

Smith, C. J., Sun, D., Hoegler, C., Roth, B. S., Zhang, X., Zhao, G., Xu, X. B., Kobari, Y., Pritchard, K., Jr., Sessa, W. C., and Hintze, T. H. (1996). Reduced gene expression of vascular endothelial NO synthase and cyclooxygenase-1 in heart failure. *Circ. Res.* **78,** 58–64.

Smith, D. J., Dunphy, M. J., Strag, L. N., and Marletta, M. A. (1991). The influence of wound healing on urinary nitrate levels in rats. *Wounds* **3,** 50–58.

Sobey, C. G., and Faraci, F. M. (1998). Subarachnoid haemorrhage—what happens to the cerebral arteries. *Clin. Exp. Pharmacol. Physiol.* **25,** 867–876.

Steudel, W., Scherrer-Crosbie, M., Bloch, K. D., Weimann, J., Huang, P. L., Jones, R. C., Picard, M. H., and Zapol, W. M. (1998). Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase 3. *J. Clin. Invest.* **101,** 2468–2477.

Taylor, B. S., Alarcon, L. H., and Billiar, T. R. (1998). Inducible nitric oxide synthase in the liver—regulation and function. *Biochemistry* (*Moscow*) **63,** 766–781.

Thornton, F. J., Schaffer, M. R., Witte, M. B., Moldawer, L. L., MacKay, S. L., Abouhamze, A., Tannahill, C. L., and Barbul, A. (1998). Enhanced collagen accumulation following direct transfection of the inducible nitric oxide synthase gene in cutaneous wounds. *Biochem. Biophys. Res. Commun.* **246,** 654–659.

Tomko, R. P., Xu, R., and Philipson, L. (1997). HCAR and MCAR: Human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3352–3356.

Toyoda, K., Ooboshi, H., Chu, Y., Fasbender, A., Davidson, B. L., Welsh, M. J., and Heistad, D. D. (1998). Cationic polymer and lipids enhance adenovirus-mediated gene transfer to rabbit carotid artery. *Stroke* **29,** 2181–2188.

Tsutsui, M., Chen, A. F. Y., O'Brien, T., Crotty, T. B., and Katusic, Z. S. (1998). Adventitial expression of recombinant nitric oxide synthase gene restores nitric oxide production in arteries without endothelium. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1231–1241.

Tzeng, E., Shears, L. L., Robbins, P. D., Pitt, B. R., Geller, D. A., Watkins, S. C., Simmons, R. L., and Billiar, T. R. (1996). Vascular gene transfer

of the human inducible nitric oxide synthase: Characterization of activity and effects on myointimal hyperplasia. *Mol. Med.* **2,** 211–225.

Tzeng, E., Kim, Y.-M., Pitt, B. R., Lizonova, A., Kovesdi, I., and Billiar, T. R. (1997). Adenoviral transfer of the inducible nitric oxide synthases gene blocks endothelial cell apoptosis. *Surgery* **122,** 255–263.

Tzeng, E., Billiar, T. R., Williams, D. L., Li, J. R., Lizonova, A., Kovesdi, I., and Kim, Y. M. (1998). Adenovirus-mediated inducible nitric oxide synthase gene transfer inhibits hepatocyte apoptosis. *Surgery* **124,** 278–283.

Varenne, O., Pislaru, S., Gillijns, H., van Pelt, N., Gerard, R. D., Zoldhelyi, P., van de Werf, F., Collen, D., and Janssens, S. P. (1998). Local adenovirus-mediated transfer of human endothelial nitric oxide synthase reduces luminal narrowing after coronary angioplasty in pigs. *Circulation* **98,** 919–926.

Venema, V. J., Ju, H., Zou, R., and Venema, R. C. (1997). Interaction of neuronal nitric oxide synthase with caveolin-3 in skeletal muscle: Identification of a novel caveolin scaffolding/inhibitor domain. *J. Biol. Chem.* **272,** 28187–28190.

Ventura, H. O., Smart, F. W., Stapleton, D. D., Toups, T., and Price, H. L. (1995). Cardiac allograft vasculopathy: Current concepts. *Am. Heart J.* **129,** 791–799.

Verma, I. M., and Somia, N. (1997). Gene therapy—promises, problems and prospects. *Nature* **389,** 239–242.

Vernet, D., Cai, L., Garban, H., Babbitt, M. L., Murray, F. T., Rajfer, J., and Gonzalez-Cadavid, N. F. (1995). Reduction of penile nitric oxide synthase in diabetic BB/WORdp (type I) and BBZ/WORdp (type II) rats with erectile dysfunction. *Endocrinology* **136,** 5709–5717.

Veves, A., Akbari, C. M., Primavera, J., Donaghue, V. M., Zacharoulis, D., Chrzan, J. S., DeGirolami, U., LoGerfo, F. W., and Freeman, R. (1998). Endothelial dysfunction and the expression of endothelial nitric oxide synthetase in diabetic neuropathy, vascular disease, and foot ulceration. *Diabetes* **47,** 457–463.

von der Leyen, H. E., Gibbons, G. H., Morishita, R., Lewis, N. P., Zhang, L., Kaneda, Y., Cooke, J. P., and Dzau, V. J. (1995). Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial nitric oxide synthase gene. *Proc. Natl. Acad. Sci. USA* **92,** 1137–1141.

von Seggern, D. J., Chiu, C. Y., Fleck, S. K., Stewart, P. L., and Nemerow, G. R. (1999). A helper-independent adenovirus vector with E1, E3, and fiber deleted: Structure and infectivity of fiberless particles. *J. Virol.* **73,** 1601–1608.

Wang, R., Ghahary, A., Shen, Y. J., Scott, P. G., and Tredget, E. E. (1996). Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms. *J. Invest. Dermatol.* **106,** 419–427.

Wegner, H. E., Knispel, H. H., Klan, R., and Miller, K. (1995). Efficacy of linsidomine chlorhydrate, a direct nitric oxide donor, in the treatment of human erectile dysfunction: Results of a double-blind cross over trial. *Int. J. Impot. Res.* **7,** 233–237.

Weller, R. (1997). Nitric oxide—a newly discovered chemical transmitter in human skin. *Br. J. Dermatol.* **137,** 665–672.

Werner, E. R., Wernerfelmayer, G., and Mayer, B. (1998). Tetrahydrobiopterin, cytokines, and nitric oxide synthasis. *Proc. Soc. Exp. Biol. Med.* **219,** 171–182.

Wever, R. M. F., Luscher, T. F., Cosentino, F., and Rabelink, T. J. (1998). Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* **97,** 108–112.

Wilson, J. M. (1996). Adenoviruses as gene-delivery vehicles. *N. Engl. J. Med.* **334,** 1185–1187.

Yamasaki, K., Edington, H. D. J., McClosky, C., Tzeng, E., Lizonova, A., Kovesdi, I., Steed, D. L., and Billiar, T. R. (1998). Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J. Clin. Invest.* **101,** 967–971.

Yap, J., O'Brien, T., Tazelaar, H. D., and McGregor, C. G. A. (1997). Immunosuppression prolongs adenoviral mediated transgene expression in cardiac allograft transplantation. *Cardiovasc. Res.* **35,** 529–535.

Yonemitsu, Y., Alton, E. W., Komori, K., Yoshizumi, T., Sugimachi, K., and Kaneda, Y. (1998). HVJ (Sendai virus) liposome-mediated gene transfer: Current status and future perspectives. *Int. J. Oncol.* **12,** 1277–1285.

Zabner, J., Freimuth, P., Puga, A., Fabrega, A., and Welsh, M. J. (1997). Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection. *J. Clin. Invest.* **100,** 1144–1149.

Zalewski, A., and Shi, Y. (1997). Vascular myofibroblasts: Lessons from coronary repair and remodeling. *Arterioscler. Thromb. Vasc. Biol.* **17,** 417–422.

Zanetti, M., Sato, J., Katusic, Z. S., and O'Brien, T. (1999). eNOS gene transfer alters vascular reactivity in alloxan-induced diabetic rabbit aorta. (Abstract) *Diabetes.* in press.

Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., Geppetti, P., and Ledda, F. (1994). Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J. Clin. Invest.* **94,** 2036–2044.

Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H. T., Donnini, S., Granger, H. J., and Bicknell, R. (1997). Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J. Clin. Invest.* **99,** 2625–2634.

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CHAPTER 34

Nitric Oxide and Vascular Endothelial Dysfunction

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THE REGULATORY FUNCTIONS OF THE ENDOTHELIUM ARE CHANGED BY INJURY OR ACTIVATION. DYSFUNCTION OF THE ENDOTHELIUM CAN BE DEFINED AS AN IMBALANCE BETWEEN RELAXING AND CONTRACTING FACTORS, BETWEEN PRO- AND ANTI-COAGULANT MEDIATORS, OR BETWEEN GROWTH-INHIBITING AND GROWTH-PROMOTING SUBSTANCES. THIS CHAPTER FOCUSES ON THE ROLE OF THE L-ARGININE–NITRIC OXIDE (NO) PATHWAY IN VASCULAR ENDOTHELIAL DYSFUNCTION. SEVERAL DISEASES, SUCH AS ATHEROSCLEROSIS, HYPERCHOLESTEROLEMIA, HYPERTENSION, DIABETES, AND HEART FAILURE, AS WELL AS AGE AND GENDER ARE ACCOMPANIED BY ALTERATIONS IN THIS PATHWAY, BOTH IN EXPERIMENTAL ANIMAL MODELS AND IN HUMANS. THE MECHANISMS OF NO-RELATED VASCULAR ENDOTHELIAL DYSFUNCTION UNDER THESE CONDITIONS WILL BE DISCUSSED, TOGETHER WITH SOME ASSOCIATED THERAPEUTIC ASPECTS.

Introduction

The regulatory functions of the endothelium are changed by injury or activation. Dysfunction of the endothelium has been defined as an imbalance between relaxing and contracting factors, between pro- and anticoagulant mediators, or between growth-inhibiting and growth-promoting substances (Rubanyi, 1993). This chapter will focus on the role of the L-arginine–nitric oxide (NO) pathway in vascular endothelial dysfunction. Several diseases, such as atherosclerosis, hypercholesterolemia, hypertension, diabetes, and heart failure, are accompanied by alterations of vascular NO production and/or bioavailability, both in experimental animal models and in humans. Thereby, vasoconstrictor responses to certain substances are augmented. Impaired endothelium-dependent vascular relaxation is only one manifestation of altered endothelial function. It may be associated with an increased adhesion of leukocytes or altered balance of profibrinolytic to prothrombotic activity and may play a role in the disease process itself. However, it is not known whether impaired endothelium-dependent relaxation always reflects this more general endothelial dysfunction.

NO-Related Vascular Endothelial Dysfunction in Atherosclerosis and Hypercholesterolemia

Observations in Animals and Humans

In vessels with overt atherosclerotic pathology or in vessels from hypercholesterolemic rabbits, monkeys, and pigs with serum cholesterol levels of 600–2000 mg/dl, relaxation to acetylcholine (ACh) and other receptor-mediated (ATP, substance P, thrombin, bradykinin) inducers of NO biosynthesis is impaired (Coene *et al.,* 1985; Verbeuren *et al.,* 1986; Freiman *et al.,* 1986; Bossaller *et al.,* 1987; Harrison *et al.,* 1987; Yamamoto *et al.,* 1987; Jayakody *et al.,* 1987; Cohen *et al.,* 1988; Förstermann *et al.,* 1988; Shimokawa and Van-

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houtte, 1988; Sellke *et al.,* 1990; Verbeuren *et al.,* 1990; Bult *et al.,* 1995) (Fig. 1). However, Merkel *et al.* (1990) demonstrated that even a modest elevation of serum cholesterol is sufficient to depress endothelium-dependent relaxations to carbamylcholine and to enhance vasoconstrictor responses to serotonin (5-HT). The NO defect is presumably the result, rather than the cause, of the vascular lesions, since endothelium-dependent responses are preserved in adjacent normal areas and arteries which do not develop fatty streaks (Verbeuren *et al.,* 1986; Senaratne *et al.,* 1991; Galle *et al.,* 1991). The latter findings are hard to reconcile with the proposal that hypercholesterolemia suppresses endothelial NO release (Minor *et al.,* 1990). However, one cannot exclude the possibility that progressive reduction of NO release aggravates fatty streak development (Cayatte *et al.,* 1994; Naruse *et al.,* 1994).

In most of these studies, histologic examination of the endothelium in blood vessels from cholesterol-fed animals revealed either no abnormality or only small changes in the endothelium. Endothelial cell denudation was minimal or absent. Therefore, hypercholesterolemia and diet-induced atherosclerosis produce a functional rather than an anatomic abnormality of the endothelium (Harrison, 1994). Endothelium-mediated relaxation in the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model of familial hypercholesterolemia (Buja *et al.,* 1983) is attenuated with progression of atherosclerosis (Kolodgie *et al.,* 1990; Ragazzi *et al.,* 1989).

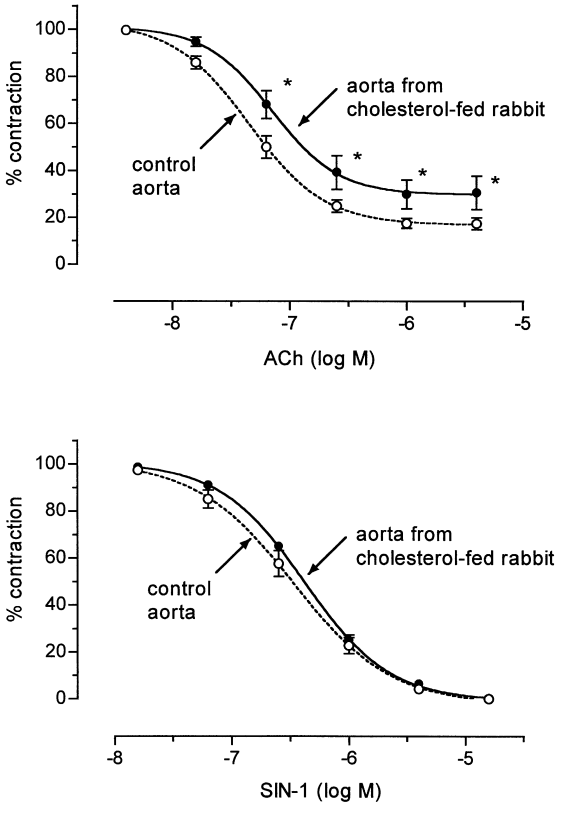


Figure 1 Example of NO-related vascular endothelial dysfunction. The figure represents relaxation curves to acetylcholine (ACh, top graph) and the NO donor SIN-1 (bottom graph) in the thoracic aorta of rabbits exposed to a control diet (◯) or a diet supplemented with cholesterol (●). In controls, ACh caused about 82% relaxation of rings contracted with phenylephrine. A cholesterol-rich diet led to both a rightward shift of the concentration–response curve and a reduction of the amplitude of the relaxation (*, different from control group, $P < 0.05$). The relaxation curve to the exogenous NO donor SIN-1 in aortic segments from hypercholesterolemic rabbits did not differ from controls, excluding the possibility that the fatty steaks developed in response to the diet interfered with the relaxing capacity of the thoracic aorta. (After Bult *et al.,* 1995.)

The early work concerning vascular endothelial dysfunction in animals involved large conduit arteries. The resistance vasculature does not develop overt atherosclerosis. Thus, it might be possible that endothelium-dependent vascular relaxation may be normal in this segment of the circulation despite the presence of atherosclerotic lesions in larger vessels. However, it has been shown that hypercholesterolemia can also alter endothelial function in the microcirculation of rabbits, primates, and pigs (Yamamoto *et al.,* 1988; Sellke *et al.,* 1990; Kuo *et al.,* 1992). Endothelium-dependent vascular relaxations to bradykinin, ACh, and the calcium-ionophore A23187 were abnormal in resistance vessels from cholesterol-fed animals. In contrast, relaxations to the endothelium-independent vasodilators nitroprusside and adenosine were not altered in these vessels. Thus, in addition to predisposing large vessels to altered vasomotion, endothelial dysfunction in atherosclerosis may also impair neurohumoral regulation of tissue perfusion at the microvascular level (Harrison, 1994).

NO contributes importantly to resting epicardial and coronary microvascular tone. Coronary vascular dilation in response to ACh is predominantly due to increased production of NO. *In vitro* studies with isolated human coronary arteries have shown that the endothelium-dependent vasodilators ACh and substance P relax nonatherosclerotic coronary segments (Bossaller *et al.,* 1987; Förtstermann *et al.,* 1988). However, in isolated atherosclerotic human coronary arteries, endothelium-dependent relaxations to ACh are abolished (Bossaller *et al.,* 1987; Förstermann *et al.,* 1988), and those to substance P (Bossaller *et al.,* 1987; Förstermann *et al.,* 1988; Chester *et al.,* 1990), histamine (Bossaller *et al.,* 1987), and bradykinin (Chester *et al.,* 1990) are partly suppressed. The response to A23187 may be completely preserved (Bossaller *et al.,* 1987) or not (Förstermann *et al.,* 1988). Some of these *in vitro* findings have been extended to the *in vivo* situation.

Ludmer *et al.* (1986) demonstrated paradoxical vasoconstriction induced by ACh both early and late in the course of coronary atherosclerosis in patients, suggesting an association of endothelial dysfunction and atherosclerosis. Furthermore, the coronary arteries of cardiac transplant patients may constrict in response to ACh even though angiographic evidence of coronary artery disease is absent (Fish *et al.,* 1987). In patients with normal coronary arteriograms and angina pectoris an impairment of endothelium-dependent vasodilation is present similar to that observed in patients with overt coronary artery atherosclerosis (Werns *et al.,* 1989; Vrints *et al.,* 1992). Using intracoronary infusions of ACh, substance P, and nitroglycerin (NTG), Bossaller *et al.* (1989) and Crossman *et al.* (1989) provided evidence that the failure of

atherosclerotic human coronary arteries to dilate in response to ACh represents a muscarinic endothelial dysfunction. Vita *et al.* (1990) showed that patients with angiographically smooth coronary arteries and few risk factors for coronary disease tend to exhibit coronary vasodilation in response to intracoronary ACh infusion, whereas patients with the same apparently normal coronary arteries and increased risk factors show segmental coronary vasoconstriction. It has also been demonstrated that most of the angiographically normal coronary arteries in subjects more than 30 years old, as well as in patients with coronary artery disease, constrict to an intracoronary injection of ACh, as do coronary arteries with evident atherosclerosis, whereas those of younger subjects dilate in response to this agent (Yasue *et al.,* 1990). Furthermore, despite the absence of angiographic evidence of atherosclerosis, exposure to coronary risk factors is associated with reduced resting and stimulated bioavailability of NO from the human coronary circulation (Quyyumi *et al.,* 1995).

Thus, endothelial dysfunction occurs early in atherosclerosis, predating clinical disease. It has also been demonstrated that endothelial dysfunction is associated with cholesterol levels in the high normal range in humans. There was a negative correlation between total cholesterol levels and maximal endothelium-dependent vasodilation (Steinberg *et al.,* 1997). In addition, a more recent study by Lewis *et al.* (1999) demonstrated that endothelium-dependent relaxation by acetylcholine is also impaired in hypertriglyceridemic humans with normal levels of plasma low density lipoprotein (LDL) cholesterol.

Evidence of endothelial dysfunction may identify young individuals most likely to develop atherosclerosis. To assess endothelial function in large populations noninvasive approaches are preferable (Creager and Selwyn, 1997). In the human brachial artery atherosclerosis is common and is significantly correlated with both coronary and carotid disease (Sorensen *et al.,* 1997). Furthermore, there is a strong correlation between flow-mediated endothelium-dependent dilation in the coronary and brachial arteries. Thus, noninvasive assessment of flow-mediated dilation in the brachial artery may serve as a ''surrogate'' measure for coronary artery endothelial function (Takase *et al.,* 1998). The diameter of a conduit artery such as the brachial artery during an endothelium-dependent stimulus (e.g., shear stress created by reactive hyperemia) can be measured by high-resolution ultrasonography (Sorensen *et al.,* 1995). In patients with hypercholesterolemia or other risk factors for atherosclerosis, and in patients with coronary artery disease, this vasodilation is impaired (Celermajer *et al.,* 1992, 1994; Tawakol *et al.,* 1997).

Mechanisms of NO-Related Vascular Endothelial Dysfunction in Atherosclerosis and Hypercholesterolemia, and Associated Therapeutic Aspects

Several lines of evidence provide arguments that the mechanisms underlying the role of NO in vascular endothelial dysfunction are diverse and likely multifactorial (Harrison, 1997). Indeed, the dysfunction may be situated at different levels of the L-arginine–NO pathway (Fig. 2): (1) dysfunctional receptor coupling and signal transduction, (2) decreased bioavailability of L-arginine, (3) altered expression and/or structure of endothelial NO synthase (eNOS), (4) altered eNOS activity, (5) increased destruction of NO, (6) changes in the balance of NO–endothelium-derived hyperpolarizing factor (EDHF), and (7) decreased sensitivity of atherosclerotic smooth muscle to NO. Each of these factors will be discussed in more detail.

Dysfunctional Receptor Coupling and Signal Transduction

A defect in membrane receptors or a defect in the signaling mechanisms activated by these receptors, for example, changes in the coupling and/or expression of G proteins, may account for a dysfunctional signal transduction of NO.

As described earlier endothelium-dependent relaxations in response to nonreceptor mediated stimuli such as the calcium ionophore A23187 are altered to a smaller extent as responses to receptor-mediated stimuli (Bossaller *et al.,* 1987). Therefore, there may be a defect in the membrane receptors and/or the signal transduction mechanism activated by these receptors as described later. However, this concept has been controversial because others have reported that the endothelium-dependent vascular relaxation to A23187 is also abnormal in vessels from hypercholesterolemic animals and humans (Förstermann *et al.,* 1988; Mügge *et al.,* 1991; Ohara *et al.,* 1993). The nature of the lesions (early or advanced) may account for these discrepancies (see later).

In the WHHL rabbit aorta progressive atherosclerosis differentially affects the activity of endothelial receptors: the most precociously altered is the P2y-purinoceptor, followed by an impairment of the muscarinic and finally of the P2U-purinoceptor (Ragazzi *et al.,* 1995).

A major mechanism for information transfer across the lipid bilayer of the cell border is the guanine nucleotide binding protein (G protein) signal transduction system. G proteins relay extracellular signals to activate or inhibit intracellular enzymes and ion channels. A number of distinct G proteins have been identified, including Gi protein, which inhibits adenylyl cyclase and activates K^+ channels, Gs protein, which can activate adenylyl cyclase and calcium channels, and Gq protein, which activates phospholipase C. Certain G proteins can be ADP-ribosylated by pertussis toxin, resulting in an irreversible inhibition of their function. The endothelial pertussis toxin-sensitive G protein has been identified as a Gi protein, and couples certain endothelial receptors to the release of NO (e.g., muscarinic receptor, serotonin receptor, α_2-receptor). Other endothelial receptors, including bradykinin and purinergic receptors, are coupled to the Gq protein, which is insensitive to pertussis toxin (Flavahan, 1992).

Alterations in G protein function represent another mechanism to explain the impaired endothelium-dependent vascular relaxation in hypercholesterolemia and after endothe-

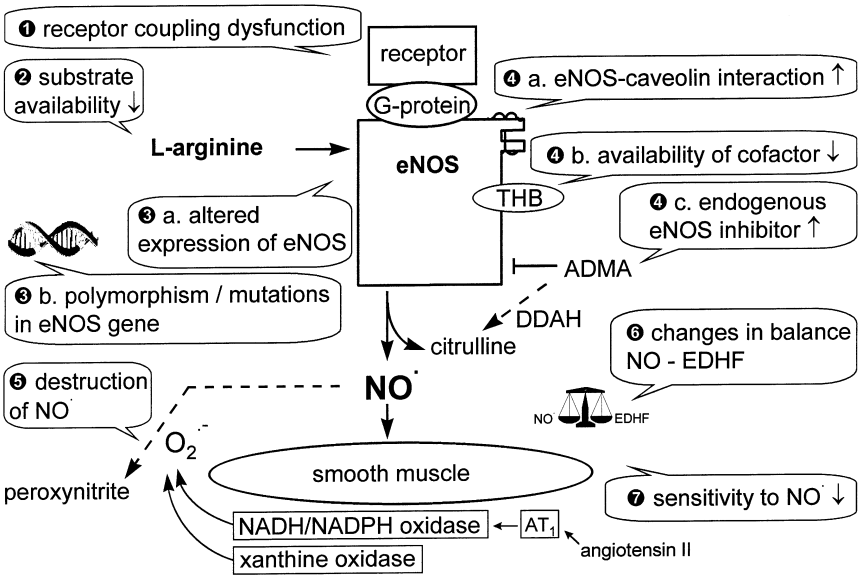


Figure 2 Mechanisms of NO-related vascular endothelial dysfunction in atherosclerosis and hypercholesterolemia. The mechanisms underlying the role of NO in vascular endothelial dysfunction are diverse and likely multifactorial. The dysfunction may be situated at different levels of the L-arginine–NO pathway: (1) dysfunctional receptor coupling and signal transduction, (2) decreased bioavailability of L-arginine, (3) altered expression and/or structure of endothelial NO synthase (eNOS), (4) altered eNOS activity, including enhanced inhibitory eNOS–caveolin interaction, decreased availability of tetrahydrobiopterin (THB), and elevated levels of an endogenous NO synthase inhibitor, (5) increased destruction of NO, (6) changes in the balance of NO to endothelium-derived hyperpolarizing factor (EDHF), and (7) decreased sensitivity of atherosclerotic smooth muscle to NO. For details, see text. ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; AT_1, angiotensin II subtype 1 receptor.

lial regeneration (Bossaller *et al.,* 1987; Shimokawa *et al.,* 1990; Flavahan, 1992). Endothelial dysfunction appears to occur in distinct phases, and the underlying mechanisms may be determined by the stage of the disease or the concentration of oxidized LDL (oxLDL).

In the early stages of the atherosclerotic process or after low concentrations of oxLDL, the endothelial dysfunction appears to be limited to the pertussis toxin-sentitive Gi protein-dependent pathway. Indeed, responses to G protein-mediated agents, such as ACh, are abnormal, whereas responses to the calcium ionophore A23187, which bypasses G protein signaling, are relatively normal (Flavahan, 1992). Furthermore, endothelial exposure to atherogenic lipids, including lysophosphatidylcholine (lysoPC), is characterized by a selective impairment of responses mediated by the pertussis toxin-sensitive Gi protein (Freeman *et al.,* 1996).

The impairment of the interaction of the G proteins with the receptors that activates eNOS may be due to changes in membrane fluidity (Briggs and Lefkowitz, 1980). Other studies showed that the expression of Gi protein was inhibited by oxLDL *in vitro* (Liao and Clark, 1995) and in human coronary arteries by hypercholesterolemia, hypertension, and age (Tsutsui *et al.,* 1994). Furthermore, G proteins are anchored in the lipid bilayer by means of covalently linked apolar hydrocarbon chains and belong to the class of geranylgeranyl or farnesyl-anchored proteins. In cholesterol-fed rabbits exogenous cholesterol downregulates mevalonic acid and subsequent farnesol biosynthesis. This may affect the G protein signal transduction pathway. Indeed, it has been shown that in hypercholesterolemic rabbits farnesol treatment restored impaired nitrergic [nonadrenergic, noncholinergic (NANC), neuronal NOS (nNOS)-mediated] relaxation of the sphincter of Oddi, presumably via a G protein related mechanism (Szilvassy *et al.,* 1998). Since the muscarinic receptor–eNOS signal transduction involves Gi proteins (Hare *et al.,* 1998; Flavahan, 1992) it is possible that farnesol also restores vascular endothelium-dependent relaxation via improvement of muscarinic receptor–eNOS signal transduction in endothelial cells of cholesterol-fed rabbits.

As atherosclerosis progresses or as the concentration of oxLDL is increased, dysfunction also occurs in endothelial mechanisms distinct from the Gi-protein-dependent pathway, such as a decrease in the bioavailability of L-arginine, a reduced sensitivity of the vascular smooth muscle cells to NO, or an increased breakdown of NO (cf. other mechanisms). This is illustrated by an impaired endothelium-dependent relaxations to A23187 (Flavahan, 1992).

Decreased Availability of L-Arginine

NO is formed from the amino acid L-arginine by NO synthase. In the blood the concentration of L-arginine is approximately 100 μM (Böger *et al.,* 1997a). L-Arginine is actively transported into the endothelial cells by a cationic amino acid transporter, which colocalizes with eNOS in

caveolae (microdomains within the plasma membrane, *vide infra*) (McDonald *et al.,* 1997). The concentration of L-arginine in endothelial cells has been estimated to be several 100 μ*M* (Hecker *et al.,* 1990), far above the K_m of NO synthase, which is about 5 μ*M* (Harrison, 1997). Moreover, endothelial cells can resynthesize L-arginine from L-citrulline (Hecker *et al.,* 1990).

Although depletion of L-arginine is thus not likely to account for endothelial dysfunction, experiments in which exogenous L-arginine was given *in vitro* to vascular segments in organ chambers or *in vivo* to hypercholesterolemic animals and humans nevertheless produced contradictory results. The fact that extracellular L-arginine administration seems to drive NO production even when intracellular levels of L-arginine are available in excess, has been called the ''arginine paradox'' (Kurz and Harrison, 1997).

In *in vitro* studies L-arginine failed to correct the endothelial dysfunction of the aorta of cholesterol-fed (Bult *et al.,* 1990; Mügge and Harrison, 1991) and WHHL rabbits (Caparrotta *et al.,* 1993), as well as in human arteries (Cooper and Heagerty, 1998). Moreover, the intracellular concentration of L-arginine in endothelial cells can be varied over 100-fold without changing the production of NO (Arnal *et al.,* 1995).

In contrast, *in vivo* administration of L-arginine improved vascular responses to endothelium-dependent vasodilators, both in hypercholesterolemic animals and in humans (Kuo *et al.,* 1992; Girerd *et al.,* 1990; Cooke *et al.,* 1991; Drexler *et al.,* 1991), though the behavior of conduit arteries with atherosclerotic plaques was different from arterioles which do not develop atherosclerosis. In patients with hypercholesterolemia, L-arginine infusion improved or restored endothelium-dependent vasodilation in the coronary and peripheral arterioles without atherosclerosis (Drexler *et al.,* 1991; Creager *et al.,* 1992; Adams *et al.,* 1997). However, this was not confirmed by others (Casino *et al.,* 1994; Dakak *et al.,* 1998; Nitenberg *et al.,* 1998). In patients with coronary artery disease or peripheral artery occlusive disease L-arginine did not improve endothelium-dependent dilatation of conduit arteries with atherosclerotic plaques.

How can these contradictory and/or paradoxical effects of L-arginine be explained?

1. An explanation for why L-arginine is not effective in *in vitro* studies is the composition of the buffers used. L-arginine has no effect on endothelium-dependent vascular relaxations in a simple Krebs buffer. When L-glutamine is present in concentrations similar to those in the plasma, L-arginine enhances endothelium-dependent relaxations, possibly by reversing the inhibitory effect of L-glutamine in receptor-mediated NO release (Arnal *et al.,* 1995).

2. The levels of L-arginine in endothelial cells may be decreased due to induction of arginase and/or an increased arginase activity, which degrades L-arginine to ornithine and urea (Buga *et al.,* 1996). In normal endothelial cells arginase and not NO synthase is the major pathway of L-arginine metabolism (Wu and Meininger, 1995).

3. In endothelial cells the intracellular L-arginine may be sequestered in pools that are poorly accessible to eNOS. It has more recently been shown that the arginine transporter [cationic amino acid transporter 1 (CAT1)], eNOS, and caveolin (see later) colocalize in plasma membrain microdomains (caveolae). A caveolar complex between CAT1 and eNOS provides a mechanism for the directed delivery of extracellular L-arginine to membrane-bound eNOS and would account for the arginine paradox, that is, that extracellular L-arginine administration seems to drive NO production even when intracellular levels of L-arginine are available in excess (McDonald *et al.,* 1997). It also explains why caveolar localization of eNOS is required for optimal NO production by endothelial cells (see later).

4. Oral L-arginine supplementation inhibits the formation of intimal lesions in hypercholesterolemic rabbits (Cooke *et al.,* 1992; Wang *et al.,* 1994), reduces intimal hyperplasia after balloon injury in the rabbit, and improves endothelium-dependent vasorelaxation (McNamara *et al.,* 1993; Tarry and Makhoul, 1994). These effects of L-arginine have been proposed to be due to its metabolism to NO, as the effect is not mimicked by D-arginine. Moreover, L-arginine attenuates both platelet reactivity in hypercholesterolemic rabbits (Tsao *et al.,* 1994a) and the enhanced endothelial adhesiveness for monocytes (Tsao *et al.,* 1994b). In hypercholesterolemic rabbits dietary L-arginine also completely blocked the progression of plaques via restoration of NO synthase substrate availability, reduction of vascular oxidative stress (Böger *et al.,* 1997b), inhibition of cell proliferation, and vascular monocyte accumulation (Böger *et al.,* 1998). The endothelial dysfunction in the atherosclerotic rabbit aorta is dependent on the size of the plaque (Bult *et al.,* 1995). Therefore, it is likely that the antiatherogenic effect of L-arginine supplementation contributes to the improvement of endothelium-dependent relaxations. However, in arterioles of hypercholesterolemic rabbits (Girerd *et al.,* 1990) and pigs (Kuo *et al.,* 1992), which do not develop atherosclerotic plaques, endothelium-dependent vasodilation was slightly enhanced during L-arginine infusion. Therefore, L-arginine seems to exert additional effects.

5. Effects of L-arginine not related to its role as a substrate for NO synthase may account for the arginine paradox as well. L-arginine has a potent hormonal (i.e., insulin) secretagogue activity (Barbul, 1990; Giugliano *et al.,* 1997) which can influence vascular reactivity in general, as suggested by the similar shift of endothelium-independent relaxations after *in vivo* treatment with L-arginine (Cooke *et al.,* 1991).

6. In addition, in humans the nature of endothelial injury associated with individual cardiovascular risk factors might be different and this might affect the response to L-arginine. Although flow-mediated dilation is impaired in hypercholesterolemic subjects, smokers, and diabetic subjects, these three risk factor groups respond differently to L-arginine. Indeed, flow-mediated dilation is improved in hypercholesterolemic subjects and smokers but unchanged in diabetic subjects (Thorne *et al.,* 1998). Moreover, in healthy long-

term smokers acute administration of L-arginine reversed the abnormal myocardial blood flow response to cold pressor test. This test evokes a mixed, adrenergically mediated vasoconstrictor and vasorelaxant response; the vasorelaxant effects are thought to be mediated through β-adrenoceptor stimulation and through NO release from endothelial cells (Campisi *et al.,* 1999). Therefore, differing underlying pathophysiologies may facilitate the design of treatment strategies for subjects with different risk factors.

ALTERATIONS IN eNOS

Altered Expression of eNOS Although eNOS is constitutively present, its expression can be regulated. Exposure of cultured endothelial cells to tumor necrosis factor α (TNF-α), hypoxia, and high concentrations of oxLDL decreases the levels of eNOS (Liao *et al.,* 1995). It has been reported that the expression of eNOS is reduced in endothelial cells overlying advanced atherosclerotic plaques (Wilcox *et al.,* 1997). *In vitro* studies demonstrated that both eNOS mRNA and protein are downregulated by atherogenic concentrations of native LDL at a transcriptional level by a specific and concentration-dependent mechanism (Vidal *et al.,* 1998). In another study a marked reduction of immunoreactive eNOS in endothelial cells was accompanied by reduced NO release in atherosclerotic human carotid artery segments, as measured with a porphyrinic microsensor and compared to normal mammary arteries (Oemar *et al.,* 1998). Thus, in clinically relevant human atherosclerosis, eNOS protein expression and NO release are markedly reduced. In this way hypercholesterolemia induces early changes in endothelial cells that could have significance in the etiopathogenesis of the atherosclerotic process, including its progression. Also mechanical forces may downregulate eNOS levels in regions prone to the development of atherosclerosis (Ziegler *et al.,* 1998).

Although there is a loss of eNOS expression by endothelial cells covering advanced atherosclerotic lesions, there is a significant increase in overall NOS synthesis by other cell types in advanced lesions. The increased expression of NOS and presumably NO in atherosclerotic plaques may be related to cell death and necrosis in these tissues (Wilcox *et al.,* 1997). Also, explanted vein grafts with an intact endothelium demonstrate reduced focal expression of eNOS specific to atherosclerotic sites, which supports the view that vascular activity of NO is impaired in atherosclerosis. Reduced expression of eNOS may make an important contribution to this phenomenon (Buttery *et al.,* 1996). It has been shown that overexpression of eNOS with an adenoviral vector improves impaired NO-mediated relaxation in atherosclerotic arteries (Ooboshi *et al.,* 1998). Furthermore, although 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) restore endothelial function by reducing serum cholesterol levels, they can also directly upregulate eNOS expression. Atorvastatin and simvastatin prevent the inhibitory action exerted by oxLDL on eNOS mRNA and protein levels. The effects on eNOS expression correlate with changes in eNOS activity. Thus, HMG CoA reductase inhibitors may have beneficial effects in atherosclerosis beyond that attributed to the lowering of serum cholesterol by increasing eNOS activity (Laufs *et al.,* 1998; Hernandez-Perera *et al.,* 1998).

In contrast, WHHL rabbit aortas show significantly impaired endothelium-dependent relaxations, although *in situ* hybridization and immunohistochemistry, respectively, exhibit enhanced expression of eNOS mRNA and protein in WHHL aortas (Kanazawa *et al.,* 1996). Similarly, low concentrations of oxLDL and lysophosphatidylcholine upregulate eNOS mRNA in bovine aortic endothelial cells (Hirata *et al.,* 1995).

Polymorphism/Mutations in the eNOS Gene A common polymorphism in exon 7 of the eNOS gene (894G→T) has been reported to be a strong risk factor for coronary artery disease. Markus *et al.* (1998) failed to find a relationship between this exon 7 polymorphism and ischemic cerebrovascular disease. In particular, it was not associated with stroke and transient ischemic attack secondary to large-vessel atherosclerosis or with the degree of carotid stenosis in patients with cerebrovascular disease. However, the missense Glu298Asp variant in exon 7 of the eNOS gene is significantly associated with myocardial infarction (Shimasaki *et al.,* 1998; Hibi *et al.,* 1998), essential hypertension (Miyamoto *et al.,* 1998), and coronary spasm (Yoshimura *et al.,* 1998). This marker-disease association may be due to the impaired effects of NO on the cardiovascular system, such as dysregulation of vascular tone, platelet aggregation and leukocyte adhesion, and smooth muscle cell proliferation. Furthermore, it has more recently been shown that the T^{-786}→C mutation in the 5'-flanking region of the eNOS gene is associated with coronary spasm and results in a significant reduction in eNOS gene promoter activity (Nakayama *et al.,* 1999).

ALTERATIONS IN eNOS ACTIVITY

Alterations of eNOS activity can be due to several factors, such as: (a) enhanced inhibitory eNOS–caveolin interaction (Fig. 3); (b) decreased availability of tetrahydrobiopterin; and (c) elevated levels of an endogenous NO synthase inhibitor.

Enhanced Inhibitory eNOS–Caveolin Interaction NO production in the vascular endothelium is promoted by diverse agonists that transiently increase intracellular Ca^{2+} concentration and activate eNOS, a Ca^{2+}/calmodulin-dependent enzyme. eNOS is acylated by the fatty acids myristate and palmitate and is targeted thereby to plasmalemmal signal-transducing domains termed caveolae. Caveolae are small invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin. eNOS enzyme activity is markedly attenuated by its interactions with caveolin, the structural scaffolding protein of caveolae. In living cells, the eNOS–caveolin heteromeric complex undergoes cycles of dissociation and reassociation

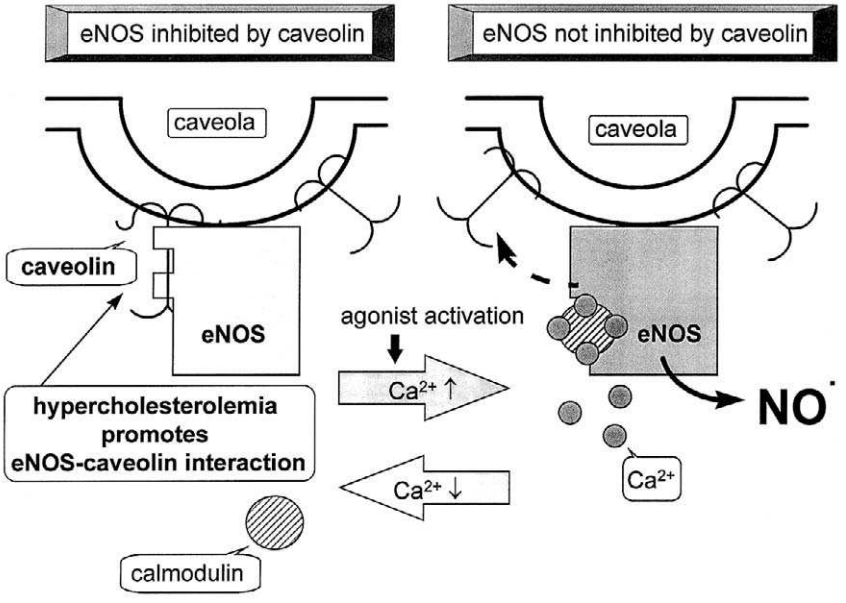


Figure 3 eNOS–caveolin interaction. Caveolae are small invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin. The interaction of eNOS with caveolin maintains the enzyme in its inactivated state. The calcium regulatory protein calmodulin, which is enriched in the caveolar fraction of plasma membranes, does not bind to eNOS in the absence of Ca^{2+}. After agonist activation or other stimuli causing a local increase in the intracellular Ca^{2+} concentration, Ca^{2+}-bound calmodulin competitively displaces caveolin from eNOS. This allows the conformational changes within eNOS required for electron transfer, leading to the synthesis of NO by the activated enzyme. Exposure of endothelial cells to the serum of hypercholesterolemic individuals or its LDL fraction upregulates caveolin abundance, without a change in eNOS protein levels. This is associated with increased inhibitory eNOS–caveolin complex formation, accompanied by an impairment of NO production. (After Michel and Feron, 1997, and Feron *et al.,* 1999.)

modulated by Ca^{2+}-mobilizing agonists. Calcium ionophore A23187 and the muscarinic cholinergic agonist carbamylcholine both promote the dissociation of eNOS from caveolin in cultured cells, associated with translocation of eNOS from caveolae. Thus, after agonist activation, the increase in $[Ca^{2+}]_i$ promotes calmodulin binding to eNOS and the dissociation of caveolin from eNOS. As $[Ca^{2+}]_i$ returns to basal levels, eNOS reassociates with caveolin, and the inhibited enzyme complex is then restored to caveolae, a process accelerated by palmitoylation of the enzyme. Taken together, in the eNOS–caveolin regulatory cycle eNOS activation is modulated by reversible protein–protein interactions controlled by Ca^{2+}/calmodulin and by enzyme palmitoylation (Michel and Feron, 1997).

More recently, it has been shown that exposure of endothelial cells to the serum of hypercholesterolemic individuals or its LDL fraction upregulates caveolin abundance, without a change in eNOS protein levels. This is associated with increased inhibitory eNOS–caveolin complex formation, accompanied by an impairment of both basal and calcium ionophore A23187-stimulated NO production (Feron *et al.,* 1999). The findings with A23187, a receptor-independent agonist for eNOS stimulation, exclude the G protein-coupled receptor signaling cascade in this effect. Since this impairment in NO release by endothelial cells occurs in the absence of any change in eNOS protein levels it may be one of the early events among the proatherogenic processes induced by hypercholesterolemia. Indeed, the early impairment of NO-mediated vascular relaxation in hypercholesterolemia is generally observed without a decrease in eNOS protein level (Kanazawa *et al.,* 1996) or angiographically or ultrasound-detectable changes of the vessel wall (Seiler *et al.,* 1993; Reddy *et al.,* 1994). Thus, in early atherogenesis eNOS activity appears to be regulated at the posttranslational level (Feron *et al.,* 1999). Furthermore, because it has been shown that an increase in shear stress is associated with an increase in NO production rate and eNOS phosphorylation, also the degree of phosphorylation of eNOS may play a role in NO-related vascular endothelial dysfunction (Corson *et al.,* 1996).

Decreased Availability of Tetrahydrobiopterin Tetrahydrobiopterin is an essential cofactor required for catalytic activity of NO synthases. The precise role of tetrahydrobiopterin in regulation of NO synthase activity is not completely understood. It may function as both an allosteric and redox cofactor. It appears to contribute to the binding of L-arginine to NO synthase (Harrison, 1997) and has antioxidative activity (Kojima *et al.,* 1995). In peripheral and cerebral arteries, administration of tetrahydrobiopterin can stimulate the pro-

duction of NO. In contrast, in arteries depleted of tetrahydrobiopterin, NO production is impaired. In the absence of tetrahydrobiopterin NO synthase transfers an electron to oxygen. Consequently, superoxide anion is formed (Pou *et al.,* 1992). Decreased availability of tetrahydrobiopterin may be responsible for a dysfunction of NO synthase leading to a shift in the balance between the production of protective NO and deleterious oxygen-derived free radicals (Cosentino and Lüscher, 1998). It has been demonstrated that tetrahydrobiopterin (or a derivative) restores endothelial function in aortic rings from diabetic rats *in vitro* (Pieper, 1997) and in hypercholesterolemic patients (forearm blood flow) (Stroes *et al.,* 1997). This effect may be explained by an increased production of NO and/or by the antioxidative capacity of tetrahydrobiopterin.

Elevated Levels of an Endogenous NO Synthase Inhibitor A circulating endogenous NO synthase inhibitor, asymmetric dimethylarginine (ADMA), has been detected in human plasma (Fickling *et al.,* 1993; Pettersson *et al.,* 1997). High levels of ADMA have been shown in the plasma of cholesterol-fed rabbits (Bode-Böger *et al.,* 1996), in the regenerating endothelium of balloon injured vessels (Azuma *et al.,* 1995), in the urine of hypertensive rats (Matsuoka *et al.,* 1997), and in patients with peripheral arterial occlusive disease (Böger *et al.,* 1997a). However, the intracellular levels of these endogenous antagonist in different cardiovascular diseases are not known. Plasma dimethylarginines arise mainly from degradation of intracellular methylated proteins and are eliminated via urinary excretion (McDermott, 1976). ADMA is metabolized to citrulline by dimethylarginine dimethylaminohydrolase (DDAH) (Ogawa *et al.,* 1989). Inhibitors of DDAH block ADMA degradation and induce a contraction of isolated vascular rings (MacAllister *et al.,* 1996), which is consistent with the concept that ADMA produced by vascular cells modulates the synthesis of NO by the endothelial cells. Taken together, plasma and/or vascular ADMA may be increased due to an increased degradation of methylated proteins, a decreased renal filtration, and/or a decreased activity of DDAH.

In vivo, L-arginine supplementation may overcome the competitive inhibition evoked by ADMA, which offers a possible explication for the restoration of endothelial NO-mediated vasodilation (Cooke and Dzau, 1997). In addition, a more recent study reveals that plasma ADMA levels are positively correlated with risk factors for atherosclerosis (mean arterial blood pressure, incremental area of plasma glucose level over time, and age) and that plasma ADMA levels are significantly correlated with intima–media thickness of the human carotid artery (Miyazaki *et al.,* 1999).

Overproduction of Oxygen-Derived Free Radicals and Destruction of NO

Several studies in animals and humans provide arguments that oxidative inactivation of NO may also be important as an explanation for vascular endothelial dysfunction. The endothelium generates superoxide anions under basal conditions and may be stimulated to generate excess superoxide anions by several factors. Indeed, infusion of the antioxidant ascorbic acid (vitamin C) improves vascular responses to ACh or metacholine in cigarette smokers, in diabetics, in patients with hypertension, in chronic heart failure, and in patients with hypercholesterolemia (Heitzer *et al.,* 1996; Ting *et al.,* 1996, 1997; Solzbach *et al.,* 1997; Hornig *et al.,* 1998a), which supports the notion that these conditions are associated with increased radical formation that in turn affects endothelium-mediated vasomotor tone.

Thus, in arteries subject to hypercholesterolemia or atherosclerosis the biological activity of NO is attenuated, whereas its synthesis might be normal. This may be caused by its inactivation in the intima by components of oxidized lipoproteins acting directly (by reaction with NO), indirectly (by simulation of release of NO scavengers), or both. Thus, in hypercholesterolemia the normal balance between NO availability and lipoprotein oxidation is shifted to favor a self-reinforcing cycle of NO depletion and accelerated lipoprotein oxidation that may ultimately lead to atherosclerosis (Jessup, 1996).

In a randomized double-blind placebo-controlled study of patients with angiographically proven coronary artery disease, the augmentation of cellular glutathione levels by L-2-oxo-4-thiazolidine carboxylate improved endothelium-derived NO action, as assessed by brachial artery flow-mediated dilation. Therefore, cellular redox state may be an important regulator of endothelium-derived NO action, and is a potential target for therapy in patients with coronary artery disease (Vita *et al.,* 1998).

In cholesterol-fed rabbits, the aortic endothelial cells produce excess quantities of nitrogen oxides with minimal vasodilator activity (Minor *et al.,* 1990), which suggests that NO is inactivated before being released from the endothelial cell. In this model chronic treatment with polyethylene glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations (Mügge *et al.,* 1991), which favors the concept that an increased superoxide anion production in hypercholesterolemia destroys endothelium-derived NO. Vascular superoxide anion production in this model is inhibited by oxypurinol (White *et al.,* 1996). Therefore, superoxide anion seems to be formed by xanthine oxidase. In the plasma of cholesterol-fed rabbits this enzyme is increased. Circulating xanthine oxidase has been shown to bind to heparin-binding sites on the vessel, where it produces superoxide anions (White *et al.,* 1996). Also the results of Warnholtz *et al.* (1999) are suggestive that vascular and/or circulating xanthine oxidase is a significant source of superoxide anion in hypercholesterolemic rabbits. An additional flavin-containing oxidase, such as NADH/NADPH oxidase, probably serves as a source of superoxide anion in hypercholesterolemic vessels (Warnholtz *et al.,* 1999). NADH/NADPH oxidase has been demonstrated to be the predominant superoxide source in both endothelial and smooth muscle cells (Mohazzab-H. *et al.,* 1994; Griendling *et al.,* 1994). In homogenates of aortas from WHHL rabbits and cholesterol-fed rabbits NADH oxidase activity was increased as com-

pared to controls (Warnholtz *et al.,* 1999). It has been shown that the activity of NADH/NADPH oxidase is regulated by angiotensin II. Activation of the angiotensin II subtype 1 (AT_1) receptor increases the activity of (membrane-associated) NADH/NADPH oxidase in smooth muscle cells (Griendling *et al.,* 1994) and endothelial cells (Lang *et al.,* 1997). In atherosclerotic lesions the expression of angiotensin converting enzyme (ACE) is increased (Diet *et al.,* 1996). Moreover, hypercholesterolemia is associated with enhanced expression of the AT_1 receptor (Nickenig *et al.,* 1997).

Endothelial dysfunction in hypercholesterolemic animals and in humans is improved by ACE-inhibitors. The TREND (trial on reversing endothelial dysfunction) trial showed that ACE inhibition with quinapril improved endothelial dysfunction in patients with coronary artery disease who were normotensive and who did not have severe hyperlipidemia or evidence of heart failure (Mancini *et al.,* 1996). How can this beneficial effect be explained in relation to the L-arginine–NO pathway? ACE inhibition inhibits the breakdown of bradykinin. It is of interest that endothelial cells can produce and release bradykinin locally, particularly in response to flow and shear stress. Locally released bradykinin may then activate B2-bradykinin receptors on endothelial cells and activate the endothelial L-arginine–NO pathway (Büsse and Lamontagne, 1991), as well as EDHF (Holzmann *et al.,* 1994). A bradykinin antagonist partly diminishes this effect. Therefore, the protective effect of ACE inhibitors has been attributed to inhibition of bradykinin breakdown, with subsequent high concentrations of NO and EDHF, rather than inhibition of angiotensin formation itself. However, improvement in endothelium-dependent vasodilation and vascular superoxide anion production can be achieved independently of bradykinin preservation. AT_1-receptor blockade inhibits NADH-oxidase activity and in parallel improves endothelial dysfunction in cholesterol-fed rabbits (Warnholtz *et al.,* 1999). Thus, the increased activity of NADH oxidase in hypercholesterolemia may be due to increased local angiotensin II generation and may be partly responsible for the enhanced superoxide production. Therefore, angiotensin II may play a crucial role in superoxide anion production and endothelial dysfunction in the early stage of atherosclerosis. Taken together, the renin–angiotensin system and the L-arginine–NO pathway are interconnected. Modulation of NO generation and degradation by, respectively, bradykinin and superoxide anions contribute to the beneficial effect of ACE inhibitors when the endothelium is dysfunctional.

CHANGES IN THE BALANCE OF NO-EDHF

In some blood vessels, such as the rabbit carotid and porcine coronary arteries, inhibition of eNOS only slightly attenuates endothelium-dependent relaxations. These relaxations are mediated by an endothelial factor distinct from NO, which has been named endothelium-derived hyperpolarizing factor (EDHF) since it hyperpolarizes the vascular smooth muscle cells, most likely by opening calcium-sensitive potassium channels. The chemical nature of EDHF is still unclear. There is evidence that in coronary, cerebral, and renal arteries EDHF is a cytochrome P-450 monooxygenase-derived arachidonic acid metabolite, an epoxyeicosatrienoic acid. The rabbit carotid artery and rabbit aorta have EDHF activity but do not produce metabolites of arachidonic acid by the cytochrome P-450 pathway. Thus, some other compound(s), possibly NO or another metabolite of arachidonic acid, may mediate the effect (Campbell and Harder, 1999). In isolated arteries proinflammatory mediators inhibit the formation of EDHF. This impairment is coincident with inducible NOS (iNOS) expression in the arterial wall and seems to be mediated through the induced generation of NO, which downregulates the EDHF-forming enzyme. Thus, a decreased formation of EDHF may contribute to the endothelial dysfunction in atherosclerosis (Kessler *et al.,* 1999). The contribution of EDHF to endothelium-dependent relaxations is significantly larger in microvessels than in large arteries. Aging and hypercholesterolemia significantly impair EDHF-mediated relaxations (Urakami-Harasawa *et al.,* 1997).

In the renal artery from hypercholesterolemic rabbits the nitro-L-arginine-resistant part of ACh-induced endothelium-dependent relaxation was enhanced, in comparison to arteries from normal animals. Possibly, differences in the balance between NO- and EDHF-mediated control of vascular tone may maintain ACh-induced vasodilation (Brandes *et al.,* 1997).

DECREASED SENSITIVITY OF ATHEROSCLEROTIC SMOOTH MUSCLE TO NO

In contrast to endothelium-dependent agonists, the relaxations in response to the endothelium-dependent exogenous NO donors NTG or sodium nitrate are normal or near normal in atherosclerotic blood vessels (Bossaller *et al.,* 1987; Freiman *et al.,* 1986; Jayakody *et al.,* 1987; Verbeuren *et al.,* 1986). The vasodilator actions of exogenous nitrovasodilators may even be augmented acutely by endothelial removal and chronically by diseases that damage the endothelial lining. Basal formation of NO may interfere with the relaxant effects to exogenous nitrovasodilators. The removal of this basal influence of NO seems to sensitize the guanylyl cyclase to agents that act by the stimulation of this pathway (Guerra *et al.,* 1989). However, as atherosclerosis progresses it may also interfere more directly with vascular smooth muscle cell relaxation. In rabbit aortae with severe fatty streak formation the relaxations induced by NTG are reduced (Verbeuren *et al.,* 1990). Moreover, the endothelium-independent relaxations evoked by atrial natriuretic factor are attenuated in thoracic aortae from hypercholesterolemic rabbits (Verbeuren *et al.,* 1990). Also the relaxations evoked by ATP are inhibited more in the atherosclerotic rabbit aortic arch than in a control aortic arch without endothelium (Verbeuren *et al.,* 1986). In addition, vessels with severe atherosclerosis taken from WHHL rabbits are less sensitive to NTG (Kolodgie *et al.,* 1990). Moreover, it has been shown that there is a substantial resistance to NO action in aortic smooth muscle cells of cholesterol-fed rabbits. This is consistent with the notion that resistance of smooth muscle cells to NO contributes to

abnormal endothelium-dependent vasodilation during hypercholesterolemia and can play a role in the pathogenesis of atherosclerosis (Weisbrod *et al.,* 1997). Moreover, the vasodilator response to exogenous NO is impaired in asymptomatic subjects with reduced endothelium-dependent dilation, consistent with smooth muscle dysfunction in adults at risk for atherosclerosis (Adams *et al.,* 1998). Taken together, these findings indicate that smooth muscle cell responsiveness to vasodilator substances is affected as atherosclerosis progresses.

Endothelium-Mediated Relaxation after Balloon Catheter De-endothelialization and Balloon Angioplasty

The mechanism of dilation by balloon angioplasty is disruption or splitting of the plaque and stretching of the underlying arterial wall to increase the intraluminal diameter of the vessel. Although the removed endothelium may regrow from the edges of the lesion, its function may not return to normal. The degree and duration of endothelial dysfunction seem to depend on the severity of the initial injury and also seem to be related to the extent of intimal thickness. The endothelium-dependent relaxation of balloon-injured vessels to ACh and to the calcium ionophore A23187 was reduced at 2 and at 4 weeks after severe injury (Weidinger *et al.,* 1990). Bosmans *et al.* (1996) demonstrated that although regeneration of endothelial cells was complete within 2 weeks, the endothelium-dependent relaxations evoked by ACh remained impaired even up to 10 weeks after dilation. Thus, although the endothelium quickly regenerated after severe balloon injury, the endothelium-dependent release of NO remained disturbed. However, angioplasty also led to a significant induction of NOS in ''nonendothelial'' cells of the artery (Bosmans *et al.,* 1996).

After moderate injury, endothelium-dependent relaxations to ACh and A23187 were reduced at 2 weeks but had normalized by 4 weeks (Weidinger *et al.,* 1990). Dysfunction of a pertussis toxin-sensitive G protein partly accounted for the endothelial abnormality in the chronic regenerated state (Shimokawa *et al.,* 1990).

Implications of Endothelial Dysfunction in Atherosclerosis

It is becoming clear that NO, in addition to regulating vasomotion, might also modulate the progression of atherosclerosis, and the restenosis process after balloon angioplasty (Bult, 1996). NO appears to inhibit atherogenesis by inhibiting leukocyte adhesion and platelet adhesion and aggregation and by inhibiting smooth muscle cell proliferation and migration. Since the renin–angiotensin system and the L-arginine–NO pathway are interconnected (see earlier) modulation of NO generation and degradation, respectively, by bradykinin and superoxide anions contribute to the beneficial effect of ACE inhibitors in atherogenesis.

Excess of LDL cholesterol leads to selective impairment of NO-dependent vasodilation (forearm blood flow measured by venous occlusion plethysmography) even in young adults, whereas adenylyl cyclase-dependent vasodilation of vascular smooth muscle and maximal dilatory capacity are preserved. In view of the antiatherogenic properties of NO, it appears desirable to detect this selective vascular dysfunction early in young adults at high risk of developing atherosclerotic lesions (Preik *et al.,* 1996a).

However, like the Roman god Janus, NO seems to have a double face in the atherogenic process. In atherosclerosis, diabetes mellitus, and high renin hypertension, there is increased production of superoxide. Superoxide rapidly reacts with NO to form the highly reactive intermediate peroxynitrite ($ONOO^-$). Peroxynitrite reacts relatively slowly with most biological molecules, making peroxynitrite a selective oxidant. Peroxynitrite modifies tyrosine in proteins to create nitrotyrosines, leaving a footprint detectable *in vivo.* Antibodies to nitrotyrosine have revealed nitration in several diseases, including human atherosclerosis and myocardial ischemia (Beckman and Koppenol, 1996). Peroxynitrite can be protonated to form peroxynitrous acid which in turn can yield the hydroxyl radical ($OH\cdot$). These reactive species can oxidize lipids, damage cell membranes, and oxidize thiol groups. NO given locally, exerts potent antiatherosclerotic effects such as inhibition of platelet aggregation, inhibition of adhesion of leukocytes, and the expression of leukocyte adhesion molecules. However, *in vivo* treatment with NO (via organic nitrates) increases rather than decreases oxidant load within endothelial cells. It remains therefore questionable whether systemic treatment with NO may have antiatherosclerotic properties or whether NO may initiate or even accelerate the atherosclerotic process (Münzel *et al.,* 1997; Bult, 1996; Leeuwenburgh *et al.,* 1997).

Manipulation of the L–arginine–NO–cGMP pathway may provide novel therapeutic approaches for limiting atherogenesis and neointimal proliferation in the future. For example, estrogens have atheroprotective properties, the mechanisms of which are not completely understood. Some of the benefits of estrogens can be ascribed to their ability to favorably alter the lipoprotein profile, that is, increase in high density lipoprotein and decrease in low density lipoprotein. In addition, estrogens have been reported to augment eNOS activity without altering gene expression, which was accompanied by a rapid increase in basal NO release (Caulin-Glaser *et al.,* 1997) (see also later). This effect may be, at least in part, mediated by a novel action of the estrogen receptor α (Chen *et al.,* 1999). Ethinylestradiol also prevents the degradation of NO by decreasing superoxide anion production (Arnal *et al.,* 1996). Increased NO bioavailability would promote vasodilation. Indeed, physiological concentrations of 17β-estradiol selectively enhance endothelium-dependent coronary vasodilation in postmenopausal women, an effect that was mediated by enhanced bioavailability of NO (Guetta *et al.,* 1997). In addition, increased NO bioavailability can inhibit proliferation of vascular smooth muscle cells, reduce platelet aggregation, and inhibit monocyte

adhesion to the endothelium and the inflammatory reaction induced by cytokines, all key contributors in the development of atherosclerosis. However, it has also been shown that prevention of fatty streak formation by 17β-estradiol is not mediated by the production of NO in apolipoprotein E-deficient mice (Elhage *et al.,* 1997).

Endothelial Dysfunction in Hypertension

Impaired release of NO occurs in most animal and human models of hypertension, contributing to the increased peripheral resistance and most likely to the development of cardiovascular complications. Many *in vitro* studies have reported endothelial dysfunction in thoracic aortae of spontaneously hypertensive rats (SHR) (Konishi and Su, 1983; Lüscher and Vanhoutte, 1986; Carvalho *et al.,* 1987; Shirasaki *et al.,* 1988), in the New Zealand hypertensive rat (Winquist *et al.,* 1984), in the deoxycorticosterone–salt model, the one-kidney, one-clip model (Van De Voorde and Leusen, 1986), and in aortic coarctation (Lockette *et al.,* 1986). Also, in patients with essential hypertension endothelium-dependent relaxation is diminished (Linder *et al.,* 1990; Panza *et al.,* 1990).

Endothelial dysfunction is largely due to reduced bioactivity of NO (Panza *et al.,* 1993a) and is not related to decreased availability of L-arginine (Panza *et al.,* 1993b). Moreover, it is not isolated to a specific defect of one endothelial cell surface receptor (Panza *et al.,* 1994). In patients with essential hypertension, endothelium-dependent vasodilation to ACh and bradykinin is impaired. These findings indicate that the endothelial dysfunction in this condition is not related to a specific defect of a single intracellular signal-transduction pathway and suggest a more generalized abnormality of endothelial vasodilator function (Panza *et al.,* 1995). Thus, these results contrast with those obtained in hypercholesterolemic patients in whom the response to ACh is impaired but the response to bradykinin is preserved (see earlier). In conjunction, these observations suggest that an NO defect of the hypertensive vasculature may lie distal to the G protein step in endothelial cell signal transduction. A reduced soluble guanylyl cyclase expression appears to contribute to vasodilator dysfunction in SHR (Bauersachs *et al.,* 1998). Moreover, in the endothelial dysfunction observed in hypertension, angiotensin II-stimulated NADH/NADPH oxidase activity and subsequent superoxide anion production plays a role as well. Indeed, in rats made hypertensive by infusions of angiotensin II, endothelium-dependent vascular relaxations are impaired. The vessels show an increased NADH/NADPH oxidase activity and produce increased amounts of superoxide (Rajagopalan *et al.,* 1996). Furthermore, in essential hypertensive patients, impaired endothelial vasodilation can be improved by the antioxidant vitamin C, as shown by forearm blood flow measurements. This effect can be reversed by the NO synthase inhibitor N^G–monomethyl-L-arginine (Taddei *et al.,* 1998). Therefore, NO inactivation by oxygen free radicals seems to contribute to endothelial dysfunction in essential hypertension. In contrast to hypercholesterolemic patients, the xanthine oxidase system does not appear to play a significant role in the impaired endothelial vasodilator function in essential hypertension (Cardillo *et al.,* 1997).

In contrast to essential hypertension, preeclampsia, which is a hypertensive disorder unique to human pregnancy, selectively impairs endothelium-dependent relaxation: responses to bradykinin are similar in vessels from normal pregnant and preeclamptic women whereas those to ACh are absent in vessels from women with preeclampsia (Pascoal *et al.,* 1998).

Another mechanism to explain reduced agonist-induced endothelium-dependent relaxations in essential hypertension is a concomitant release of vasoconstrictor prostanoids (endoperoxides and thromboxane A2), which is associated with aging. These prostanoids may be produced in the vascular smooth muscle rather than in the endothelium (Shimokawa, 1998). Enhanced production of an endothelial contractile factor is apparently not involved in the depressed endothelium-dependent responses of aortae from renal hypertensive rats (Van De Voorde and Leusen, 1986). In these vessels endothelium-dependent hyperpolarization is largely depressed; this might contribute to the impaired endothelium-dependent relaxation in hypertension (Van De Voorde *et al.,* 1992).

Endothelial dysfunction appears to occur in large but not small arteries in human and animal hypertension. Indeed, in contrast with the findings in isolated conduit arteries, endothelium-dependent relaxation is unimpaired in mesenteries from transgenic hypertensive rats. However, there are differences in the relative contributions of NO and EDHF, such that inhibition of either NO or EDHF alone in mesenteries from transgenic hypertensive rats has less impact as compared to mesenteries from normotensive controls. The relationship between NO and EDHF has more recently been shown to be dynamic, with EDHF activity becoming upregulated on loss of basal or coreleased NO due to a cGMP-mediated modulation at the smooth muscle cells (McCulloch *et al.,* 1997). Thus, this reciprocal relationship between NO and EDHF may be upregulated in the mesenteries from transgenic hypertensive rats. EDHF may be more effective at compensating for the loss of NO in the transgenic hypertensive rats (Randall and March, 1998).

ACE inhibitors prevent the impairment of NO-mediated vasodilation in experimental hypertension in animals. Because this endothelial protective effect can be obtained with different ACE inhibitors, it is likely to be a drug class effect. Moreover, in SHR endothelial dysfunction and subendothelial monocyte macrophage infiltration are associated (Clozel *et al.,* 1991). There is an association between improvement of endothelial function and decrease of monocyte macrophage infiltration shortly after short-term treatment with the ACE inhibitor cilazapril (Clozel *et al.,* 1991). The improvement in endothelial function by cilazapril might be responsible for the decrease in monocyte macrophage infiltration because quiescent endothelial cells inhibit leukocyte adherence and chemotactic activity (Zimmermann *et al.,* 1985). In

contrast to animal models, the ACE inhibitors captopril and enalapril were unable to improve or even normalize impaired endothelium-dependent vasodilation in hypertensive patients (Creager and Roddy, 1994). This lack of effect may be related to (1) the duration of therapy, (2) the start of the treatment in relation the to stage of hypertension (in animals models treatment is usually started before hypertension develops), (3) the lipophilicity and tissue selectivity of the ACE inhibitors used, and (4) the processing of angiotensin I to angiotensin II by other enzymes (Ruschitzka *et al.,* 1998). In NO-deficient hypertensive rats, long-term but not short-term therapy with the calcium antagonist verapamil improves impaired endothelium-dependent relaxations (Takase *et al.,* 1996). In SHR, chronic therapy with the calcium channel blocker nifedipine or isradipine ameliorates impaired endothelium-dependent relaxations to ACh (Tschudi *et al.,* 1994).

In hypertensive patients dilation of resistance arteries in response to infusion of NO donors is impaired, and the degree of this impairment depends critically on the severity of arterial hypertension. The reduced effectiveness of NO appears to be independent of the class of NO donor and thus of the mode of intravascular NO generation (Preik *et al.,* 1996b).

Endothelial Dysfunction in Diabetes

Observations in Animals and Humans

Diabetes mellitus is associated with early development of cardiovascular complications. Evidence for endothelial dysfunction in diabetes comes from studies that measured endothelial substances that mediate fibrinolysis and coagulation. In both type I and type II diabetes, fibrinolytic activity is decreased and plasminogen activator inhibitor activity increased (Auwerx *et al.,* 1988). Moreover, von Willebrand factor, which is synthesized within endothelial cells and may be a marker of endothelial damage, circulates in increased concentrations in diabetics (Bensoussan *et al.,* 1975). There is also substantial evidence that endothelium-dependent vasodilation is abnormal in both conduit arteries and resistance vessels of diabetic animals (Meraji *et al.,* 1987; Durante *et al.,* 1988; Mayhan, 1989; Tesfamariam *et al.,* 1989; Abiru *et al.,* 1990; Lash and Bohlen, 1991). However, not all studies have confirmed abnormal endothelium-dependent vasodilation in diabetic animals (Fortes *et al.,* 1983; Harris and MacLeod, 1988). The reasons for the discrepancies may be related to the duration of diabetes, species, gender, the inductor of diabetes, or the choice of the artery (Johnstone *et al.,* 1993). Also endothelium-dependent hyperpolarization is reduced by diabetes, and this would, in part, account for the impaired endothelium-dependent relaxations in mesenteric arteries from diabetic rats (Fukao *et al.,* 1997).

In patients with insulin-dependent diabetes mellitus (IDDM) endothelium-dependent vasodilation is abnormal in forearm resistance vessels (Johnstone *et al.,* 1993). Calver *et al.* (1992) reported impaired basal release of NO in these vessels. In patients with noninsulin-dependent diabetes mellitus (NIDDM) the dilator responses to both ACh and NTG are reduced (McVeigh *et al.,* 1992), but endothelium-derived basal NO formation is not impaired (Catalano *et al.,* 1997). However, in these individuals the ability of the vascular smooth muscle cells to respond to vasodilator stimuli may be disturbed, because these patients are generally older than insulin-dependent diabetics and are often affected by hypertension and hyperlipidemia (Johnstone *et al.,* 1993).

Decreased NO activity in diabetes can be caused by (1) deficiency of the NOS substrate L-arginine, (2) abnormalities in signal transduction due to decreased expression of inhibitory G proteins, reduced phosphoinositol metabolism, and increased activation of protein kinase C (Gawler *et al.,* 1987; Greene *et al.,* 1987; Lee *et al.,* 1989), (3) a decreased availability of one or more cofactors essential for optimal functioning of NOS, (4) generation and release of vasoconstrictor prostanoids that counteract the effect of NO (Mayhan, 1989; Tesfamariam *et al.,* 1989), and (5) accelerated inactivation of NO due to high levels of oxygen-derived free radicals, advanced glycosylation end products (AGEs, see later), and transport barriers such as thickened basement membranes (Pieper and Gross, 1988; Tesfamariam, 1994; Bucala *et al.,* 1991; Siperstein *et al.,* 1968). We will discuss some of these possibilities in more detail.

Mechanisms of NO-Related Vascular Endothelial Dysfunction

Bioavailability of L-Arginine

In diabetic rats the concentration of L-arginine in plasma and aortic tissue are both decreased. In aortic rings from diabetic rats acute L-arginine supplementation *in vitro* restores endothelium-dependent relaxation by augmenting cGMP production (Pieper and Dondlinger, 1997; Pieper *et al.,* 1997a). Similarly, acute dietary supplementation with L-arginine *in vivo* in diabetic rats reverses the defective endothelium-dependent relaxation in diabetic blood vessels assessed *ex vivo* (Pieper *et al.,* 1996a). In contrast, as already discussed above, flow-mediated dilation in humans is impaired in hypercholesterolemic subjects, smokers, and diabetic subjects. These three risk factor groups responded differently to L-arginine. Flow-mediated dilation is improved in hypercholesterolemic subjects and smokers but unchanged in diabetic subjects (Thorne *et al.,* 1998). This discrepancy may be related to the stage of diabetes. Indeed, in experimental diabetes a defect in substrate/supply for NO synthesis is acutely reversed by L-arginine supplementation at early but not at later stages of diabetes (Pieper *et al.,* 1996b).

Signal Transduction

Impaired NO-mediated vascular relaxation in diabetes may also arise from abnormalities in signal transduction due to decreased expression of inhibitory G proteins, reduced phosphoinositol metabolism, and increased activation of

protein kinase C (Gawler *et al.,* 1987; Greene *et al.,* 1987; Lee *et al.,* 1989). Increased activation of protein kinase C may occur by increased synthesis of diacylglycerol under hyperglycemic conditions (Lee *et al.,* 1989). The abnormal NO-mediated relaxation obtained in this way may in part be due to an increased production of vasoconstrictor prostanoids (see later) (Tesfamariam *et al.,* 1991).

Tetrahydrobiopterin Availability

As discussed above, tetrahydrobiopterin is a cofactor for NO synthase. In low concentrations of this cofactor, NO synthase is known to produce less NO and, correspondingly, enhanced quantities of the oxidant species hydrogen peroxide (H_2O_2). An exogenous tetrahydrobiopterin derivative (6-methyl-5,6,7,8-tetrahydropterin) improves eNOS activity in diabetic endothelium. Thus, also tetrahydrobiopterin availability can play a role in the regulation of NO production by diabetic endothelium (Pieper, 1997).

Generation and Release of Vasoconstrictor Prostanoids That Counteract the Effect of NO

In the aorta from diabetic rabbits impaired relaxations to ACh are restored by inhibitors of cyclooxygenase. Moreover, the release of thromboxane A_2 and prostaglandin E_2 is significantly increased in this model. Therefore, these contractile prostanoids could counteract NO-mediated relaxations (Mayhan, 1989; Tesfamariam *et al.,* 1989).

Oxidative Stress

As in atherosclerosis, reactive oxygen species are considered to play an important role in diabetes mellitus as well. In rats, diabetic endothelium produces increases in both superoxide anion and H_2O_2 leading to enhanced intracellular production of hydroxyl radicals (Pieper *et al.,* 1997b). It has been demonstrated that inactivation of endothelium-derived NO by oxygen-derived free radicals contributes to abnormal vascular reactivity in animal models of diabetes mellitus. However, at the onset of diabetes acute endothelium-mediated vasodilation is not impaired (Brands and Fitzgerald, 1998). Vasoactivity during acute hyperglycemia seems to depend on the superoxide anion scavenging properties of the vascular wall. In acute hyperglycemia and early stages of diabetes, radical scavenging capacity may be suitable to protect NO degradation, resulting in an enhanced vasodilation. In contrast, decreased free radical scavenging properties of the vasculature in prolonged hyperglycemia and in later stages of diabetes might promote NO degradation by an overshoot of superoxide anions, resulting in an attenuation of endothelium-dependent vasodilation (Graier *et al.,* 1997).

In diabetic patients, responses of angiographically normal coronary arteries to cold pressor test and to flow increase are impaired. Abnormal responses are not improved by L-arginine, suggesting that a deficit in substrate for NO synthesis is not involved. Deferoxamine restores a vasodilator response to the two tests, suggesting that inactivation of NO by oxygen species might be partly responsible for the impairment of coronary artery dilation in diabetic patients. (Nitenberg *et al.,* 1998). Moreover, in patients with insulin-dependent diabetes mellitus, the antioxidant vitamin C selectively restores the impaired endothelium-dependent vasodilation in the forearm resistance vessels. Thus, NO degradation by oxygen-derived free radicals contributes to abnormal vascular reactivity in humans with insulin-dependent diabetes mellitus (Timimi *et al.,* 1998; Ting *et al.,* 1996).

Another way in which oxidative stress can be enhanced in diabetes involves the polyol pathway. Under hyperglycemic conditions, one-third of the metabolism of glucose is shunted through this pathway, in which glucose is metabolized first to sorbitol by aldose reductase and subsequently to fructose by sorbitol dehydrogenase. This polyol pathway may increase oxidative stress since aldose reductase activity may deplete NADPH, which is required for the antioxidant activity of gluthation reductase (Gonzalez *et al.,* 1986).

In diabetic patients impairment of endothelial function occurs even under normocholesterolemic conditions with normal LDL levels (Cohen, 1993). An important metabolic effect of diabetes mellitus is glycation of lipoproteins: LDL, like other proteins, are nonenzymatically glycated. Glycation of LDL occurs under hyperglycemic conditions in a time and glucose concentration-dependent way and favors the oxidation of LDL (Kawamura *et al.,* 1994). Glycated and oxLDL impairs endothelium-dependent vascular relaxations more potently than oxLDL, most likely due to increased formation of superoxide anions, resulting in inactivation of NO (Galle *et al.,* 1998). This mechanism may be relevant to the high prevalence of vascular disease, including atherosclerosis and microangiopathy, that occurs in diabetic patients. NO may indeed inhibit platelet aggregation, monocyte adhesion to endothelial cells, and vascular smooth muscle cell proliferation. Nonenzymatic glycation of LDL is not identical with formation of advanced glycation end products (AGEs). AGEs arise from glucose-derived Amadori products at a later state during the glycosylation process (Bucala and Vlassara, 1995). These products have been shown to inactivate NO *in vitro* (Bucala *et al.,* 1991). Furthermore, high glucose and AGEs suppress constitutive NO synthase expression in retinal vascular endothelial cells (Chakravarthy *et al.,* 1998).

Therapy

Exercise training, but not food restriction, prevents endothelial dysfunction in NIDDM rats, presumably due to the exercise-induced increase in the production of NO (Sakamoto *et al.,* 1998).

In IDDM patients acute administration of the ACE inhibitor, enalaprilat, enhances NO-mediated endothelial function, with further improvement evident after 4 weeks of enalapril therapy (O'Driscoll *et al.,* 1997).

Gliclazide and glibenclamide, two therapeutic agents belonging to the group of sulfonylureas, are commonly used in the treatment of diabetes. They effectively reduce blood sugar in NIDDM by augmenting insulin release. In addition,

gliclazide scavenges superoxide anions, hydroxyl radicals, and NO in a dose-dependent manner, whereas glibenclamide is without effect. Thus, gliclazide not only is effective in reducing blood sugar, but also may be beneficial by inhibition of lipid and protein denaturation, which leads to the development of diabetic complications (Noda *et al.,* 1997).

Implications of Endothelial Dysfunction in Diabetes

The fact that diabetes mellitus is associated with early development of cardiovascular complications may be related to impaired NO-mediated vascular endothelial dysfunction. Furthermore, in women with gestational diabetes, maternal vascular endothelial dysfunction may contribute to the increased incidence of cardiovascular disorders (Knock *et al.,* 1997). The reduced blood flow and increased vascular resistance observed in diabetic pregnancies may be due to decreased NO synthesis and activity in the stem villous vessels which are the major determinants of resistance in the fetal–placental vasculature (Dollberg *et al.,* 1997).

Endothelial dysfunction in diabetes may also lead to sexual impotence. Penile erection is a neurovascular event, requiring relaxation of the trabecular muscle of the corpora cavernosa, which has been shown to be mediated by NO (Holmqvist *et al.,* 1991). The formation of NO and prostacyclin (PGI_2), and adenylyl cyclase activity but not guanylyl cyclase, are impaired in the corpus cavernosum of diabetic rabbits. Because NO and PGI_2 are produced by the endothelium, these studies consolidate the view that endothelial dysfunction is a major contributor to erectile dysfunction in diabetes mellitus (Sullivan *et al.,* 1998). These findings may provide a rationale for new types of treatment of diabetic men with impotence (Wennmalm, 1994).

Endothelial Dysfunction in Heart Failure

Another disease that is associated with endothelial dysfunction, including impaired flow-dependent endothelium-mediated dilation, is chronic heart failure (Drexler, 1998). In a model of heart failure in dogs expression of eNOS is reduced (Smith *et al.,* 1996). There is also evidence that chronic heart failure is associated with increased oxidative stress, which is apparently due to increased superoxide production by mitochondria and NADH oxidoreductases (Belch *et al.,* 1991). The antioxidant vitamin C improves endothelial dysfunction in patients with chronic heart failure (Hornig *et al.,* 1998a). Furthermore, in patients with chronic heart failure the ACE inhibitor quinapril, which has a high affinity for tissue ACE, improves flow-dependent endothelium-mediated dilation as the result of increased availability of NO. The ACE-inhibitor enalapril, which has a low affinity to tissue ACE, does not show this effect (Hornig *et al.,* 1998b). Also physical training improves the impaired endothelium-dependent relaxations in patients with chronic heart failure (Hornig *et al.,* 1996). This seems to be related to repetitive increases in blood flow and consequently intermittent enhanced shear stress leading to an increased expression and activity of eNOS (Nishida *et al.,* 1992; Sessa *et al.,* 1994), upregulation of the radical scavenger enzyme Cu/Zn superoxide dismutase (Inoue *et al.,* 1996), and a decreased expression of ACE (Rieder *et al.,* 1997).

Age and Gender

Also age and gender play a role in vascular endothelial dysfunction. Endothelium-dependent vasodilation in forearm resistance vessels declines steadily with increasing age in healthy human subjects. Age is a strong univariate and multivariate predictor of endothelium-dependent vasodilation. This finding may be a marker for more widespread endothelial dysfunction (Gerhard *et al.,* 1996). Using forearm blood flow measurements it was shown that in normotensive and hypertensive humans, similar mechanisms, including dysfunction of the NO pathway and production of cyclooxygenase-dependent vasoconstrictors, cause age-related impairment of endothelium-dependent vasodilation. Only their earlier appearance characterizes hypertensive disease. Therefore, the endothelial dysfunction that occurs in hypertension seems to represent an accelerated form of dysfunction that occurs in aging (Taddei *et al.,* 1997).

Basal release of NO is larger in female animals than in males (Hayashi *et al.,* 1992). In cholesterol-fed rabbits the impairment of endothelium-dependent vasorelaxation is dependent on gender. A cholesterol-rich diet induces a more severe impairment of maximal relaxation to ACh in males compared to females, which may be due to a greater degradation of extracellular NO in the vessels wall of males (Kojda *et al.,* 1998). Moreover, intimal thickening following balloon denudation (White *et al.,* 1997), cuff placement (Akishita *et al.,* 1997; Moroi *et al.,* 1998), or feeding a cholesterol-rich diet (Hayashi *et al.,* 1995) are always smaller in female animals as compared to males. Although the precise mechanism for the slower rate of development of atherosclerosis in female rabbits is not completely clear, the greater basal release of NO in females before they were fed a hyperlipidemic diet, besides other factors, may be involved.

Endothelial dysfunction has also been found to be less severe in female than in male SHR, which could contribute to the gender differences observed in the extent and rate of progression of hypertension in SHR. Release of cyclooxygenase-derived constricting factors appeared to be more pronounced in male than in female SHR. In addition, the relative role of NO in endothelium-dependent arterial relaxation seemed to be higher in female than in male SHR, and relaxation induced by an NO donor also was more pronounced in female than in male SHR (Kahonen *et al.,* 1998).

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References

Abiru, T., Watanabe, Y., Kamata, K., Miyata, N., and Kasuya, Y. (1990). Decrease in endothelium-dependent relaxation and levels of cyclic nucleotides in aorta from rabbits with alloxan-induced diabetes. *Res. Commun. Chem. Pathol. Pharmacol.* **68,** 13–25.

Adams, M. R., McCredie, R., Jessup, W., Robinson, J., Sullivan, D., and Celermajer, D. S. (1997). Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. *Atherosclerosis* **129,** 261–269.

Adams, M. R., Robinson, J., McCredie, R., Seale, J. P., Sorensen, K. E., Deanfield, J. E., and Celermajer, D. S. (1998). Smooth muscle dysfunction occurs independently of impaired endothelium-dependent dilation in adults at risk of atherosclerosis. *J. Am. Coll. Cardiol.* **32,** 123–127.

Akishita, M., Ouchi, Y., Miyoshi, H., Kozaki, K., Inoue, S., Ishikawa, M., Eto, M., Toba, K., and Orimo, H. (1997). Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: Effects on migration and proliferation of vascular smooth muscle cells. *Atherosclerosis* **130,** 1–10.

Arnal, J. F., Münzel, T., Venema, R. C., James, N. L., Bai, C. L., Mitch, W. E., and Harrison, D. G. (1995). Interactions between L-arginine and L-glutamine change endothelial NO production. An effect independent of NO synthase substrate availability. *J. Clin. Invest.* **95,** 2565–2572.

Arnal, J. F., Clamens, S., Pechet, C., Negre-Salvayre, A., Allera, C., Girolami, J. P., Salvayre, R., and Bayard, F. (1996). Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4108–4113.

Auwerx, J., Bouillon, R., Collen, D., and Geboers, J. (1988). Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus. *Arteriosclerosis* **8,** 68–72.

Azuma, H., Sato, J., Hamasaki, H., Sugimoto, A., Isotani, E., and Obayashi, S. (1995). Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *Br. J. Pharmacol.* **115,** 1001–1004.

Barbul, A. (1990). Physiology and pharmacology of arginine. *In* "Nitric Oxide from L-Arginine, a Bioregulatory System" (S. Moncada and E. A. Higgs, eds.), pp. 317–329. Elsevier, Amsterdam.

Bauersachs, J., Bouloumie, A., Mulsch, A., Wiemer, G., Fleming, I., and Busse, R. (1998). Vasodilator dysfunction in aged spontaneously hypertensive rats, changes in NO synthase III and soluble guanylyl cyclase expression, and in superoxide anion production. *Cardiovasc. Res.* **37,** 772–779.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Belch, J. J., Bridges, A. B., Scott, N., and Chopra, M. (1991). Oxygen free radicals and congestive heart failure. *Br. Heart J.* **65,** 245–248.

Bensoussan, D., Levy-Toledano, S., Passa, P., Caen, J., and Canivet, J. (1975). Platelet hyperaggregation and increased plasma level of von Willebrand factor in diabetic patients with retinopathy. *Diabetologia* **11,** 307–312.

Bode-Böger, S. M., Böger, R. H., Kienke, S., Junker, W., and Frolich, J. C. (1996). Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. *Biochem. Biophys. Res. Commun.* **219,** 598–603.

Böger, R. H., Bode-Böger, S. M., Thiele, W., Junker, W., Alexander, K., and Frolich, J. C. (1997a). Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* **95,** 2068–2074.

Böger, R. H., Bode-Böger, S. M., Brandes, R. P., Phivthong-ngam, L., Bohme, M., Nafe, R., Mugge, A., and Frolich, J. C. (1997b). Dietary L-arginine reduces the progression of atherosclerosis in cholesterol-fed rabbits, comparison with lovastatin. *Circulation* **96,** 1282–1290.

Böger, R. H., Bode-Böger, S. M., Kienke, S., Stan, A. C., Nafe, R., and Frolich, J. C. (1998). Dietary L-arginine decreases myointimal cell proliferation and vascular monocyte accumulation in cholesterol-fed rabbits. *Atherosclerosis* **136,** 67–77.

Bosmans, J. M., Bult, H., Vrints, C. J., Kockx, M. M., and Herman, A. G. (1996). Balloon angioplasty and induction of non-endothelial nitric oxide synthase in rabbit carotid arteries. *Eur. J. Pharmacol.* **310,** 163–174.

Bossaller, C., Habib, G. B., Yamamoto, H., Williams, C., Wells, S., and Henry, P. D. (1987). Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. *J. Clin. Invest.* **79,** 170–174.

Bossaller, C., Hehlert-Friedrich, C., Jost, S., Rafflenbeul, W., and Lichtlen, P. (1989). Angiographic assessment of human coronary artery endothelial function by measurement of endothelium-dependent vasodilation. *Eur. Heart J.* **10**(Suppl. F), 44–48.

Brandes, R. P., Behra, A., Lebherz, C., Böger, R. H., Bode-Böger, S. M., Phivthong-Ngam, L., and Mugge, A. (1997). *N*(G)-nitro-L-arginine- and indomethacin-resistant endothelium-dependent relaxation in the rabbit renal artery, effect of hypercholesterolemia. *Atherosclerosis* **135,** 49–55.

Brands, M. W., and Fitzgerald, S. M. (1998). Acute endothelium-mediated vasodilation is not impaired at the onset of diabetes. *Hypertension* **32,** 541–547.

Briggs, M. M., and Lefkowitz, R. J. (1980). Parallel modulation of catecholamine activation of adenylate cyclase and formation of the high-affinity agonist–receptor complex in turkey erythrocyte membranes by temperature and *cis*-vaccenic acid. *Biochemistry* **19,** 4461–4466.

Bucala, R., and Vlassara, H. (1995). Advanced glycosylation end products in diabetic renal and vascular disease. *Am. J. Kidney Dis.* **26,** 875–888.

Bucala, R., Tracey, K. J., and Cerami, A. (1991). Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J. Clin. Invest.* **87,** 432–438.

Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkinson, C. P., Cederbaum, S. D., and Ignarro, L. J. (1996). Arginase activity in endothelial cells, inhibition by N^G-hydroxy-L-arginine during high-output NO production. *Am. J. Physiol.* **271,** H1988–H1998.

Buja, L. M., Kita, T., Goldstein, J. L., Watanabe, Y., and Brown, M. S. (1983). Cellular pathology of progressive atherosclerosis in the WHHL rabbit, an animal model of familial hypercholesterolaemia. *Arteriosclerosis* **3,** 87–101.

Bult, H. (1996). Nitric oxide and atherosclerosis, possible implications for therapy. *Mol. Med. Today* **2,** 510–518.

Bult, H., Buyssens, N., De Meyer, G. R. Y., Jordaens, F. H., and Herman, A. G. (1990). Effects of chronic treatment with a source of exogenous nitric oxide on EDRF release by aortae from normal and hypercholesterolaemic rabbits. *In* "Nitric Oxide from L-Arginine, a Bioregulatory System" S. Moncada and E. A. Higgs, eds.), pp. 101–106. Elsevier, Amsterdam.

Bult, H., De Meyer, G. R. Y., and Herman, A. G. (1995). Influence of chronic treatment with a nitric oxide donor on fatty streak development and reactivity of the rabbit aorta. *Br. J. Pharmacol.* **114,** 1371–1382.

Büsse, R., and Lamontagne, D. (1991). Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **344,** 126–129.

Buttery, L. D., Chester, A. H., Springall, D. R., Borland, J. A., Michel, T., Yacoub, M. H., and Polak, J. M. (1996). Explanted vein grafts with an intact endothelium demonstrate reduced focal expression of endothelial nitric oxide synthase specific to atherosclerotic sites. *J. Pathol.* **179,** 197–203.

Calver, A. C., Collier, J. G., and Vallance, P. J. T. (1992). Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J. Clin. Invest.* **90,** 2548–2554.

Campbell, W. B., and Harder, D. R. (1999). Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circ. Res.* **84,** 484–488.

Campisi, R., Czernin, J., Schoder, H., Sayre, J. W., and Schelbert, H. R. (1999). L-Arginine normalizes coronary vasomotion in long-term smokers. *Circulation* **99,** 491–497.

Caparrotta, L., Pandolfo, L., Chinellato, A., Ragazzi, E., Froldi, G., Aliev, G., and Fassina, G. (1993). L-Arginine does not improve endotheluim-dependent relaxation in in vitro Watanabe rabbit thoracic aorta. *Amino Acids* **5,** 403–411.

Cardillo, C., Kilcoyne, C. M., Cannon III, R. O., Quyyumi, A. A., and Panza, J. A. (1997). Xanthine oxidase inhibition with oxypurinol improves endothelial vasodilator function in hypercholesterolemic but not in hypertensive patients. *Hypertension* **30,** 57–63.

Carvalho, M. H. C., Scivoletto, R., Fortes, B., Nigro, D., and Cordellini, S. (1987). Reactivity of aorta and mesenteric microvessels to drugs in spontaneously hypertensive rats: Role of the endothelium. *J. Hypertens.* **5,** 377–382.

Casino, P. R., Kilcoyne, C. M., Quyyumi, A. A., Hoeg, J. M., and Panza, J. A. (1994). Investigation of decreased availability of nitric oxide precursor as the mechanism responsible for impaired endothelium-dependent vasodilation in hypercholesterolemic patients. *J. Am. Coll. Cardiol.* **23,** 844–850.

Catalano, M., Carzaniga, G., Perilli, E., Jun, T., Scandale, G., Andreoni, S., and Carotta, M. (1997). Basal nitric oxide production is not reduced in patients with noninsulin-dependent diabetes mellitus. *Vasc. Med.* **2,** 302–305.

Caulin-Glaser, T., Garcia-Cardena, G., Sarrel, P., Sessa, W. C., and Bender, J. R. (1997). 17 Beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ. Res.* **81,** 885–892.

Cayatte, A. J., Palacino, J. J., Horten, K., and Cohen, R. A. (1994). Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler. Throm.* **14,** 753–759.

Celermajer, D. S., Sorensen, K. E., Gooch, V. M., Spiegelhalter, D. J., Miller, O. I., Sullivan, I. D., Lloyd, J. K., and Deanfield, J. E. (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* **340,** 1111–1115.

Celermajer, D. S., Sorensen, K. E., Bull, C., Robinson, J., and Deanfield, J. E. (1994). Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J. Am. Coll. Cardiol.* **24,** 1468–1474.

Chakravarthy, U., Hayes, R. G., Stitt, A. W., McAuley, E., and Archer, D. B. (1998). Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* **47,** 945–952.

Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M. E., and Shaul, P. W. (1999). Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J. Clin. Invest.* **103,** 401–406.

Chester, A. H., O'Neil, G., Moncada, S., Tadjkarimi, S., and Yacoub, M. H. (1990). Low basal and stimulated release of nitric oxide in atherosclerotic epicardial coronary arteries. *Lancet* **336,** 897–900.

Clozel, M., Kuhn, H., Hefti, F., and Baumgartner, H. R. (1991). Endothelial dysfunction and subendothelial monocyte macrophages in hypertension. Effect of angiotensin converting enzyme inhibition. *Hypertension* **18,** 132–141.

Coene, M.-C., Herman, A. G., Jordaens, F., Van Hove, C., and Verbeuren, T. J. (1985). Endothelium-dependent relaxations in isolated arteries of control and hypercholesterolaemic rabbits. *Br. J. Pharmacol.* **85,** 276P.

Cohen, R. A. (1993). Dysfunction of vascular endothelium in diabetes mellitus. *Circulation* **87**(Suppl. V), V67–V76.

Cohen, R. A., Zitnay, K. M., Haudenschild, C. C., and Cunningham, L. D. (1988). Loss of selective endothelial cell vasoactive functions caused by hypercholesterolemia in pig coronary arteries. *Circ. Res.* **63,** 903–910.

Cooke, J. P., and Dzau, V. J. (1997). Derangements of the nitric oxide synthase pathway, L-arginine, and cardiovascular diseases. *Circulation* **96,** 379–382.

Cooke, J. P., Andon, N. A., Girerd, X. J., Hirsch, A. T., and Creager, M. A. (1991). Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta. *Circulation* **83,** 1057–1062.

Cooke, J. P., Singer, A. H., Tsao, P., Zera, P., Rowan, R. A., and Billingham, M. E. (1992). Anti-atherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J. Clin. Invest.* **90,** 1168–1172.

Cooper, A., and Heagerty, A. M. (1998). Endothelial dysfunction in human intramyocardial small arteries in atherosclerosis and hypercholesterolemia. *Am. J. Physiol.* **275,** H1482–H1488.

Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C., and Harrison, D. G. (1996). Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ. Res.* **79,** 984–991.

Cosentino, F., and Lüscher, T. F. (1998). Tetrahydrobiopterin and endothelial function. *Eur. Heart J.* **19** (Suppl. G), G3–G8.

Creager, M. A., and Roddy, M. A. (1994). Effect of captopril and enalapril on endothelial function in hypertensive patients. *Hypertension* **24,** 499–505.

Creager, M. A., and Selwyn, A. (1997). When 'normal' cholesterol levels injure the endothelium. *Circulation* **96,** 3255–3257.

Creager, M. A., Gallagher, S. J., Girerd, X. J., Coleman, S. M., Dzau, V. J., and Cooke, J. P. (1992). L-Arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J. Clin. Invest.* **90,** 1248–1253.

Crossman, D. C., Larkin, S. W., Dashwood, N., Davies, G. J., Yacoub, M., and Maseri, A. (1989). Atherosclerotic human coronary vessels preserve a dilator response to substance P in vivo. *Br. Heart J.* **61,** 455–456.

Dakak, N., Husain, S., Mulcahy, D., Andrews, N. P., Panza, J. A., Waclawiw, M., Schenke, W., and Quyyumi, A. A. (1998). Contribution of nitric oxide to reactive hyperemia: Impact of endothelial dysfunction. *Hypertension* **32,** 9–15.

Diet, F., Pratt, R. E., Berry, G. J., Momose, N., Gibbons, G. H., and Dzau, V. J. (1996). Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation* **94,** 2756–2767.

Dollberg, S., Brockman, D. E., and Myatt, L. (1997). Nitric oxide synthase activity in umbilical and placental vascular tissue of gestational diabetic pregnancies. *Gynecol. Obstet. Invest.* **44,** 177–181.

Drexler, H. (1998). Endothelium as a therapeutic target in heart failure. *Circulation* **98,** 2652–2655.

Drexler, H., Zeiher, A. M., Meinzer, K., and Just, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* **338,** 1546–1550.

Durante, W., Sen, A. K., and Sunahara, F. A. (1988). Impairment of endothelium dependent relaxation in aortae from spontaneously diabetic rats. *Br. J. Pharmacol.* **94,** 463–468.

Elhage, R., Bayard, F., Richard, V., Holvoet, P., Duverger, N., Fievet, C., and Arnal, J. F. (1997). Prevention of fatty streak formation of 17beta-estradiol is not mediated by the production of nitric oxide in apolipoprotein E-deficient mice. *Circulation* **96,** 3048–3052.

Feron, O., Dessy, C., Moniotte, S., Desager, J. P., and Balligand, J. L. (1999). Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J. Clin. Invest.* **103,** 897–905.

Fickling, S. A., Williams, D., Vallance, P., Nussey, S. S., and Whitley, G. S. (1993). Plasma concentrations of endogenous inhibitor of nitric oxide synthesis in normal pregnancy and pre-eclampsia. *Lancet* **342,** 242–243.

Fish, R. D., Nabel, E. G., Selwyn, A. P., Ludmer, P. L., Mudge, G. H., Kirshenbaum, J. M., Schoen, F. J., Alexander, R. W., and Ganz, P. (1987). Responsiveness of coronary arteries of cardiac transplant patients to acetylcholine. *J. Clin. Invest.* **81,** 21–31.

Flavahan, N. A. (1992). Atherosclerosis or lipoprotein-induced endothelial dysfunction. Potential mechanisms underlying reduction in EDRF/nitric oxide activity. *Circulation* **85,** 1927–1938.

Förstermann, U., Mügge, A., Alheid, U., Haverich, A., and Frölich, J. C. (1988). Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. *Circ. Res.* **62,** 185–190.

Fortes, Z. B., Leme, J. Q., and Scivoletto, R. (1983). Vascular reactivity in diabetes mellitus, role of the endothelial cell. *Br. J. Pharmacol.* **79,** 771–781.

Freeman, J. E., Kuo, W. Y., Drenger, B., Barnett, T. N., Levine, M. A., and Flavahan, N. A. (1996). Analysis of lysophophatidylcholine-induced endothelial dysfunction. *J. Cardiovasc. Pharmacol.* **28,** 345–352.

Freiman, P. C., Mitchell, G. G., Heistad, D. D., Armstrong, M. L., and Harrison, D. G. (1986). Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ. Res.* **58,** 783–789.

Fukao, M., Hattori, Y., Kanno, M., Sakuma, I., and Kitabatake, A. (1997). Alterations in endothelium-dependent hyperpolarization and relaxation in mesenteric arteries from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.* **121,** 1383–1391.

Galle, J., Busse, R., and Bassenge, E. (1991). Hypercholesterolemia and atherosclerosis change vascular reactivity in rabbits by different mechanisms. *Arterioscler. Thromb.* **11,** 1712–1718.

Galle, J., Schneider, R., Winner, B., Lehmann-Bodem, C., Schinzel, R., Munch, G., Conzelmann, E., and Wanner, C. (1998). Glyc-oxidized LDL impair endothelial function more potently than oxidized LDL: Role of enhanced oxidative stress. *Atherosclerosis* **138,** 65–77.

Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G., and Houslay, M. D. (1987). Abolition of the expression of inhibitory guanine nucleotide regulatory protein G_i activity in diabetes. *Nature* **327,** 229–232.

Gerhard, M., Roddy, M. A., Creager, S. J., and Creager, M. A. (1996). Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. *Hypertension* **27,** 849–853.

Girerd, X. J., Hirsch, A. T., Cooke, J. P., Dzau, V. J., and Creager, M. A. (1990). L-Arginine augments endothelium-dependent vasodilation in cholesterol-fed rabbits. *Circ. Res.* **67,** 1301–1308.

Giugliano, D., Marfella, R., Verrazzo, G., Acampora, R., Coppola, L., Cozzolino, D., and D'Onofrio, F. (1997). The vascular effects of L-arginine in humans. The role of endogenous insulin. *J. Clin. Invest.* **99,** 433–438.

Gonzalez, A. M., Sochor, M., Hothersall, J. S., and McLean, P. (1986). Effect of aldose reductase inhibitor (sorbinil) on integration of polyol pathway, pentose phosphate pathway, and glycolytic route in diabetic rat lens. *Diabetes* **35,** 1200–1205.

Graier, W. F., Posch, K., Wascher, T. C., and Kostner, G. M. (1997). Role of superoxide anions in changes of endothelial vasoactive response during acute hyperglycemia. *Horm. Metab. Res.* **29,** 622–626.

Greene, D. A., Lattimer, S. A., and Sima, A. A. F. (1987). Sorbitol, phosphoinositides, and sodium-ATPase in the pathogenesis of diabetic complications. *N. Engl. J. Med.* **316,** 599–606.

Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D., and Alexander, R. W. (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ. Res.* **74,** 1141–1148.

Guerra, R., Brotherton, A. F. A., Goodwin, P. J., Clark, C. R., Armstrong, M. L., and Harrison, D. G. (1989). Mechanisms of abnormal endothelium-dependent vascular relaxation in atherosclerosis, implications for altered autocrine and paracrine functions of EDRF. *Blood Vessels* **26,** 300–314.

Guetta, V., Quyyumi, A. A., Prasad, A., Panza, J. A., Waclawiw, M., and Cannon III, R. O. (1997). The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation* **96,** 2795–2801.

Hare, J. M., Kim, B., Flavahan, N. A., Ricker, K. M., Peng, X., Colman, L., Weiss, R. G., and Kass, D. A. (1998). Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart. *J. Clin. Invest.* **101,** 1424–1431.

Harris, K. H., and MacLeod, K. M. (1988). Influence of the endothelium on contractile responses of arteries from diabetic rats. *Eur. J. Pharmacol.* **153,** 55–64.

Harrison, D. G. (1994). Endothelial dysfunction in atherosclerosis. *Basic Res. Cardiol.* **89**(Suppl. 1), 87–102.

Harrison, D. G. (1997). Cellular and molecular mechanisms of endothelial cell dysfunction. *J. Clin. Invest.* **100,** 2153–2157.

Harrison, D. G., Freiman, P. C., Armstrong, M. L., Marcus, M. L., and Heistad, D. D. (1987). Alterations of vascular reactivity in atherosclerosis. *Circ. Res.* **61**(Suppl. II), 74–80.

Hayashi, T., Fukuto, J. M., Ignarro, L. J., and Chaudhuri, G. (1995). Gender differences in atherosclerosis: Possible role of nitric oxide. *J. Cardiovasc. Pharmacol.* **26,** 792–802.

Hayashi, T., Fukuto, J. M., Ignarro, L. J., and Chaudhuri, G. (1992). Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: Implications for atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 11259–11263.

Hecker, M., Sessa, W. C., Harris, H. J., Ånggard, E. E., and Vane, J. R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: Cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 8612–8616.

Heitzer, T., Just, H., and Münzel, T. (1996). Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation* **94,** 6–9.

Hernandez-Perera, O., Perez-Sala, D., Navarro-Antolin, J., Sanchez-Pascuala, R., Hernandez, G., Diaz, C., and Lamas, S. (1998). Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J. Clin. Invest.* **101,** 2711–2719.

Hibi, K., Ishigami, T., Tamura, K., Mizushima, S., Nyui, N., Fujita, T., Ochiai, H., Kosuge, M., Watanabe, Y., Yoshii, Y., Kihara, M., Kimura, K., Ishii, M., and Umemura, S. (1998). Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. *Hypertension* **32,** 521–526.

Hirata, K., Miki, N., Kuroda, Y., Sakoda, T., Kawashima, S., and Yokoyama, M. (1995). Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine upregulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells. *Circ. Res.* **76,** 958–962.

Holmqvist, F., Hedlund, H., and Andersson, K. E. (1991). L-N^G-nitro arginine inhibits non-adrenergic, non-cholinergic relaxation of human isolated corpus cavernosum. *Acta Physiol. Scand.* **141,** 441–442.

Holzmann, S., Kukovetz, W. R., Windischhofer, W., Paschke, E., and Graier, W. F. (1994). Pharmacologic differentiation between endothelium-dependent relaxations sensitive and resistant to nitro-L-arginine in coronary arteries. *J. Cardiovasc. Pharmacol.* **23,** 747–756.

Hornig, B., Maier, V., and Drexler, H. (1996). Physical training improves endothelial function in patients with chronic heart failure. *Circulation* **93,** 210–214.

Hornig, B., Arakawa, N., Kohler, C., and Drexler, H. (1998a). Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. *Circulation* **97,** 363–368.

Hornig, B., Arakawa, N., Haussmann, D., and Drexler, H. (1998b). Differential effects of quinaprilat and enalaprilat on endothelial function of conduit arteries in patients with chronic heart failure. *Circulation* **98,** 2842–2848.

Inoue, N., Ramasamy, S., Fukai, T., Nerem, R. M., and Harrison, D. G. (1996). Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. *Circ. Res.* **79,** 32–37.

Jayakody, L., Senaratne, M., Thomson, A., and Kappagoda, T. (1987). Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. *Circ. Res.* **60,** 251–264.

Jessup, W. (1996). Oxidized lipoproteins and nitric oxide. *Curr. Opin. Lipidol.* **7,** 274–280.

Johnstone, M. T., Creager, S. J., Scales, K. M., Cusco, J. A., Lee, B. K., and Creager, M. A. (1993). Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation* **88,** 2510–2516.

Kahonen, M., Tolvanen, J. P., Sallinen, K., Wu, X., and Porsti, I. (1998). Influence of gender on control of arterial tone in experimental hypertension. *Am. J. Physiol.* **275,** H15–H22.

Kanazawa, K., Kawashima, S., Mikami, S., Miwa, Y., Hirata, K., Suematsu, M., Hayashi, Y., Itoh, H., and Yokoyama, M. (1996). Endothelial constitutive nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired endothelium-dependent vascular relaxation. *Am. J. Pathol.* **148,** 1949–1956.

Kawamura, M., Heinecke, J. W., and Chait, A. (1994). Pathophysiological concentrations of glucose promote oxidative modification of low density lipoprotein by a superoxide-dependent pathway. *J. Clin. Invest.* **94,** 771–778.

Kessler, P., Popp, R., Busse, R., and Schini-Kerth, V. B. (1999). Proinflammatory mediators chronically downregulate the formation of the endothelium-derived hyperpolarizing factor in arteries via a nitric oxide/cyclic GMP-dependent mechanism. *Circulation* **99,** 1878–1884.

Knock, G. A., McCarthy, A. L., Lowy, C., and Poston, L. (1997). Association of gestational diabetes with abnormal maternal vascular endothelial function. *Br. J. Obstet. Gynaecol.* **104,** 229–234.

Kojda, G., Husgen, B., Hacker, A., Perings, D., Schnaith, E. M., Kottenberg, E., and Noack, E. (1998). Impairment of endothelium-dependent vasorelaxation in experimental atherosclerosis is dependent on gender. *Cardiovasc. Res.* **37,** 738–747.

Kojima, S., Ona, S., Iizuka, I., Arai, T., Mori, H., and Kubota, K. (1995). Antioxidative activity of 5,6,7,8-tetrahydrobiopterin and its inhibitory effect on paraquat-induced cell toxicity in cultured rat hepatocytes. *Free Radical Res.* **23,** 419–430.

Kolodgie, F. D., Virmani, R., Rice, H. E., and Mergner, W. V. (1990). Vascular reactivity during the progression of atherosclerotic plaque. A study in Watanabe heritable hyperlipidemic rabbits. *Circ. Res.* **66,** 1112–1126.

Konishi, M., and Su, C. (1983). Role of endothelium in dilator responses of spontaneously hypertensive rat arteries. *Hypertension* **5,** 881–886.

Kuo, L., Davis, M. J., Cannon, M. S., and Chilian, W. M. (1992). Pathophysiological consequences of atherosclerosis extend into the coronary microcirculation. Restoration of endothelium-dependent responses by L-arginine. *Circ. Res.* **70,** 465–476.

Kurz, S., and Harrison, D. G. (1997). Insulin and the arginine paradox. *J. Clin. Invest.* **99,** 369–370.

Lang, D., Shakesby, A. C., Mosfer, S., and Lewis, M. J. (1997). Angiotensin II upregulates NADH/NADPH oxidase mediated superoxide anion production by guinea pig coronary microvascular endothelial cells. *Circulation* **96**(Suppl. I), I-44 (Abstract).

Lash, J. M., and Bohlen, H. G. (1991). Structural and functional origins of suppressed acetylcholine vasodilation in diabetic rat intestinal arterioles. *Circ. Res.* **69,** 1259–1268.

Laufs, U., La-Fata, V., Plutzky, J., and Liao, J. K. (1998). Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* **97,** 1129–1135.

Lee, T.-S., MacGregor, L. C., Fluharty, S. J., and King, G. L. (1989). Differential regulation of protein kinase C and (Na, K)-adenosine triphosphatase activities by elevated glucose levels of retinal capillary endothelial cells. *J. Clin. Invest.* **83,** 90–94.

Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P., and Heinecke, J. W. (1997). Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J. Biol. Chem.* **272,** 1433–1436.

Lewis, T. V., Dart, A. M., and Chin-Dusting, J. P. (1999). Endothelium-dependent relaxation by acetylcholine is impaired in hypertriglyceridemic humans with normal levels of plasma LDL cholesterol. *J. Am. Coll. Cardiol.* **33,** 805–812.

Liao, J. K., and Clark, S. L. (1995). Regulation of G-protein alpha i2 subunit expression by oxidized low-density lipoprotein. *J. Clin. Invest.* **95,** 1457–1463.

Liao, J. K., Shin, W. S., Lee, W. Y., and Clark, S. L. (1995). Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J. Biol. Chem.* **270,** 319–324.

Linder, L., Kiowski, W., Bühler, F. R., and Lüscher, T. F. (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response to essential hypertension. *Circulation* **81,** 1762–1767.

Lockette, W., Otsuka, Y., and Carretero, O. (1986). Loss of endothelium-dependent vascular relaxation in hypertension. *Hypertension* **8**(Suppl. II), II61–II66.

Ludmer, P. L., Selwyn, A. P., Shook, T. L., Wayne, R. R., Mudge, G. H., Alexander, R. W., and Ganz, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N. Engl. J. Med.* **315,** 1046–1050.

Lüscher, T. F., and Vanhoutte, P. M. (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension* **8,** 344–348.

MacAllister, R. J., Parry, H., Kimoto, M., Ogawa, T., Russell, R. J., Hodson, H., Whitley, G. S., and Vallance, P. (1996). Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. *Br. J. Pharmacol.* **119,** 1533–1540.

McCulloch, A. I., Bottrill, F. E., Randall, M. D., and Hiley, C. R. (1997). Characterization and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric arterial bed. *Br. J. Pharmacol.* **120,** 1431–1438.

McDermott, J. R. (1976). Studies on the catabolism of N^G-methylarginine and N^G, N^G-dimethylarginine in the rabbit. *Biochem. J.* **154,** 179–184.

McDonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric oxide synthase may explain the ''arginine paradox.'' *J. Biol. Chem.* **272,** 31213–31216.

McNamara, D. B., Bedi, B., Aurora, H., Tena, L., Ignarro, L. J., Kadowitz, P. J., and Akers, D. L. (1993). L-Arginine inhibits balloon cathether-induced intimal hyperplasia. *Biochem. Biophys. Res. Commun.* **193,** 291–296.

McVeigh, G. E., Brennan, G. M., Johnston, G. D., McDermott, B. J., McGrath, L. T., Henry, W. R., Andrews, J. W., and Hayes, J. R. (1992). Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* **35,** 771–776.

Mancini, G. B., Henry, G. C., Macaya, C., O'Neill, B. J., Pucillo, A. L., Carere, R. G., Wargovich, T. J., Mudra, H., Lüscher, T. F., Klibaner, M. I., Haber, H. E., Uprichard, A. C., Pepine, C. J., and Pitt, B. (1996). Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. The TREND (Trial on Reversing ENdothelial Dysfunction). Study. *Circulation* **94,** 258–265.

Markus, H. S., Ruigrok, Y., Ali, N., and Powell, J. F. (1998). Endothelial nitric oxide synthase exon 7 polymorphism, ischemic cerebrovascular disease, and carotid atheroma. *Stroke* **29,** 1908–1911.

Matsuoka, H., Itoh, S., Kimoto, M., Kohno, K., Tamai, O., Wada, Y., Yasukawa, H., Iwami, G., Okuda, S., and Imaizumi, T. (1997). Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* **29,** 242–247.

Mayhan, W. G. (1989). Impairment of endothelium-dependent dilatation of cerebral arterioles during diabetes mellitus. *Am. J. Physiol.* **256,** H621–H625.

Meraji, S., Jayakody, L., Senaratne, M. P. J., Thomson, A. B. R., and Kappagoda, T. (1987). Endothelium-dependent relaxation in aorta of BB rat. *Diabetes* **36,** 978–981.

Merkel, L. A., Rivera, L. M., Bilder, G. E., and Perrone, M. H. (1990). Differential alteration of vascular reactivity in rabbit aorta with modest elevation of serum cholesterol. *Circ. Res.* **67,** 550–555.

Michel, T., and Feron, O. (1997). Nitric oxide synthases, which, where, how, and why? *J. Clin. Invest.* **100,** 2146–2152.

Minor, R. L. J., Myers, P. R., Guerra, R. J., Bates, J. N., and Harrison, D. G. (1990). Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J. Clin. Invest.* **86,** 2109–2116.

Miyamoto, Y., Saito, Y., Kajiyama, N., Yoshimura, M., Shimasaki, Y., Nakayama, M., Kamitani, S., Harada, M., Ishikawa, M., Kuwahara, K., Ogawa, E., Hamanaka, I., Takahashi, N., Kaneshige, T., Teraoka, H., Akamizu, T., Azuma, N., Yoshimasa, Y., Yoshimasa, T., Itoh, H., Masuda, I., Yasue, H., and Nakao, K. (1998). Endothelial nitric oxide synthase gene is positively associated with essential hypertension. *Hypertension* **32,** 3–8.

Miyazaki, H., Matsuoka, H., Cooke, J. P., Usui, M., Ueda, S., Okuda, S., and Imaizumi, T. (1999). Endogenous nitric oxide synthase inhibitor, a novel marker of atherosclerosis. *Circulation* **99,** 1141–1146.

Mohazzab-H., K. M., Kaminski, P. M., and Wolin, M. S. (1994). NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am. J. Physiol.* **266,** H2568–H2572.

Moroi, M., Zhang, L., Yasuda, T., Virmani, R., Gold, H. K., Fishman, M. C., and Huang, P. L. (1998). Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J. Clin. Invest.* **101,** 1225–1232.

Mügge, A., and Harrison, D. G. (1991). L-Arginine does not restore endothelial dysfunction in atherosclerotic rabbit aorta in vitro. *Blood Vessels* **28,** 354–357.

Mügge, A., Elwell, J. H., Peterson, T. E., Hofmeyer, T. G., Heistad, D. D., and Harrison, D. G. (1991). Chronic treatment with polyethyleneglycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ. Res.* **69,** 1293–1300.

Münzel, T., Heitzer, T., and Harrison, D. G. (1997). The physiology and pathophysiology of the nitric oxide/superoxide system. *Herz* **22,** 158–172.

Nakayama, M., Yasue, H., Yoshimura, M., Shimasaki, Y., Kugiyama, K., Ogawa, H., Motoyama, T., Saito, Y., Ogawa, Y., Miyamoto, Y., and Nakao, K. (1999). $T^{-786} \rightarrow C$ mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation* **99,** 2864–2870.

Naruse, K., Shimizu, K., Maramatsu, M., Toki, Y., Miyazaki, Y., Okumura, K., Hashimoto, H., and Ito, T. (1994). Long-term inhibitio of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta. PGH2 does not contribute to impaired endothelium-dependent relaxation. *Arterioscler. Thromb.* **14,** 746–752.

Nickenig, G., Jung, O., Strehlow, K., Zolk, O., Linz, W., Scholkens, B. A., and Bohm, M. (1997). Hypercholesterolemia is associated with enhanced angiotensin AT1-receptor expression. *Am. J. Physiol.* **272,** H2701–H2707.

Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W., and Murphy, T. J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.* **90,** 2092–2096.

Nitenberg, A., Paycha, F., Ledoux, S., Sachs, R., Attali, J. R., and Valensi, P. (1998). Coronary artery responses to physiological stimuli are improved by deferoxamine but not by L-arginine in non-insulin-dependent diabetic patients with angiographically normal coronary arteries and no other risk factors. *Circulation* **97,** 736–743.

Noda, Y., Mori, A., and Packer, L. (1997). Gliclazide scavenges hydroxyl, superoxide and nitric oxide radicals, an ESR study. *Res. Commun. Mol. Pathol. Pharmacol.* **96,** 115–124.

O'Driscoll, G., Green, D., Rankin, J., Stanton, K., and Taylor, R. (1997). Improvement in endothelial function by angiotensin converting enzyme inhibition in insulin-dependent diabetes mellitus. *J. Clin. Invest.* **100,** 678–684.

Oemar, B. S., Tschudi, M. R., Godoy, N., Brovkovich, V., Malinski, T., and Lüscher, T. F. (1998). Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation* **97,** 2494–2498.

Ogawa, T., Kimoto, M., and Sasaoka, K. (1989). Purification and properties of a new enzyme, N^G, N^G-dimethylarginine dimethylaminohydrolase, from rat kidney. *J. Biol. Chem.* **264,** 10205–10209.

Ohara, Y., Peterson, T. E., and Harrison, D. G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* **91,** 2546–2551.

Ooboshi, H., Toyoda, K., Faraci, F. M., Lang, M. G., and Heistad, D. D. (1998). Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1752–1758.

Panza, J. A., Quyyumi, A. A., Brush, J. E., and Epstein, S. E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N. Engl. J. Med.* **323,** 22–27.

Panza, J. A., Casino, P. R., Kilcoyne, C. M., and Quyyumi, A. A. (1993a). Role of nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation* **87,** 1468–1474.

Panza, J. A., Casino, P. R., Badar, D. M., and Quyyumi, A. A. (1993b). Effect of increased availability of endothelium-derived nitric oxide precursor on endothelium-dependent vascular relaxation in normal subjects and in patients with essential hypertension. *Circulation* **87,** 1475–1481.

Panza, J. A., Casino, P. R., Kilcoyne, C. M., and Quyyumi, A. A. (1994). Impaired endothelium-dependent vasodilation in patients with essential hypertension, evidence that the abnormality is not at the muscarinic receptor level. *J. Am. Coll. Cardiol.* **23,** 1610–1616.

Panza, J. A., Garcia, C. E., Kilcoyne, C. M., Quyyumi A. A., and Cannon III, R. O. (1995). Impaired endothelium-dependent vasodilation in patients with essential hypertension. Evidence that nitric oxide abnormality is not localized to a single signal transduction pathway. *Circulation* **91,** 1732–1738.

Pascoal, I. F., Lindheimer, M. D., Nalbantian-Brandt, C., and Umans, J. G. (1998). Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. *J. Clin. Invest.* **101,** 464–470.

Pettersson, A., Uggla, L., and Backman, V. (1997). Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. *J. Chromatogr. B, Biomed. Sci. Appl.* **692,** 257–262.

Pieper, G. M. (1997). Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor tetrahydrobiopterin. *J. Cardiovasc. Pharmacol.* **29,** 8–15.

Pieper, G. M., and Dondlinger, L. A. (1997). Plasma and vascular tissue arginine are decreased in diabetes, acute arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. *J. Pharmacol. Exp. Ther.* **283,** 684–691.

Pieper, G. M., and Gross, G. J. (1988). Oxygen-derived free radicals abolish endothelium-dependent relaxation in diabetic rat aorta. *Am. J. Physiol.* **255,** H825–H833.

Pieper, G. M., Siebeneich, W., and Dondlinger, L. A. (1996a). Short-term oral administration of L-arginine reverses defective endothelium-dependent relaxation and cGMP generation in diabetes. *Eur. J. Pharmacol.* **317,** 317–320.

Pieper, G. M., Jordan, M., Adams, M. B., and Roza, A. M. (1996b). Restoration of vascular endothelial function in diabetes. *Diabetes. Res. Clin. Pract.* **31**(Suppl.), S157–S162.

Pieper, G. M., Siebeneich, W., Moore-Hilton, G., and Roza, A. M. (1997a). Reversal by L-arginine of a dysfunctional arginine/nitric oxide pathway in the endothelium of the genetic diabetic BB rat. *Diabetologia* **40,** 910–915.

Pieper, G. M., Langenstroer, P., and Siebeneich, W. (1997b). Diabetic-induced endothelial dysfunction in rat aorta, role of hydroxyl radicals. *Cardiovasc. Res.* **34,** 145–156.

Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H., and Rosen, G. M. (1992). Generation of superoxide by purified brain nitric oxide synthase. *J. Biol. Chem.* **267,** 24173–24176.

Preik, M., Kelm, M., Schoebel, F., Schottenfeld, Y., Leschke, M., and Strauer, B. E. (1996a). Selective impairment of nitric oxide dependent vasodilation in young adults with hypercholesterolaemia. *J. Cardiovasc. Risk* **3,** 465–471.

Preik, M., Kelm, M., Feelisch, M., and Strauer, B. E. (1996b). Impaired effectiveness of nitric oxide-donors in resistance arteries of patients with arterial hypertension. *J. Hypertens.* **14,** 903–908.

Quyyumi, A. A., Dakak, N., Andrews, N. P., Husain, S., Arora, S., Gilligan, D. M., Panza, J. A., and Cannon III, R. O. (1995). Nitric oxide activity in the human coronary circulation. Impact of risk factors for coronary atherosclerosis. *J. Clin. Invest.* **95,** 1747–1755.

Ragazzi, E., Chinellato, A., De Biasi, M., Pandolfo, L., Prosdocimi, M., Norido, F., Caparrotta, L., and Fassina, G. (1989). Endothelium-dependent relaxation, cholesterol content and high energy metabolite balance in Watanabe hyperlipemic rabbit aorta. *Atherosclerosis* **80,** 125–134.

Ragazzi, E., Chinellato, A., Pandolfo, L., Froldi, G., Caparrotta, L., Aliev, G., Prosdocimi, M., and Fassina, G. (1995). Endothelial nucleotide-mediated aorta relaxation in aged Watanabe heritable hyperlipidemic rabbits. *J. Cardiovasc. Pharmacol.* **26,** 119–126.

Rajagopalan, S., Kurz, S., Münzel, T., Tarpey, M., Freeman, B. A., Griendling, K. K., and Harrison, D. G. (1996). Angiotensin II-mediated

hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.* **97,** 1916–1923.

Randall, M. D., and March, J. E. (1998). Characterization of endothelium-dependent relaxations in mesenteries from transgenic hypertensive rats. *Eur. J. Pharmacol.* **358,** 31–40.

Reddy, K. G., Nair, R. N., Sheehan, H. M., and Hodgson, J. M. (1994). Evidence that selective endothelial dysfunction may occur in the absence of angiographic or ultrasound atherosclerosis in patients with risk factors for atherosclerosis. *J. Am. Coll. Cardiol.* **23,** 833–843.

Rieder, M. J., Carmona, R., Krieger, J. E., Pritchard, K. A., Jr., and Greene, A. S. (1997). Suppression of angiotensin-converting enzyme expression and activity by shear stress. *Circ. Res.* **80,** 312–319.

Rubanyi, G. M. (1993). The role of endothelium in cardiovascular homeostasis and diseases. *J. Cardiovasc. Pharmacol.* **22**(Suppl. 4), S1–S4.

Ruschitzka, F. T., Noll, G., and Lüscher, T. F. (1998). Combination of ACE inhibitors and calcium antagonists, a logical approach. *J. Cardiovasc. Pharmacol.* **31**(Suppl. 2), S5–S16.

Sakamoto, S., Minami, K., Niwa, Y., Ohnaka, M., Nakaya, Y., Mizuno, A., Kuwajima, M., and Shima, K. (1998). Effect of exercise training and food restriction on endothelium-dependent relaxation in the Otsuka Long-Evans Tokushima Fatty rat, a model of spontaneous NIDDM. *Diabetes* **47,** 82–86.

Seiler, C., Hess, O. M., Buechi, M., Suter, T. M., and Krayenbuehl, H. P. (1993). Influence of serum cholesterol and other coronary risk factors on vasomotion of angiographically normal coronary arteries. *Circulation* **88,** 2139–2148.

Sellke, F. W., Armstrong, M. L., and Harrison, D. G. (1990). Endothelium-dependent vascular relaxation is abnormal in the coronary microcirculation of atherosclerotic primates. *Circ. Res.* **81,** 1586–1593.

Senaratne, M. P., Thomson, A. B., and Kappagoda, C. T. (1991). Lovastatin prevents the impairment of endothelium dependent relaxation and inhibits accumulation of cholesterol in the aorta in experimental atherosclerosis in rabbits. *Cardiovasc. Res.* **25,** 568–578.

Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J., and Hintze, T. H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* **74,** 349–353.

Shimasaki, Y., Yasue, H., Yoshimura, M., Nakayama, M., Kugiyama, K., Ogawa, H., Harada, E., Masuda, T., Koyama, W., Saito, Y., Miyamoto, Y., Ogawa, Y., and Nakao, K. (1998). Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with myocardial infarction. *J. Am. Coll. Cardiol.* **31,** 1506–1510.

Shimokawa, H. (1998). Endothelial dysfunction in hypertension. *J. Atheroscler. Thromb.* **4,** 118–127.

Shimokawa, H., and Vanhoutte, P. M. (1988). Dietary cool-liver oil improves endothelium-dependent responses in hypercholesterolemic and atherosclerotic porcine coronary arteries. *Circulation* **78,** 1421–1430.

Shimokawa, H., Flavahan, N. A., and Vanhoutte, P. M. (1990). Natural course of the impairment of endothelium-dependent relaxations in regenerating porcine endothelial cells, possible dysfunction of a pertussis toxin-sensitive G protein. *Circ. Res.* **65,** 740–753.

Shirasaki, Y., Kolm, P., Nickols, G. A., and Lee, T. J. F. (1988). Endothelial regulation of cyclic GMP and vascular responses in hypertension. *J. Pharmacol. Exp. Ther.* **245,** 53–58.

Siperstein, M. D., Unger, R. H., and Madison, L. L. (1968). Studies of muscle capillary basement membranes in normal subjects, diabetics and pro-diabetic patients. *J. Clin. Invest.* **47,** 1973–1999.

Smith, C. J., Sun, D., Hoegler, C., Roth, B. S., Zhang, X., Zhao, G., Xu, X. B., Kobari, Y., Pritchard, K., Jr., Sessa, W. C., and Hintze, T. H. (1996). Reduced gene expression of vascular endothelial NO synthase and cyclooxygenase-1 in heart failure. *Circ. Res.* **78,** 58–64.

Solzbach, U., Hornig, B., Jeserich, M., and Just, H. (1997). Vitamin C improves endothelial dysfunction of epicardial coronary arteries in hypertensive patients. *Circulation* **96,** 1513–1519.

Sorensen, K. E., Celermajer, D. S., Spiegelhalter, D. J., Georgakopoulos, D., Robinson, J., Thomas, O., and Deanfield, J. E. (1995). Non-invasive measurement of human endothelium dependent arterial responses: Accuracy and reproducibility. *Br. Heart J.* **74,** 247–253.

Sorensen, K. E., Kristensen, I. B., and Celermajer, D. S. (1997). Atherosclerosis in the human brachial artery. *J. Am. Coll. Cardiol.* **29,** 318–322.

Steinberg, H. O., Bayazeed, B., Hook, G., Johnson, A., Cronin, J., and Baron, A. D. (1997). Endothelial dysfunction is associated with cholesterol levels in the high normal range in humans. *Circulation* **96,** 3287–3293.

Stroes, E., Kastelein, J., Cosentino, F., Erkelens, W., Wever, R., Koomans, H., Lüscher, T., and Rabelink, T. (1997). Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J. Clin. Invest.* **99,** 41–46.

Sullivan, M., Thompson, C. S., Mikhailidis, D. P., Morgan, R. J., Angelini, G. D., and Jeremy, J. Y. (1998). Differential alterations of prostacyclin, cyclic AMP and cyclic GMP formation in the corpus cavernosum of the diabetic rabbit. *Br. J. Urol.* **82,** 578–584.

Szilvassy, Z., Sari, R., Nemeth, J., Nagy, I., Csati, S., and Lonovics, J. (1998). Improvement of nitrergic relaxation by farnesol of the sphincter of Oddi from hypercholesterolaemic rabbits. *Eur. J. Pharmacol.* **353,** 75–78.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Fasolo, C. B., Sudano, I., and Salvetti, A. (1997). Hypertension causes premature aging of endothelial function in humans. *Hypertension* **29,** 736–743.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., and Salvetti, A. (1998). Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* **97,** 2222–2229.

Takase, B., Uehata, A., Akima, T., Nagai, T., Nishioka, T., Hamabe, A., Satomura, K., Ohsuzu, F., and Kurita, A. (1998). Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. *Am. J. Cardiol.* **82,** 1535–1539, A7–8.

Takase, H., Moreau, P., Kung, C. F., Nava, E., and Lüscher, T. F. (1996). Antihypertensive therapy prevents endothelial dysfunction in chronic nitric oxide deficiency. Effect of verapamil and trandolapril. *Hypertension* **27,** 25–31.

Tarry, W. C., and Makhoul, R. G. (1994). L-Arginine improves endothelium-dependent vasorelaxation and reduces intimal hyperplasia after balloon angioplasty. *Arterioscler. Thromb.* **14,** 938–943.

Tawakol, A., Omland, T., Gerhard, M., Wu, J. T., and Creager, M. A. (1997). Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation* **95,** 1119–1121.

Tesfamariam, B. (1994). Free radicals in diabetic endothelial cell dysfunction. *Free Radicals Biol. Med.* **16,** 383–391.

Tesfamariam, B., Jakubowski, J. A., and Cohen, R. A. (1989). Contraction of diabetic rabbit aorta caused by endothelium-derived PGH2-TXA2. *Am. J. Physiol.* **257,** H1327–H1333.

Tesfamariam, B., Brown, M. L., and Cohen, R. A. (1991). Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J. Clin. Invest.* **87,** 1643–1648.

Thorne, S., Mullen, M. J., Clarkson, P., Donald, A. E., and Deanfield, J. E. (1998). Early endothelial dysfunction in adults at risk from atherosclerosis, different responses to L-arginine. *J. Am. Coll. Cardiol.* **32,** 110–116.

Timimi, F. K., Ting, H. H., Haley, E. A., Roddy, M. A., Ganz, P., and Creager, M. A. (1998). Vitamin C improves endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *J. Am. Coll. Cardiol.* **31,** 552–557.

Ting, H. H., Timimi, F. K., Boles, K. S., Creager, S. J., Ganz, P., and Creager, M. A. (1996). Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J. Clin. Invest.* **97,** 22–28.

Ting, H. H., Timimi, F. K., Haley, E. A., Roddy, M. A., Ganz, P., and Creager, M. A. (1997). Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. *Circulation* **95,** 2617–2622.

Tsao, P. S., Theilmeier, G., Singer, A. M., Leung, L. L.-K., and Cooke, J. P. (1994a). L-Arginine attenuates platelet reactivity in hypercholesterolemic rabbits. *Arterioscler. Thromb.* **14,** 1529–1533.

Tsao, P. S., McEvoy, L. M., Drexler, H., Butcher, E. C., and Cooke, J. P. (1994b). Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation* **89,** 2176–2182.

Tschudi, M. R., Criscione, L., Novosel, D., Pfeiffer, K., and Lüscher, T. F. (1994). Antihypertensive therapy augments endothelium-dependent relaxations in coronary arteries of spontaneously hypertensive rats. *Circulation* **89,** 2212–2218.

Tsutsui, M., Shimokawa, H., Tanaka, S., Kuwaoka, I., Hase, K., Nogami, N., Nakanishi, K., and Okamatsu, S. (1994). Endothelial Gi protein in human coronary arteries. *Eur. Heart J.* **15,** 1261–1266.

Urakami-Harasawa, L., Shimokawa, H., Nakashima, M., Egashira, K., and Takeshita, A. (1997). Importance of endothelium-derived hyperpolarizing factor in human arteries. *J. Clin. Invest.* **100,** 2793–2799.

Van De Voorde, J., and Leusen, I. (1986). Endothelium-dependent and independent relaxation of aortic rings from hypertensive rats. *Am. J. Physiol.* **250,** H711–H717.

Van De Voorde, J., Vanheel, B., and Leusen, I. (1992). Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. *Circ. Res.* **70,** 1–8.

Verbeuren, T. J., Jordaens, F. H., Zonnekeyn, L. L., Van Hove, C. E., Coene, M.-C., and Herman, A. G. (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ. Res.* **58,** 552–564.

Verbeuren, T. J., Jordaens, F. H., Van Hove, C. E., Van Hoydonck, A.-E., and Herman, A. G. (1990). Release and vascular activity of endothelium-derived relaxing factor in atherosclerotic rabbit aorta. *Eur. J. Pharmacol.* **191,** 173–184.

Vidal, F., Colome, C., Martinez-Gonzalez, J., and Badimon, L. (1998). Atherogenic concentrations of native low-density lipoproteins down-regulate nitric-oxide-synthase mRNA and protein levels in endothelial cells. *Eur. J. Biochem.* **252,** 378–384.

Vita, J. A., Treasure, C. B., Nabel, E. G., McLenachan, J. M., Fish, R. D., Yeung, A. C., Vekshtein, V. I., Selwyn, A. P., and Ganz, P. (1990). Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. *Circulation* **81,** 491–497.

Vita, J. A., Frei, B., Holbrook, M., Gokce, N., Leaf, C., and Keaney, J. F., Jr. (1998). L-2-Oxothiazolidine-4-carboxylic acid reverses endothelial dysfunction in patients with coronary artery disease. *J. Clin. Invest.* **101,** 1408–1414.

Vrints, C. J. M., Bult, H., Hitter, E., Herman, A. G., and Snoeck, J. P. (1992). Impaired endothelium-dependent cholinergic coronary vasodilation in patients with angina and normal coronary arteriograms. *J. Am. Coll. Cardiol.* **19,** 21–31.

Wang, B.-Y., Singer, A. H., Tsao, P. S., Drexler, H., Kosek, J., and Cooke, J. P. (1994). Dietary arginine prevents atherogenesis in the coronary artery of the hypercholesterolemic rabbit. *J. Am. Coll. Cardiol.* **23,** 452–458.

Warnholtz, A., Nickenig, G., Schulz, E., Macharzina, R., Brasen, J. H., Skatchkov, M., Heitzer, T., Stasch, J. P., Griendling, K. K., Harrison, D. G., Bohm, M., Meinertz, T., and Münzel, T. (1999). Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: Evidence for involvement of the renin-angiotensin system. *Circulation* **99,** 2027–2033.

Weidinger, F. F., McLenachan, J. M., Cybulsky, M. I., Gordon, J. B., Rennke, H. G., Hollenberg, N. K., Fallar, J. T., Ganz, P., and Cooke, J. P. (1990). Persistent dysfunction of regenerated endothelium after balloon angioplasty of rabbit iliac artery. *Circ. Res.* **81,** 1667–1679.

Weisbrod, R. M., Griswold, M. C., Du, Y., Bolotina, V. M., and Cohen, R. A. (1997). Reduced responsiveness of hypercholesterolemic rabbit aortic smooth muscle cells to nitric oxide. *Arterioscler. Thromb. Vasc. Biol.* **17,** 394–402.

Wennmalm, A. (1994). Endothelial nitric oxide and cardiovascular disease. *J. Intern. Med.* **235,** 317–327.

Werns, S. W., Walton, J. A., Hsia, H. H., Nabel, E. G., Sanz, M. L., and Pitt, B. (1989). Evidence of endothelial dysfunction in angiographically normal coronary arteries of patients with coronary artery disease. *Circulation* **79,** 287–291.

White, C. R., Darley-Usmar, V., Berrington, W. R., McAdams, M., Gore, J. Z., Thompson, J. A., Parks, D. A., Tarpey, M. M., and Freeman, B. A. (1996). Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8745–8749.

White, C. R., Shelton, J., Chen, S. J., Darley-Usmar, V., Allen, L., Nabors, C., Sanders, P. W., Chen, Y. F., and Oparil, S. (1997). Estrogen restores endothelial cell function in an experimental model of vascular injury. *Circulation* **96,** 1624–1630.

Wilcox, J. N., Subramanian, R. R., Sundell, C. L., Tracey, W. R., Pollock, J. S., Harrison, D. G., and Marsden, P. A. (1997). Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler. Thromb. Vasc. Biol.* **17,** 2479–2488.

Winquist, R. J., Bunting, P. B., Baskin, E. P., and Wallace, A. A. (1984). Decreased endothelium-dependent relaxation in New Zealand genetic hypertensive rats. *J. Hypertens.* **2,** 541–545.

Wu, G., and Meininger, C. J. (1995). Impaired arginine metabolism and NO synthesis in coronary endothelial cells of the spontaneously diabetic BB rat. *Am. J. Physiol.* **269,** H1312–H1318.

Yamamoto, H., Bossaller, C., Cartwright, J., Jr., and Henry, P. D. (1988). Videomicroscopic demonstration of defective cholinergic arteriolar vasodilation in atherosclerotic rabbit. *J. Clin. Invest.* **81,** 1752–1758.

Yamamoto, Y., Tomoike, H., Egashira, K., and Nakamura, M. (1987). Attenuation of endothelium-related relaxation and enhanced responsiveness of vascular smooth muscle to histamine in spastic coronary arterial segments from miniature pigs. *Circ. Res.* **61,** 772–778.

Yasue, H., Matsuyama, K., Matsuyama, K., Okumura, K., Morikami, Y., and Ogawa, H. (1990). Responses of angiographically normal human coronary arteries to intracoronary injection of acetylcholine by age and segment. Possible role of early coronary atherosclerosis. *Circulation* **81,** 482–490.

Yoshimura, M., Yasue, H., Nakayama, M., Shimasaki, Y., Sumida, H., Sugiyama, S., Kugiyama, K., Ogawa, H., Ogawa, Y., Saito, Y., Miyamoto, Y., and Nakao, K. (1998). A missense $Glu^{298}Asp$ variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese. *Hum. Genet.* **103,** 65–69.

Ziegler, T., Bouzourene, K., Harrison, V. J., Brunner, H. R., and Hayoz, D. (1998). Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18,** 686–692.

Zimmermann, G. A., Wiseman, G. A., and Hill, H. R. (1985). Human endothelial cells modulate granulocyte adherence and chemotaxis. *J. Immunol.* **134,** 1866–1874.

CHAPTER 35

Endothelium-Derived Hyperpolarizing Factor and Its Interaction with NO

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LOCAL VASCULAR TONE IS GENERALLY DETERMINED BY EXTRINSIC AND INTRINSIC MECHANISMS SUCH AS AUTONOMIC NERVE ACTIVITY, CIRCULATING VASOACTIVE COMPOUNDS, TISSUE METABOLITES, THE MYOGENIC RESPONSE, AND ENDOTHELIUM-DERIVED AUTACOIDS. THE BEST CHARACTERIZED AUTACOIDS ARE THE POTENT VASODILATORS NITRIC OXIDE (NO) AND PROSTACYCLIN (PGI_2) AND THE VASOCONSTRICTOR PEPTIDE ENDOTHELIN. SEVERAL STUDIES HAVE, HOWEVER, CONVINCINGLY DEMONSTRATED THE EXISTENCE OF AN NO/PGI_2-INDEPENDENT COMPONENT OF ENDOTHELIUM-DEPENDENT RELAXATION IN VARIOUS ARTERIAL BEDS, MOST NOTABLY IN MESENTERIC, CAROTID, CORONARY, AND RENAL ARTERIES. SINCE THIS NO/PGI_2-INDEPENDENT VASODILATION WAS COINCIDENT WITH VASCULAR SMOOTH MUSCLE HYPERPOLARIZATION, THE TERM ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR, OR EDHF, WAS COINED. THIS CHAPTER IS INTENDED TO PROVIDE AN OVERVIEW INTO THE FIELD OF EDHF AND TO DELINEATE THE INTERACTION OF NO WITH THE ONLY WELL-CHARACTERIZED EDHF, NAMELY, THAT GENERATED IN THE CORONARY AND RENAL VASCULATURE.

Relationship between Membrane Potential and Relaxation

Hyperpolarization and Relaxation

The mechanism by which hyperpolarization elicits relaxation is controversial. The most direct and obvious explanation is that hyperpolarization of the smooth muscle cell membrane reduces the open probability of voltage-dependent Ca^{2+} channels, activates Ca^{2+} sequestration and removal mechanisms so that smooth muscle $[Ca^{2+}]_i$ is lowered, and relaxation can take place.

Although this mechanism may operate in some vascular preparations, it is not universal. For example, the rabbit thoracic aorta is not depolarized during contraction induced by norepinephrine and the contraction induced is minimally affected by Ca^{2+} entry blockers such as nifedipine. Nevertheless, under such conditions K^+_{ATP} channel openers are potent relaxants, indicating that the opening of K^+ channels may also induce relaxation by mechanisms other than inhibition of voltage-dependent Ca^{2+} channels. However, although hyperpolarization is not always essential for relaxation, it may be determinant in systems in which NO/cyclic GMP production is attenuated or inhibited.

Endothelium-Dependent Smooth Muscle Cell Hyperpolarization

Receptor-dependent Ca^{2+}-elevating agonists (e.g., acetylcholine, bradykinin, substance P, histamine, ADP, and

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endothelin) stimulate endothelial cells and induce the hyperpolarization of vascular smooth muscle cells. As this response is resistant to nitric oxide synthase (NOS) and cyclooxygenase (COX) inhibitors it is generally attributed to the release of an EDHF.

While the definition of EDHF implies that a factor released from the endothelium hyperpolarizes vascular smooth muscle cells, many studies have been performed in organ chambers without the concomitant measurement of membrane potential. Thus, in a large number of the investigations performed to elucidate the nature of EDHF, the predominant factor studied may be an as-yet-unidentified endothelium-derived relaxing factor (EDRF), that is, a nonhyperpolarizing NO/PGI_2-independent factor.

Under normal physiological conditions, the time course of smooth muscle hyperpolarization is more transient than that of relaxation. Under conditions of NOS/COX inhibition, a situation in which agonist-induced endothelium-dependent relaxation is thought to be mediated by the EDHF-induced hyperpolarization of smooth muscle cells, relaxations are much more transient than those mediated by NO and have a time course similar to that of hyperpolarization (Fig. 1). Such observations could indicate a cause-and-effect relationship.

The range of membrane potentials in which an EDHF can be expected to open K^+_{Ca} channels and markedly affect membrane potential is relatively narrow. Very negative membrane potentials, that is, potentials close to the equilibrium potential for K^+, are unlikely to be influenced by the opening of K^+_{Ca} channels. Conversely, in depolarized cells the conductance for K^+ is already high and the opening of additional K^+ channels is unlikely to exert much of an effect.

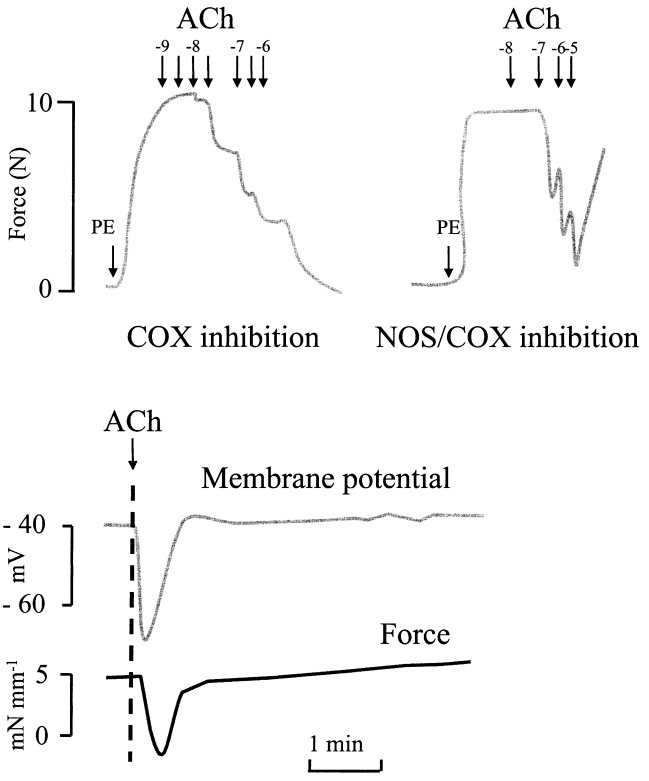


Figure 1 The kinetics of the NO- and EDHF-mediated relaxations of a mesenteric artery are distinct. Small mesenteric arteries, precontracted under isometric conditions with phenylephrine (PE), were relaxed by the cumulative addition of the Ca^{2+}-elevating endothelial agonist acetylcholine (ACh). The top left tracing shows the response obtained when COX is inhibited and the relaxation is mediated mainly by NO, and the top right tracing shows the EDHF-mediated relaxation assessed in the presence of COX and NOS inhibitors. Lower tracings show the simultaneous changes in smooth muscle cell membrane potential and vascular tone in response to the application of a maximal concentration of ACh, in the presence of COX and NOS inhibitors.

Smooth Muscle Cell Membrane Potential

The membrane potential is determined by ionic concentration gradients and permeabilities according to the Goldmann equation. The transmembrane ion distribution in vascular smooth muscle cells is similar to other excitable cells. The diffusion of ions through the membrane is driven by their electrochemical gradient and the permeability coefficient (P), which represents the ease with which an ion diffuses through the corresponding channel. The relative permeability of the membrane to K^+, Na^+, Ca^{2+}, and Cl^- is $P_K = 1 > P_{Cl} = 0.366 > P_{Ca} = 0.201 > P_{Na} = 0.037$; therefore, the membrane potential is mainly determined by the opening and closing of K^+ and, to a lesser extent, Cl^- channels.

The resting membrane potential of smooth muscle cells in normal pressurized arteries and arterioles *in vivo* is between −55 and −35 mV, reflecting the fact that arteries exist in a partially contracted state from which they can constrict or dilate. The working point is therefore positioned in the steep, linear range of the activation curve and shifted well to the right of the working point in resting skeletal muscle (approximately −90 mV).

As the membrane potential determines the activation of voltage-dependent ion channels, the consequences of K^+ channel gating (rate of opening) are thus twofold: (i) to set the membrane potential and influence the amplitude of inward Ca^{2+} currents that produce contraction and (ii) to carry the outward current that repolarizes the cell, thus modifying the kinetics of contraction.

The membrane potential is also affected by ion transporters. The activity of the Na^+/K^+ pump hyperpolarizes the plasmalemma, whereas the electrogenic Na^+/Ca^{2+} exchanger also contributes to the membrane potential by translocating three Na^+ ions into the cell and one Ca^{2+} out of the cell during each cycle.

The K^+ permeability of the smooth muscle cell membrane is, in principle, determined by four different types of K^+ channels, each of which has different characteristics and is activated under different conditions.

- Ca^{2+}-activated K^+ channels (K^+_{Ca})
- ATP-sensitive K^+ channels (K^+_{ATP})
- Inwardly rectifying K^+ channels (K^+_{IR})
- Delayed rectifying voltage-dependent K^+ channels (K^+_{dir})

K^+_{Ca} channels are, as the name suggests, all activated by an increase in intracellular Ca^{2+} and are generally voltage-dependent. Vascular smooth muscle cells derived from the systemic circulation are somewhat depolarized, and $[Ca^{2+}]_i$ is slightly elevated; thus, it follows that outward current is carried to a large extent by K^+_{Ca} channels. The opening of only a very few K^+ channels is sufficient to bring about an effective membrane hyperpolarization, and thus K^+_{Ca} channels can hyperpolarize smooth muscle cells at low levels of activation. For example, the opening of only three K^+ channels in a cell containing 50,000 K^+ channels induces a membrane hyperpolarization of 2 mV. This weak hyperpolarization simultaneously decreases the open probability of voltage-dependent Ca^{2+} channels by approximately 30% and opposes the development of tension.

K^+_{Ca} channels are grouped into three main types according to their biophysical and pharmacological properties. Those channels exhibiting a large unitary conductance are often called ''big'' Ca^{2+}-dependent K^+ channels (BK_{Ca}) and are activated (i.e., open state probability increases) by increases in intracellular Ca^{2+} and membrane depolarization. Iberiotoxin and noxiustoxin appear to be highly selective inhibitors of these channels in arterial smooth muscle cells. There are two other classes of K^+_{Ca} channels. Those exhibiting a much lower K^+ conductance are usually referred to as ''small'' K^+_{Ca} channels (SK_{Ca}) and are insensitive to charybdotoxin but are selectively blocked by apamin and scillatoxin. The other class is an intermediate or medium conductance, charybdotoxin-sensitive K^+_{Ca} channel (IK_{Ca}).

Three main groups of K^+_{Ca}:

Channel type	Conductance (pS)	Inhibitors
High conductance channel (BK_{Ca})	100–250	iberiotoxin, noxiustoxin
Intermediate conductance channel (IK_{Ca})	20–60	charybdotoxin
Small conductance channel (SK_{Ca})	5–15	apamin, scillatoxin

K^+_{ATP} channels (conductance of 10–30 pS) are inhibited as the concentration of ATP at the internal membrane is increased. A fall in intracellular ATP may not be the most important physiological activator of these channels, which are also regulated by an increase in ADP, GDP, GTP, or an acidic pH. The antidiabetic sulfonylurea glibenclamide has been shown to selectively block K^+_{ATP} channels. On the other hand, a number of synthetic K^+_{ATP} channel openers, for example, cromakalim, effect vasodilation via membrane hyperpolarization following K^+_{ATP} activation.

Inwardly rectifying K^+ channels (conductance of 4–10 pS) are present mainly in small arteries and arterioles and exhibit an increasing conductance with membrane potentials steeply negative to the K^+ equilibrium potential. The inwardly rectifying channel closes when arterioles are tonically depolarized, so that the loss of K^+ ions during moderate depolarization is minimized.

Delayed rectifying voltage-dependent K^+ channels (conductance of 5–55 pS) facilitate an outward K^+ current in response to vascular smooth muscle hyperpolarization. The activity of these channels decreases on maintained depolarization, and therefore they act to repolarize the membrane potential. This again contributes significantly to the control of the membrane potential in resistance arteries.

WHICH K^+ CHANNELS ARE ACTIVATED BY EDHF?

Initially, it was generally assumed that EDHF activates K^+_{Ca} channels in vascular smooth muscle cells. The evidence for this is based on the following observations:

1. The EDHF-mediated vasodilation of an arterial bed in response to endothelial stimulation with a receptor-dependent agonist such as acetylcholine or bradykinin is associated with an increase in K^+ efflux, and the membrane conductance of smooth muscle is increased during hyperpolarization.
2. The EDHF-mediated vasodilation is inhibited by alterations in extracellular $[K^+]$ or other manipulations that influence the open probability of K^+ channels (Fig. 2).
3. K^+_{ATP} channel blockers (for example, glibenclamide) do not inhibit EDHF-mediated smooth muscle hyperpolarization and relaxation.
4. The endothelium-dependent hyperpolarization of vascular smooth muscle segments in intact vascular segments is sensitive to K^+_{Ca} channel inhibitors (such as apamin, charybdotoxin, or iberiotoxin) but insensitive to inhibitors of K^+_{IR} or K^+_{ATP}.
5. In patch clamp bioassay systems, in which the membrane potential is monitored in smooth muscle cells positioned close to an endothelium-intact EDHF donor, the open probability of K^+ channels dramatically increases in response to an increase in Ca^{2+}, whereas decreasing intracellular Ca^{2+} blocks K^+ channel activity (Fig. 3).

It is important to keep in mind that both endothelial and smooth muscle cells possess a spectrum of ion channels, and none of the available inhibitors can differentiate between a target channel on endothelial cells and the same (or similar) target on smooth muscle cells. Indeed, although EDHF is generally reported to activate a K^+_{Ca} channel on smooth muscle cells, there is evidence to suggest that the K^+_{Ca} channel activated is an endothelial K^+_{Ca} channel. There are other observations which suggest that the K^+ channel activated by EDHF is not a typical BK_{Ca} channel. The classic BK_{Ca} channel is selectively inhibited by either iberiotoxin or noxiustoxin. However, the characteristics of the channel activated by EDHF are somewhat different, and in most vascular beds, EDHF-mediated (i.e., NO/PGI_2-independent) relaxation and hyperpolarization are only partially sensitive to iberiotoxin, charybdotoxin, or apamin but are abolished by the combination of charybdotoxin and apamin. Furthermore, the binding of charybdotoxin to a membrane preparation known to contain BK_{Ca} is unaffected by iberiotoxin but is increased by apamin. This rather unexpected effect of apamin and charybdotoxin which is a characteristic of EDHF-mediated

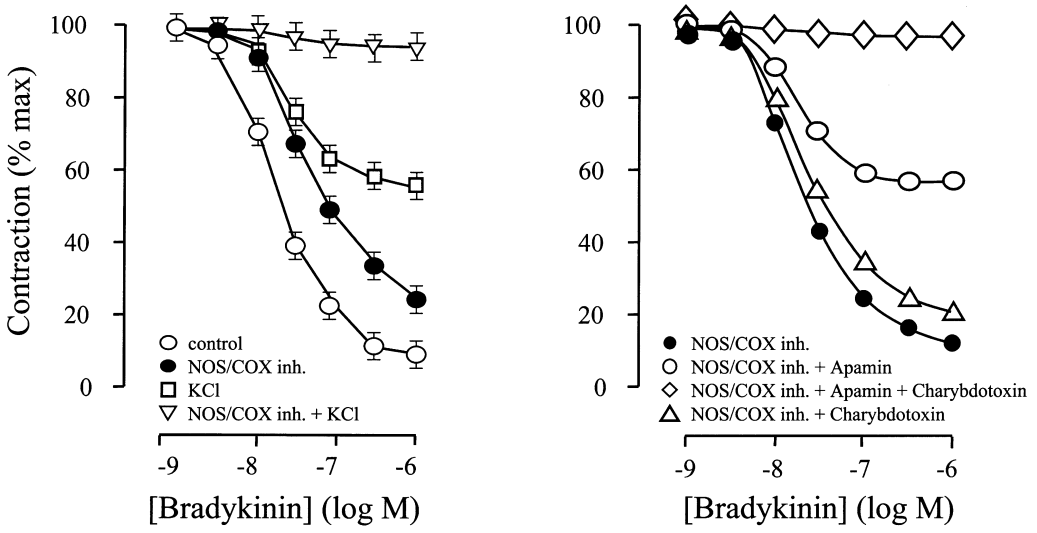


Figure 2 The EDHF-mediated vasodilation is inhibited by alterations in extracellular [K^+] or other manipulations that influence the open probability of K^+ channels. Both graphs show concentration–relaxation curves to the Ca^{2+}-elevating endothelial agonist bradykinin obtained using contracted rings of porcine coronary artery. Relaxation was monitored under a number of conditions: control, in normal physiological salt solution conditions in which relaxation is mediated mainly by NO; NOS/COX inh., in the combined presence of COX and NOS inhibitors to reveal which proportion of the response is attributable to EDHF; KCl, in the presence of KCl (~40 m*M*), which is used to depolarize vascular cells and prevent hyperpolarization by EDHF; apamin, in the presence of apamin, an inhibitor of SK_{Ca}; and charybdotoxin, in the presence of charybdotoxin, an inhibitor of IK_{Ca}. Complete inhibition of relaxation in the combined presence of apamin and charybdotoxin is a characteristic of EDHF-mediated responses in numerous arterial beds.

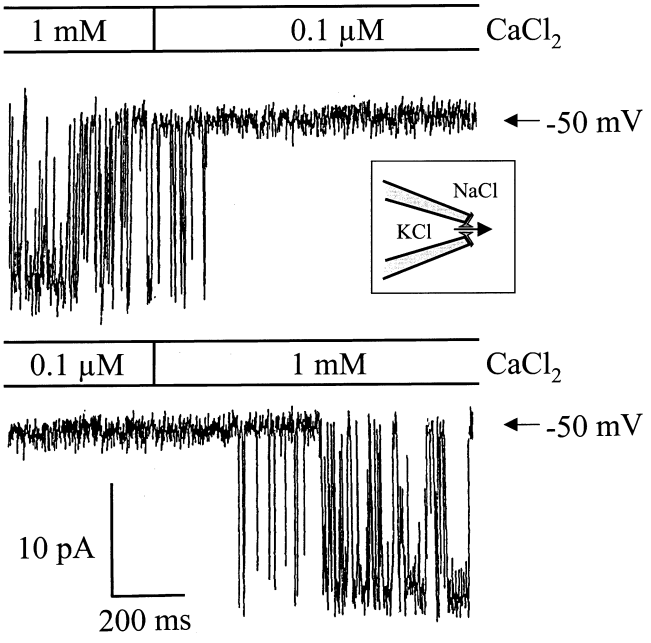


Figure 3 K^+_{Ca} channel opening in a membrane patch excised from a smooth muscle cell, stimulated by the EDHF-containing effluent from a bradykinin-stimulated porcine coronary artery in a patch clamp bioassay system. To demonstrate the Ca^{2+} dependency of these channels, the upper tracing shows the effect on channel current of decreasing the concentration of Ca^{2+} in contact with the excised patch. The lower tracing shows the opposite situation in which the K^+_{Ca} channel is activated by an increase in Ca^{2+} (modified from Popp *et al.,* 1996).

responses, could be an indication for the existence of a novel K^+_{Ca} channel in either endothelial or smooth muscle cells which is structurally related to the classic BK_{Ca}. Alternatively, the combination of these two K^+_{Ca} inhibitors may affect other cell signaling processes such as gap junctional communication (see below) or the activation of a key enzyme or effector kinase cascade.

EDHF and Endothelial Cell Hyperpolarization

Although endothelial cells are nonexcitable, the membrane potential remains an important determinant of endothelial cell function. Indeed, endothelial cells are hyperpolarized by many of the agents that elicit the hyperpolarization of vascular smooth muscle cells. Endothelial cell hyperpolarization has been attributed to the opening of K^+_{Ca} on the endothelial cell membrane, as a consequence of an agonist-induced increase in $[Ca^{2+}]_i$. Because endothelial cells lack voltage-dependent Ca^{2+} channels, the influx of Ca^{2+} into endothelial cells is determined mainly by the electrochemical gradient for Ca^{2+}. Endothelial hyperpolarization, which is a consequence of K^+ efflux via the K^+_{Ca} channel, counteracts the depolarizing effects of Ca^{2+} and enhances its driving force, thereby contributing to the sustained entry of Ca^{2+}. There is some evidence to suggest that EDHF may exert an autocrine effect on, and may be involved in regulating Ca^{2+} signaling in endothelial cells.

Which Vessels Have a Prominent EDHF?

Not all arteries generate an EDHF or exhibit an NO/PGI_2-independent relaxation on agonist stimulation. A general

rule of thumb has been that the importance of EDHF as a vasodilator increases with decreasing vessel size. The reasons for this are currently unclear and are unlikely to be resolved until the EDHF-generating enzymes in each arterial bed have been characterized. There are at least three alternative explanations for the apparent predominance of EDHF-mediated relaxation in small arteries. The first is that these arteries have many more gap junctions than larger arteries. This hypothesis complements findings suggesting that myo-endothelial gap junctional communication plays a major role in EDHF-mediated relaxation of medium- and small-sized mesenteric arteries (see later). The second hypothesis states that EDHF-mediated relaxation is predominant in vessels in which contraction depends on the entry of Ca^{2+} into vascular smooth muscle cells through L-type Ca^{2+} channels. Indeed, the closure of L-type Ca^{2+} channels appears to be the major mechanism of EDHF-mediated relaxation. However, hyperpolarizing factors may also induce relaxation by additional mechanisms, probably including a reduction in the sensitivity of contractile elements to Ca^{2+}. The third, and most probable, explanation is that the endothelium of one vessel or vascular bed is not identical with that of another, and there are specific endothelial enzymes that are considered to be differentially expressed by the endothelium from one organ to the next. For example, the intracellular localization of eNOS differs in endothelial cells within the coronary system, as does the expression of cytochrome P-450 (CYP) enzymes.

Putative Nature of EDHF

Is EDHF a Transferable Humoral Factor or an Electrical Phenomenon Associated with Gap Junctional Communication?

The existence of a humoral hyperpolarizing factor synthesized by endothelial cells was originally proposed on the basis of an endothelium-dependent hyperpolarization of vascular smooth muscle cells in an arterial segment. However, it has proved difficult to substantiate the release of a diffusible hyperpolarizing factor using classic bioassay techniques to detect the transfer of a biological mediator between two separate vascular preparations in close opposition. Evidence that an EDHF can be transferred from one vessel to another has been obtained in patch clamp bioassay systems by monitoring the membrane potential of detector vascular smooth muscle cells situated downstream from, and electrically isolated from, donor endothelial cells. It should be emphasized that, although it is possible to bioassay a hyperpolarizing factor, there has been no convincing demonstration of a transfer of a hyperpolarizing and relaxing factor other than NO and PGI_2 to date. Thus although the factor released from porcine coronary artery segments, for example, does hyperpolarize vascular smooth muscle cells, it is not necessarily identical to the factor responsible for the NO/PGI_2-independent relaxations.

The vascular endothelium contains microscopically identifiable gap junctions that allow metabolic communication between cells and that are reportedly involved in the propagation of vasomotor signals along the vessel wall. There is evidence that, at least in small arteries, gap junctions exist between endothelial and smooth muscle cells—so-called myo-endothelial gap junctions. Such a direct pathway for cellular communication between endothelial and smooth muscle cells not only electrically couples these cells but also facilitates the rapid and direct transfer of signaling molecules such as inositol 1,4,5-trisphosphate, Ca^{2+}, and cyclic nucleotides.

Myo-endothelial gap junctions were initially described as unidirectional (i.e., dye transfer from smooth muscle to endothelial cells was marked, whereas there was little evidence to suggest the movement of dye in the opposite direction), but a bidirectional electrical transfer has since been demonstrated. There are also pronounced differences in data obtained in cultured cells and in intact arterial preparations, a fact most probably related to differences in the expression of connexin proteins *in situ* and *in vitro.*

The connexin proteins that form gap junctions have intracellular amino and carboxyl termini and four membrane spanning domains that form two extracellular loops and one intracellular loop. Connexins are assembled into hexamers or connexons somewhere between the endoplasmic reticulum (ER)-Golgi apparatus and the *trans*-Golgi network, prior to insertion into the plasma membrane. Connexons in one cell dock with connexons in adjacent cells through non-covalent interactions that involve the intracellular loops (Fig. 4). This arrangement facilitates the formation of an aqueous pore or channel (gap junction) which allows the passive intercellular exchange of small molecular mass molecules, ions, and second messengers ($<$1000 Da). The different connexin proteins build channels with unique functional properties, including differences in the size and charge of the molecules and ions that can transverse the channel. Channel gating can be modified by physiological parameters (such as pH and Ca^{2+}) and pharmacological agents (such as 12-*O*-tetradecanoylphorbol 13-acetate and retinoic acid).

The hypothesis that EDHF is, at least partially, a phenomenon which can be explained by electrical spreading through gap junctions may account for the inability of numerous investigators to assay the transfer of a factor which both hyperpolarizes and relaxes. Although other hypotheses have become more fashionable, the possibility that EDHF is in fact not a diffusible factor has remained in the background. The idea that gap junctional communication is involved in the manifestation of EDHF-mediated responses has recently been revived by observations showing that a number of compounds (such as 18α-glycyrrhetinic acid and heptanol) that uncouple gap junctions are able to attenuate EDHF-mediated relaxations (Fig. 5). Moreover, peptides raised against the extracellular loops of connexin 43 have been demonstrated to attenuate EDHF-mediated relaxations in the rabbit mesenteric artery. This process is thought to represent heterocellular coupling, as connexin 43 is rarely expressed in unstimulated native endothelial cells and is the major connexin in the endothelial–smooth muscle junction.

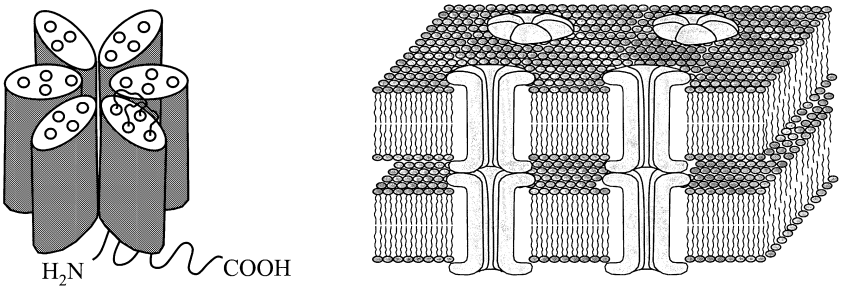


Figure 4 Model of a single connexon, consisting of six connexins (left), and a cluster of gap junctions (right).

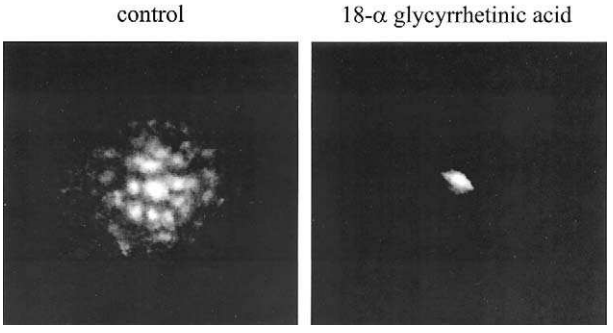


Figure 5 Fluorescent dye coupling between endothelial cells in a confluent monolayer is evident under basal conditions, demonstrating the existence of a continual gap junctional communication. Uncoupling agents such as 18α-glycyrrhetinic acid completely inhibit the cell-to-cell transfer of this dye. (Micrograph kindly provided by R. Brandes; reprinted by permission from Nature (399:601–605) © 1999 Macmillan Magazines Ltd.)

The possibility that EDHF is a substance that is capable of affecting gap junctional communication is not necessarily at odds with the observations made using bioassay models, as it is conceivable that this factor may spill over into the extracellular fluid.

Is NO an EDHF?

High concentrations of NO have been reported to directly activate Ca^{2+}-activated K^+ (K^+_{Ca}) channels and to induce smooth muscle hyperpolarization under certain experimental conditions. However, whether physiological concentrations of NO are able to induce the hyperpolarization of vascular smooth muscle cells, which is generally attributed to the actions of EDHF, was initially controversial. While an NO/PGI_2-mediated hyperpolarization can be evidenced in patch clamp experiments, a significant portion (<60%) of the hyperpolarization elicited by the intraluminal solution from bradykinin-stimulated porcine coronary segments is insensitive to combined NOS/COX blockade. This residual hyperpolarization is unaffected by either oxyhemoglobin or selective soluble guanylyl cyclase inhibitors, which abrogate NO-mediated dilation. Moreover, the production of EDHF is actually inhibited by basally produced NO, and only under conditions of impaired NO synthesis can a distinct EDHF-like response be demonstrated. The most damning evidence against the ''NO is an EDHF hypothesis'' is the fact that agonist-induced activation of the endothelium induces a COX inhibitor-insensitive, charybdotoxin/apamin-sensitive vasodilation of microvessels in eNOS knockout mice, that is, mice lacking the eNOS gene (Fig. 6).

These considerations, taken together with the pharmacological characterization discussed later, clearly indicate that the endothelium-dependent hyperpolarization observed in response to agonists and mechanical stimulation is elicited by a hyperpolarizing factor that is distinct from NO and PGI_2.

Is K^+ an EDHF?

Small increases in the concentration of extracellular K^+ (within the range 6–20 m*M*) are known to elicit vasorelaxation and hyperpolarization by activating an inwardly rectifying K^+ current [$I_{K(IR)}$] in smooth muscle cells. Although the K^+_{IR} channel is not ubiquitously expressed, it has been proposed that in some arteries K^+ may be an EDHF that effluxes from endothelial cells through K^+_{Ca} channels in response to agonist stimulation. The K^+ released in this manner would then accumulate in the myoendothelial space and activate both the K^+_{IR} and the Na^+/K^+-ATPase in smooth muscle cells, thereby inducing hyperpolarization (Fig. 7). Additional observations, showing that Ba^{2+} [which inhibits $I_{K(IR)}$] and ouabain (an inhibitor of the Na^+/K^+-ATPase) attenuate both K^+- and acetylcholine-induced hyperpolarizations of rat mesenteric and hepatic arteries, were taken as evidence for K^+ being an EDHF. The combination of Ba^{2+} and ouabain, however, only induced a slight shift in the concentration–relaxation curve of these rat mesenteric arteries to both K^+ and acetylcholine, suggesting that the majority of the NO/PGI_2-independent relaxation is mediated by a completely different mechanism that may not be accompanied with a smooth muscle hyperpolarization.

Is Anandamide an EDHF?

The endogenous cannabinoid anandamide was proposed to be an EDHF in rat mesenteric vessels. This hypothesis was based on the observation that a CB1 cannabinoid inverse agonist (a compound that inhibits the activity of a receptor which is basally active in unstimulated conditions),

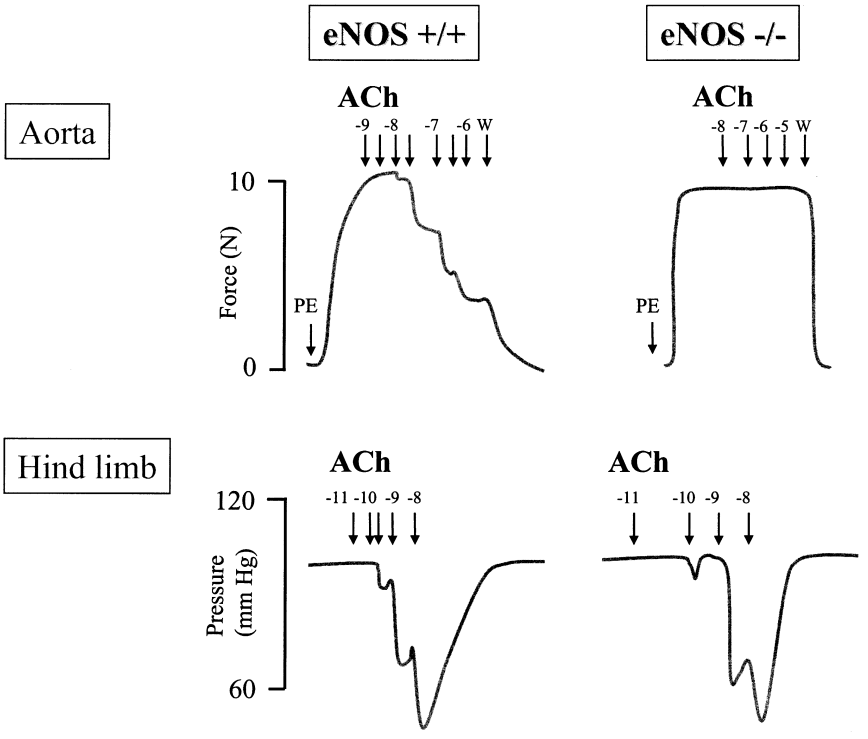


Figure 6 Tracings representing acetylcholine (ACh)-induced, endothelium-dependent relaxation of the aorta and vasodilation of hind limb arteries from control mice (eNOS +/+) and mice lacking the eNOS gene (eNOS −/−). Although in the aorta NO is the principal endothelium-derived relaxing factor and ACh cannot relax aortae from eNOS −/− mice, a comparable ACh-induced, endothelium-dependent vasodilation occurs in the hind limb of eNOS +/+ and eNOS −/− mice. (Tracings kindly provided by R. Brandes).

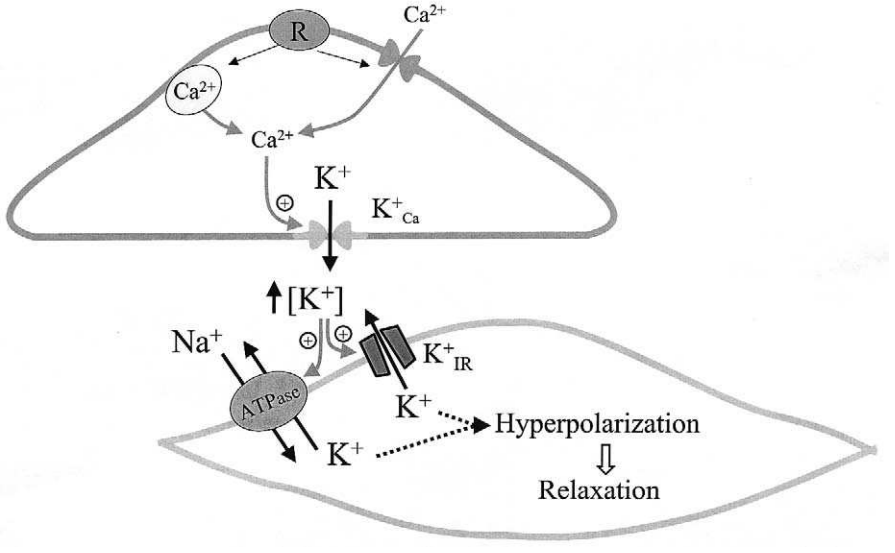


Figure 7 Scheme showing how K^+ has been proposed to act as an EDHF. An agonist stimulates its receptor (R) on an endothelial cell and elicits the release of Ca^{2+} from intracellular stores as well as the influx of Ca^{2+} from the extracellular space. The resulting increase in endothelial $[Ca^{2+}]_i$ activates K^+_{Ca} channels and thus K^+ efflux from the endothelial cell. $[K^+]$ in the myo-endothelial space increases and activates both the K^+_{IR} and the Na^+/K^+-ATPase in smooth muscle cells, thereby inducing hyperpolarization and relaxation (based on Edwards *et al.,* 1998).

attenuated relaxations mediated by ''authentic EDHF.'' However, although anandamide and additional CB1 agonists, such as Δ^9-tetrahydrocannabinol, induce the relaxation of a range of vessels by a COX-dependent mechanism, these compounds do not induce an EDHF-like (NOS/COX inhibitor-insensitive, K^+_{Ca} channel-mediated) relaxation. The observed effects of anandamide may be attributed to the generation of additional arachidonic metabolites, including EETs, as anandamide can be enzymatically synthesized from, and may well prove to be a carrier of arachidonic acid, the putative precursor of EDHF. More recently, the CB1 inverse agonist used to apparently inhibit the generation of EDHF was reported to be a potent inhibitor of gap junctional communication.

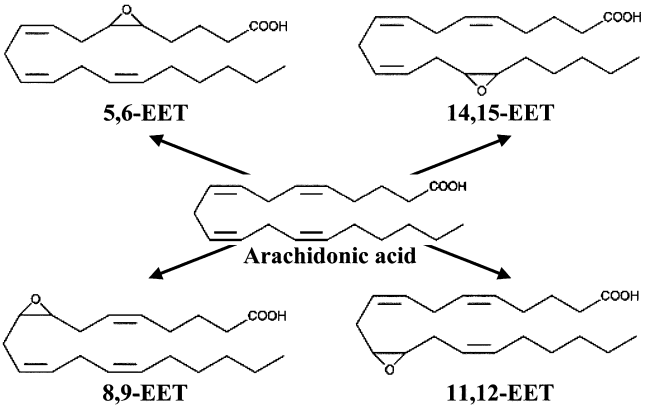


Figure 8 Structure of the epoxyeicosatrienoic acids (EETs), 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, synthesised from arachidonic acid by cytochrome P-450 (CYP) epoxygenases.

Is EDHF an Arachidonic Acid Metabolite?

The hyperpolarizing factor produced by coronary and renal arteries from humans, pigs, cows, dogs, rats, and rabbits displays characteristics similar to those of a cytochrome P-450 (CYP)-derived metabolite of arachidonic acid. This hypothesis was developed initially based on experimental observations showing that the application of exogenous arachidonic acid to isolated vascular segments induced relaxation. In addition, in many arteries EDHF-mediated responses were inhibited in the presence of compounds that inhibit phospholipase A_2 (PLA_2), the enzyme responsible for the liberation of arachidonic acid from membrane phospholipids. As EDHF and PGI_2 were known to be distinct, an arachidonic acid metabolizing enzyme distinct from COX was proposed to be responsible for the generation of EDHF. Therefore either a lipoxygenase or CYP product became a likely candidate.

That EDHF may be a CYP-dependent metabolite of arachidonic acid was implied on the basis of the fact that CYP inhibitors, such as clotrimazole, miconazole, and 17-octadecynoic acid, markedly attenuated EDHF-mediated hyperpolarization and relaxation in a number of vascular preparations. However, these conclusions were limited by the facts that these CYP inhibitors cannot discriminate between different CYP isoforms (over 300 isozymes have been reported), and can directly interfere with K^+ channels, which are the main cellular targets of EDHF. The CYP products proposed to be EDHFs were the epoxyeicosatrienoic acids (EETs) 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET (Fig. 8). Indeed, EETs are generated by endothelial cells and mediate part of the dilator effect of arachidonic acid. 11,12-EET and 5,6-EET induce K^+_{Ca} channel inhibitor-sensitive relaxations of endothelium-denuded arteries and activate large conductance K^+_{Ca} channels in native and cultured smooth muscle cells (Fig. 9).

It is only more recently that convincing evidence for the existence of a CYP-derived EDHF has been provided. Coronary endothelial cells have been found to express CYP epoxygenases (EET-producing CYP isozymes) including CYP 2C8, CYP 2C9, and CYP 2J2. Enhancing the expression of the CYP 2Cs using β-naphthoflavone enhances the synthesis of 11,12-EET by endothelial cells, as well as EDHF-mediated hyperpolarization and relaxation of intact arterial segments. Perhaps the most convincing evidence obtained to date that the EDHF synthase in porcine coronary arteries is a CYP enzyme has been provided using antisense oligonucleotides against the coding region of CYP 2C8. Incubation of porcine coronary arteries with antisense, but not sense or scrambled, oligonucleotides markedly attenuated bradykinin-induced, EDHF-mediated relaxation without affecting responsiveness to endogenously produced NO or an NO donor (Fig. 10). This inhibitory effect of antisense oligonucleotides provided the first nonpharmacological evidence that the EDHF produced by porcine coronary arteries is a CYP-dependent metabolite of arachidonic acid, or that a CYP metabolite is an essential permissive factor for EDHF-mediated vascular responses.

The reason for the caution in stating that the coronary EDHF is an EET is that it is currently unclear how endothelium-derived EETs are able to access smooth muscle cells to elicit hyperpolarization. EETs are by nature lipophilic, and simple diffusion from the endothelium to the media down a concentration gradient would be too slow to account for the rapidly initiated EDHF-mediated hyperpolarization and relaxation. One possibility, which would also link many of the observations made in different vessels, is that EETs are able to either pass through gap junctions or influence gap junctional communication of a second hyperpolarizing agent.

It must be stressed, however, that although the evidence that EDHF-mediated responses in the coronary and renal artery require the generation of an EET, several different EDHFs exist in different species. For example, the hyperpolarizing factors released from the human gastroepiploic artery, guinea pig carotid and rat mesenteric and hepatic arteries, or the rat portal vein exhibit pharmacological characteristics clearly distinct to those of the ''CYP-related EDHF.''

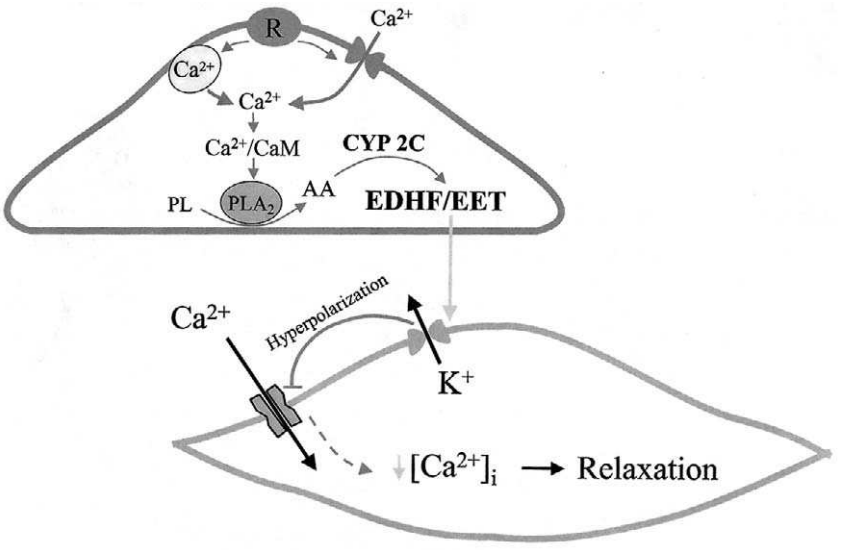


Figure 9 Scheme showing how epoxyeicosatrienoic acids (EETs) have been proposed to act as an EDHF. An agonist stimulates its receptor (R) on an endothelial cell and elicits the release of Ca^{2+} from intracellular stores as well as the influx of Ca^{2+} from the extracellular space. The resulting increase in endothelial $[Ca^{2+}]_i$ activates the phospholipase A_2 (PLA_2) which liberates arachidonic acid (AA), the substrate for the cytochrome P-450 2C (CYP 2C) isoform which is the EDHF synthase in coronary arteries. The EETs formed then activate K^+_{Ca} channels on vascular smooth muscle cells to induce hyperpolarization, inhibit voltage-dependent Ca^{2+} channels, and attenuate $[Ca^{2+}]_i$, thus inducing smooth muscle relaxation.

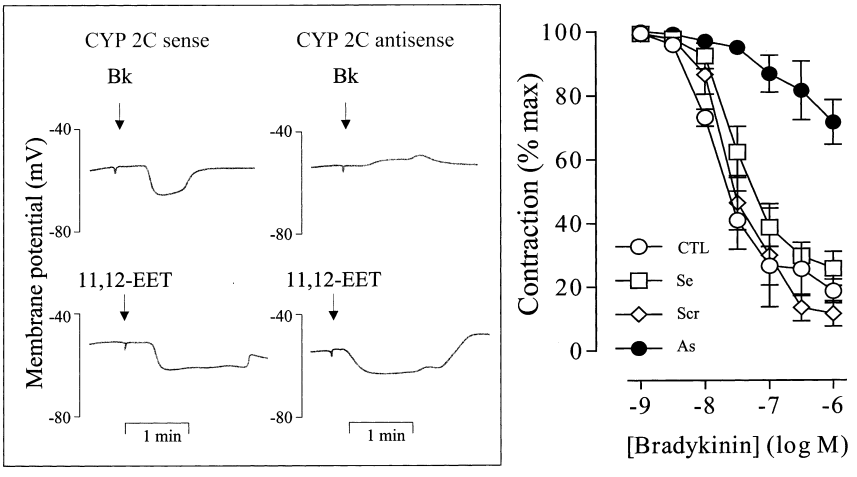


Figure 10 EDHF-mediated hyperpolarization and relaxation of the porcine coronary artery are inhibited by decreasing cytochrome P-450 2C (CYP 2C) protein using antisense oligonucleotides. (Left) Tracings represent the bradykinin-induced, EDHF-mediated hyperpolarization of artery smooth muscle cells following incubation with either CYP 2C sense oligonucleotides or CYP 2C antisense oligonucleotides. The antisense treatment selectively inhibits the generation of EDHF and does not affect the hyperpolarization produced by 11,12-epoxyeicosatrienoic acid (11,12-EET), a putative EDHF and CYP 2C product. (Right) Concentration–relaxation curves to bradykinin obtained using rings of porcine coronary artery incubated in the absence (CTL) and presence of either sense (Se), scrambled (Scr), or antisense (As) CYP 2C oligonucleotides. Only the CYP 2C As oligonucleotides attenuated EDHF-mediated relaxation. To be attributable to EDHF, hyperpolarization and relaxation were assessed under conditions of combined NOS/COX blockade (modified from Fisslthaler *et al.,* 1999).

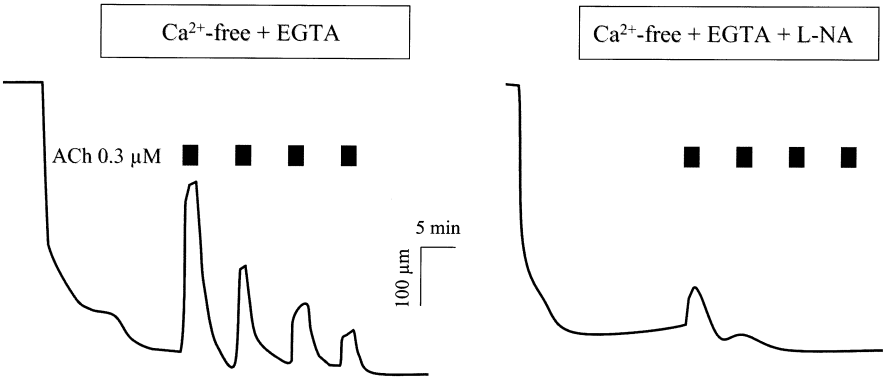


Figure 11 The generation of EDHF is Ca^{2+} dependent, and the production of EDHF is more sensitive than that of NO to the removal of extracellular Ca^{2+}. The tracings illustrate acetylcholine (ACh)-induced dilation of the rabbit carotid artery following the removal of extracellular Ca^{2+} in the continuous presence of a COX inhibitor and in the absence (left) and presence (right) of a NOS inhibitor (L-NA, N^{ω}-nitro-L-arginine).

SUBCELLULAR LOCALIZATION OF THE CYP-LIKE EDHF SYNTHASE

Whereas the intracellular site of EDHF synthesis is likely to have a number of implications on its sphere of influence and thus biological activity, the cellular distribution of the EDHF-generating enzyme and the relationship between localization and enzymatic activity are unclear. Although CYP enzymes are synthesized on polyribosomes bound to the endoplasmic reticulum and are cotransitionally inserted into the endoplasmic reticulum membrane, their exact localization is thought to be isoform specific. There is an extensive flow of vesicles to the Golgi apparatus as well as a microtubule-dependent transport from the Golgi to the plasma membrane, and at least one CYP enzyme, CYP 2B, is thought to traffic between the Golgi apparatus and the plasma membrane. Although the subcellular localization of the endothelially expressed CYP epoxygenases that could generate EDHF (CYP 2C8/9 or CYP 2J2) remains to be elucidated, the fungal toxin brefeldin A, which disrupts the Golgi apparatus and Golgi–plasma membrane trafficking, inhibits the production of the CYP-dependent EDHF.

It is only possible to speculate on the mechanism by which brefeldin A inhibits EDHF synthesis. This mechanism, however, does not appear to be related to a decreased availability of arachidonic acid, as the lack of effect of brefeldin A on PGI_2 formation suggests that the liberation of arachidonic acid is not inhibited. Brefeldin A treatment does not result in the global inhibition of CYP activity, since this agent did not affect the ability of endothelial cells to dealkylate the substrate 7-ethoxyresorufin. Rather, the effects of brefeldin A on the trafficking of proteins between the endoplasmic reticulum, Golgi apparatus, and the plasma membrane may be responsible for the observed inhibition of EDHF release.

Signal Transduction Leading to Activation of the EDHF Synthase: The CYP-Related EDHF Synthase

Ca^{2+} Dependency

Given that the exact chemical identity of EDHF remains to be elucidated, it is difficult to investigate the signal transduction cascade that results in the activation of the ''EDHF synthase.'' Current opinion is that the synthesis of EDHF is secondary to the activation of PLA_2 and to the liberation of arachidonic acid. Indeed, the synthesis of EDHF is thought to be Ca^{2+} dependent by virtue of the reported Ca^{2+} dependency of PLA_2. Thereafter, the signaling pathway is obscure, and although EDHF may be generated from arachidonic acid by CYP enzymes in some vascular beds, this is almost certainly not the case in others.

The Ca^{2+} dependency of EDHF production has been demonstrated in a number of experiments in which a maintained, extracellular Ca^{2+}-dependent hyperpolarization was demonstrated in response to the Ca^{2+} ionophore. The Ca^{2+} dependency of EDHF-mediated relaxations can be demonstrated by monitoring the vasodilator responsiveness to the repetitive administration of acetylcholine in the presence and absence of extracellular Ca^{2+} and NOS/COX inhibitors (Fig. 11). Such experiments reveal that the EDHF response is rapidly abolished following Ca^{2+} removal, whereas acetylcholine-induced NO production is more resistant. These observations are consistent with biochemical data reporting that the threshold requirement for Ca^{2+} is lower for eNOS than for PLA_2.

The formation of a Ca^{2+}/calmodulin complex has also been proposed to be involved in the activation of the EDHF synthase as the calmodulin antagonist calmidazolium inhibits the EDHF-mediated hyperpolarization of smooth muscle

cells as well as EDHF-mediated relaxation in porcine and canine coronary arteries (Fig. 12).

Stimuli Resulting in the Production of EDHF

As EDHF-mediated hyperpolarization and relaxation is Ca^{2+} dependent, it is clear that the stimuli required to elicit an increase in the production of EDHF requires an increase in endothelial $[Ca^{2+}]_i$. The agonists generally used to elicit the synthesis of EDHF and to characterize its actions, for example, bradykinin, acetylcholine, and Ca^{2+} ionophores, induce marked changes in $[Ca^{2+}]_i$. Although some of these receptor-dependent agonists can be detected in the vicinity of the endothelium *in situ,* this generally occurs in proinflammatory situations. The physiologically relevant stimuli for the continuous generation of the endothelium-derived autacoids and the moment-to-moment regulation of local vascular tone are mechanical forces such as fluid shear stress and pulsatile stretch. Besides the continuously acting fluid shear stress, which is generated by blood flowing over the endothelial cell surface, the periodic distension and compression of arteries during the cardiac cycle (most prominent in coronary arteries), which is a direct consequence of the pumping action of the heart and the periodic ejection of blood into the arterial system, is known to enhance the formation of NO and PGI_2.

The lack of a selective inhibitor of EDHF production has hampered the study of the physiological stimuli regulating the production of EDHF, but pulsatile changes in transmural pressure do result in a NOS/COX inhibitor-insensitive, K^+_{Ca} channel inhibitor-sensitive increase in the compliance (distensibility) of coronary arteries, indicating that an EDHF mediates this response. It is highly plausible that the profile of EDHF release observed following the initiation of rhythmic vessel distension is determined by mechanically stimulated increases in the endothelial $[Ca^{2+}]_i$, and that peak and steady-state levels of EDHF production mirror endothelial Ca^{2+} levels.

It is difficult to state whether or not changes in fluid shear stress are able to alter the generation of EDHF, as, at least in cultured endothelial cells, shear stress does not always elicit an increase in endothelial $[Ca^{2+}]_i$ that is large enough to activate PLA_2 and liberate the putative EDHF precursor, arachidonic acid. It is possible, however, that shear stress induces more pronounced alterations or oscillations in $[Ca^{2+}]_i$ in endothelial cells *in situ.* Indeed, spontaneous transient outward K^+ currents have been reported in shear stress-stimulated endothelial cells, and the combination of charybdotoxin and apamin significantly inhibited the EDHF-mediated component of the shear stress-induced relaxation of rat resistance mesenteric arteries.

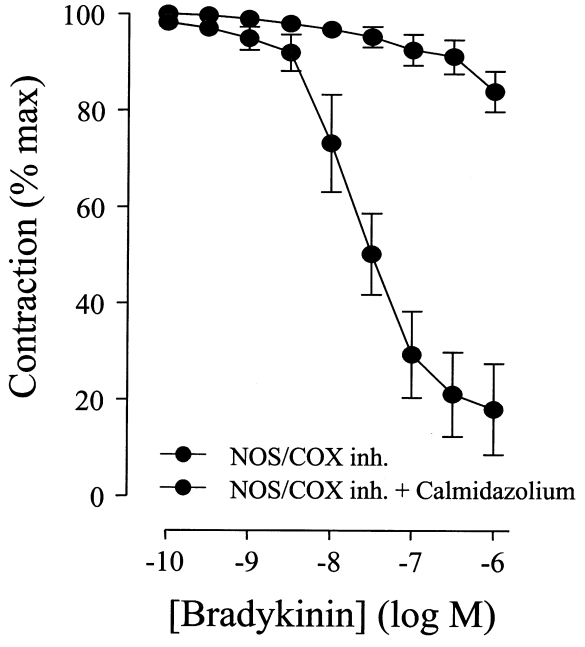


Figure 12 EDHF-mediated relaxation of porcine coronary arteries is inhibited by the calmodulin antagonist, calmidazolium, consistent with the concept that an increase in endothelial $[Ca^{2+}]_i$ and the formation of a Ca^{2+}/CaM complex is a prerequisite for the activation of the EDHF synthase.

Actions of the CYP-Dependent EDHF

Preformed Pools of EET

One distinctive feature of the EETs is their ability to become esterified to several glycerophospholipids, especially to phosphatidylcholine and phosphatidylethanolamine. This process involves the epoxidation of arachidonic acid (to generate the EET), ATP-dependent activation to the corresponding EET-CoA derivatives, and EET enantiomer-selective lysolipid acylation. Among the eicosanoids, the esterification of EETs is unique and may well be an indication that an intracellular pool of preformed EETs could exist within certain cells. Although such an EET processing has been proposed to exist in endothelial cells, the eventual effect on biological function is unclear. The biosynthesis of EET-containing cellular phospholipids may provide the molecular basis for some of the biological actions attributed to the EETs. Many of these biological activities can be interpreted in terms of the ability of the EETs to become incorporated into cellular lipids and consequently to alter cell membrane permeability and enzyme activity.

K^+_{Ca} Activation

EDHF and EETs are reported to activate K^+_{Ca} in numerous vascular preparations as well as in cultured vascular smooth muscle and endothelial cells. In smooth muscle cells, hyperpolarization is brought about by an increase in the conductance of K^+ and is abolished in the presence of depolarizing concentrations of KC1. As mentioned previously, inhibitors of K^+_{Ca} channels, especially the combination of charybdotoxin/apamin, virtually abolish the EDHF-mediated

hyperpolarization and relaxation, whereas glibenclamide, an inhibitor of K^+_{ATP}, usually has no inhibitory effect. Patch clamp experiments performed in the cell-attached mode, that is, when the channel under investigation is shielded from the extracellular medium by the pipette, have shown that EDHF is still able to activate K^+_{Ca} channels. Thus it would appear that this factor activates K^+ channels in an indirect manner, possibly involving membrane-associated second messengers. Indeed, EETs activate K^+_{Ca} channels in coronary smooth muscle cells via a $G\alpha_s$-mediated mechanism (a phenomenon observed only in the presence of added GTP or GTP and ATP) and may alter the sensitivity of these channels to Ca^{2+} (Fig. 13). The activation of protein kinase A by 11,12-EET is reported to be an important mechanism responsible for afferent arteriole vasodilation, and there is evidence suggesting that K^+_{Ca} channels are modulated by cyclic AMP and protein kinase A.

20-Hydroxyeicosatetraenoic acid (20-HETE), an ω-hydroxylation product of arachidonic acid catalyzed by the ω-hydroxylase (CYP4A4), has been proposed to be involved in the development of myogenic tone. 20-HETE is endogenously produced by smooth muscle cells from small renal and cerebral vessels and, once formed, increases smooth muscle tone by inhibiting large conductance K^+_{Ca} channels, inducing depolarization and increasing $[Ca^{2+}]_i$, probably by activating L-type Ca^{2+} channels (Fig. 14). It is tempting to speculate that the open probability of large conductance K^+_{Ca} channels in renal arteries is determined by the balance in the vascular production of 20-HETE and EDHF/NO, and that EDHF and NO affect vascular tone by counteracting the 20-HETE-induced inhibition of K^+_{Ca} channels.

EDHF-Mediated Regulation of $[Ca^{2+}]_i$ in Vascular Cells

The signal transduction cascade initiated following activation of endothelial cells by receptor-dependent agonists, such as bradykinin, is associated with an elevation in $[Ca^{2+}]_i$, characterized by a transient peak followed by a steady or oscillating plateau phase. The initial component reflects, at least in part, the inositol trisphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores, whereas the second phase is characterized by a more prolonged transmembraneous Ca^{2+} influx. These two components of the Ca^{2+} response are related in such a manner that the degree of intracellular store depletion determines the extent of the subsequent transmembraneous or "capacitative" Ca^{2+} influx. The essence of this model of Ca^{2+} entry is that the information about the IP_3-mediated

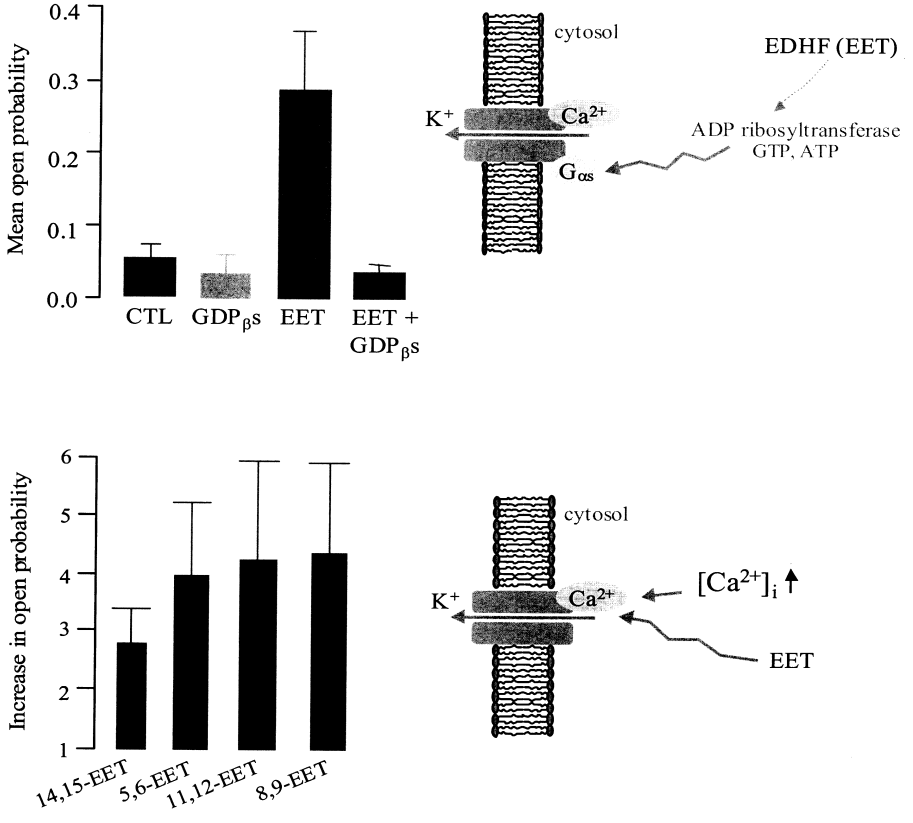


Figure 13 EDHF and EETs increase the open probability of K^+_{Ca} channels in vascular cells by two mechanisms. The top panel shows that EETs activate K^+_{Ca} channels via a $G_{\alpha s}$ protein, possibly as a result of its ADP ribosylation, as the effect of the EET is prevented in the presence of the G-protein inhibitor GTPβS. The lower panel indicates that although K^+_{Ca} channels are activated by an increase in cytosolic Ca^{2+}, EETs appear to enhance channel open probability at basal Ca^{2+} levels (modified from Li and Campbell, 1997, and Baron *et al.,* 1997).

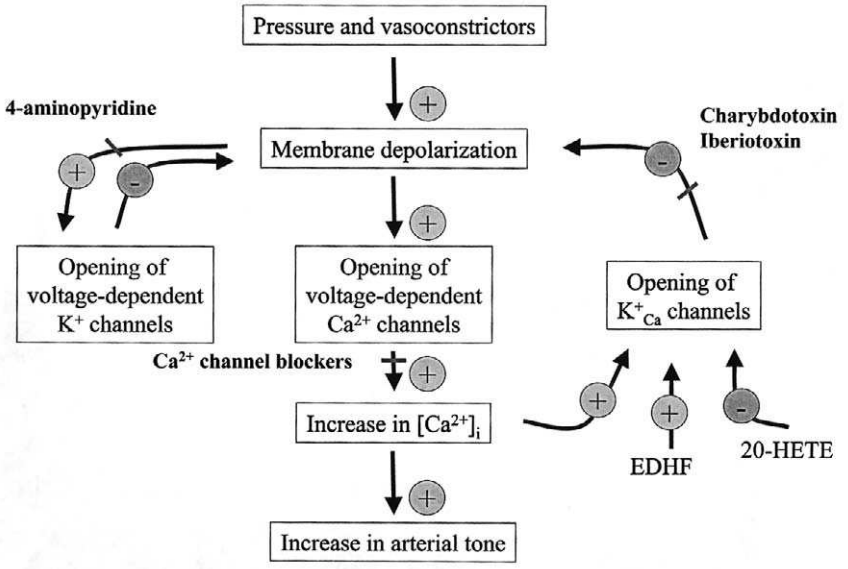


Figure 14 Mechanisms involved in the regulation of K^+_{Ca} channels and vascular tone. The open probability of large conductance K^+_{Ca} channels is determined by the balance of 20-hydroxyeicosatetraenoic acid (20-HETE), and EDHF.

emptying of intracellular Ca^{2+} stores is transferred to the plasma membrane by one or more diffusible signal molecules that facilitate the activation of a pathway for store replenishment from the extracellular space. In principle, there are two ways by which an agonist can induce capacitative Ca^{2+} entry. The activation of the transmembraneous Ca^{2+} influx pathway could be attributed to an indirect effect, that is, instigation of capacitative Ca^{2+} entry following the mobilization of intracellularly stored Ca^{2+}, or a direct effect, that is, activation of a Ca^{2+} influx regulatory signaling molecule/protein.

Activation of CYP epoxygenases, the formation of EETs (especially 5,6-EET), and the subsequent tyrosine phosphorylation of cellular proteins may play a crucial role in regulating capacitative Ca^{2+} entry into endothelial cells. Indeed, endothelial CYP epoxygenases are activated by Ca^{2+} store depletion to produce products that hyperpolarize endothelial cells, and a number of CYP inhibitors and tyrosine kinase inhibitors selectively attenuate transmembraneous Ca^{2+} influx following agonist stimulation. Moreover, the induction of some CYP isoforms with dexamethasone and clofibrate enhances CYP activity, agonist-induced membrane hyperpolarization, and capacitative Ca^{2+} entry. There may also be a role for superoxide (O_2^-), which is a by-product of CYP activation, in endothelial Ca^{2+} signaling.

14,15-EET is also reported to enhance Ca^{2+} influx, but in this case into vascular smooth muscle cells, by activating voltage-dependent L-type Ca^{2+} channels. At first sight this observation completely contradicts the previously mentioned point that EETs inhibit L-type channels by inducing membrane hyperpolarization. However, the vascular smooth muscle response to the application of 14,15-EET is very much dependent on the state of contraction. Thus, an EET-induced contraction can be shown in segments maintained under a passive tension, while only hyperpolarization and relaxation are detectable in precontracted vascular segments.

Activation of Protein Kinases by EDHF

By analogy with NO, which is a potent modulator of vascular gene expression, it is conceivable that the action of EDHF extends beyond its function as a vasodilator and is involved in the regulation of vascular cell proliferation.

CYP and lipoxygenase metabolites of arachidonic acid, including EETs, 20-hydroxyeicosatetraenoic acid (20-HETE), and 12(S)-HETE, activate the extracellular regulated protein kinases 1 and 2 (Erk1/2) and increase [^{3}H]thymidine incorporation into various cell types, including vascular smooth muscle cells and endothelial cells. There is also evidence to suggest that the overexpression of the coronary EDHF synthase, CYP 2C8/9, in endothelial cells enhances cell proliferation. The precise steps between the activation of the EDHF synthase/CYP 2C8 and the initiation of cell proliferation remain to be elucidated, but the activation of Erk1/2 is more than likely to be a key step in this process, as the phosphorylation and activation of Erk1/2 are generally accepted to be essential for both migration and proliferation of vascular cells. 11,12-EET has also been reported to exert an anti-inflammatory effect in endothelial cells by inhibiting the activation of the transcription factor, nuclear factor-κB (NF-κB) and decreasing the cytokine-induced expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). Overexpression of CYP 2J2, another EET-generating epoxygenase, into bovine endothelial cells also attenuates the tumor necrosis factor α (TNF-α)-induced activation of NF-κB, although to a lesser extent than the exogenously applied EET. Such a difference may be accounted

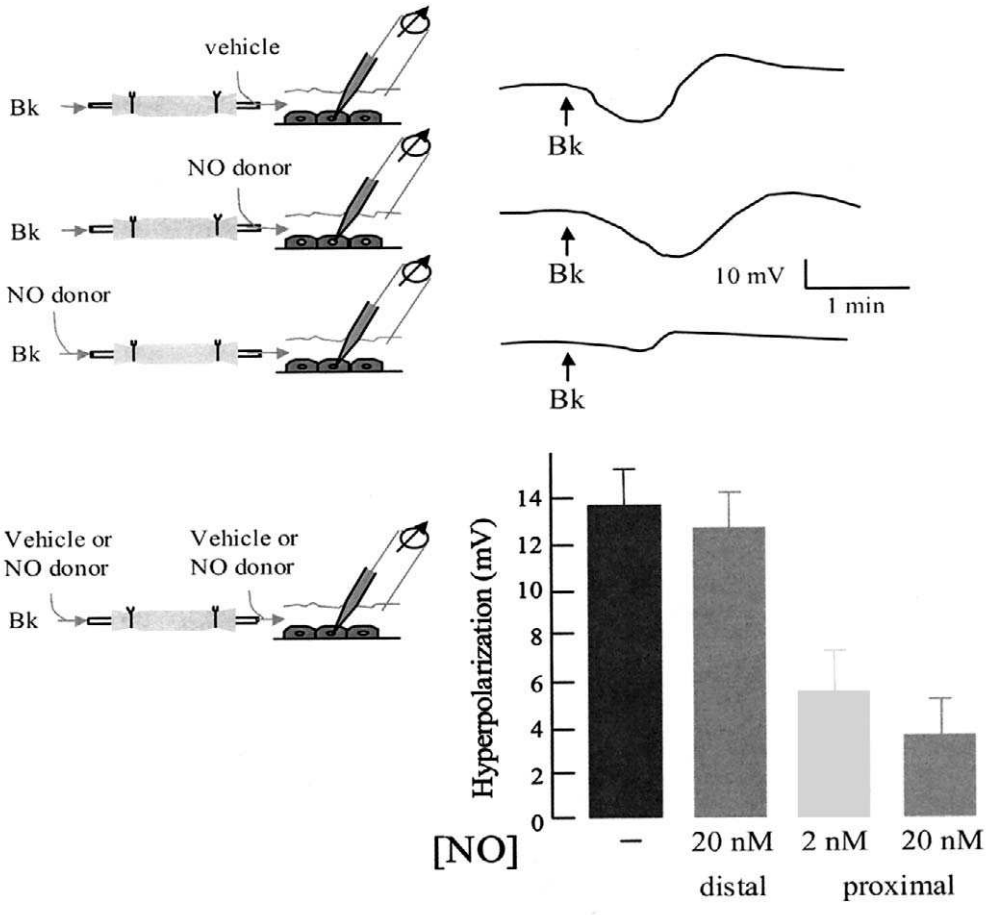


Figure 15 NO attenuates the synthesis of the CYP-dependent EDHF in a patch clamp bioassay, in which the generation of EDHF by a bradykinin (Bk)-stimulated artery is assessed as hyperpolarization of cultured vascular smooth muscle cells. (Top) As NO only inhibited EDHF-induced hyperpolarization when perfused with bradykinin through the donor artery, NO attenuates the production of EDHF rather than interfering with its ability to activate K^+_{Ca} channels on smooth muscle cells. (Bottom) The concentrations of NO that interfere with the generation of EDHF are very low and are equivalent to those produced by endothelial cells.

for by the fact that, in addition to EETs, CYP epoxygenases generate oxygen-derived free radicals, which also have marked effects on the activation of redox-sensitive transcription factors, including NF-κB. Since there is a growing body of evidence to suggest that reactive oxygen intermediates can influence cell growth, it is feasible that some of the effects of CYP 2C overexpression can be attributed to the generation of CYP-derived free radicals.

Interactions between NO and EDHF

The interference of one endothelial autacoid with the synthesis of another is difficult to study, since a pure EDHF-mediated response can be observed only in the combined presence of NOS and COX inhibitors and, until relatively recently, no selective inhibitor of an EDHF synthase had been described. Given that EDHF-mediated responses are most prominent after NOS inhibition, it seemed logical to propose that the continuous production of physiological concentrations of NO by endothelial cells could dampen the generation of EDHF and/or the activity of the EDHF synthase. Once again, since only the coronary EDHF synthase has been adequately characterized, the following discussion must be restricted to the CYP-like EDHF synthase.

NO attenuates the synthesis of the CYP-dependent EDHF, rather than interfering with its ability to activate K^+_{Ca} channels and elicit hyperpolarization. The EDHF-mediated hyperpolarization and relaxation of coronary arteries can be inhibited by very low concentrations of NO donor agents, which have been demonstrated to generate concentrations of NO similar to that produced by the stimulated endothelium (Fig. 15). Moreover, CYP activity in isolated microsomes and in endothelial cells can be abolished by NO donors. Several potential mechanisms have been proposed to account for the NO-induced inactivation of CYP enzymes, such as the liberation of heme from CYP proteins, inhibition of CYP protein synthesis, the reaction of NO with superoxide anions to form peroxynitrite (which in turn affects the nitration of critical amino acid residues), and the binding of NO to the heme moiety of CYP, forming nitrosyl–heme adducts, with subsequent oxidation of free thiols in CYP enzymes. In addition, since NO interacts with COX to enhance

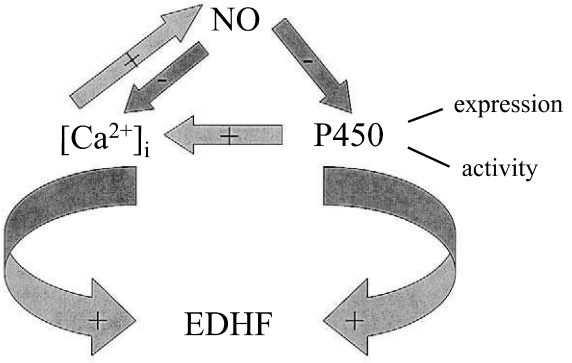


Figure 16 Potential interactions between the CYP-like EDHF synthase/CYP-dependent EDHF and NO.

PGI_2 synthesis, NO may attenuate any basal EDHF production by channeling free arachidonic acid into prostanoid synthesis. A combination of several of the above mechanisms probably accounts for the NO-mediated inhibition of CYP-dependent EDHF production (Fig. 16).

On the whole, there is much evidence that the production of EDHF is attenuated by NO under physiological conditions and that this tonic inhibition is alleviated when NO synthesis is impaired so that EDHF can act as a back-up vasodilator system. A number of observations support this concept, including the finding that the generation of EET is markedly enhanced following the attenuation of bioavailable endothelium-derived NO and the induction of hypertension by salt loading. Also, the concentration CYP products produced by the vasculature is greater following endothelial injury in a model of coronary artery stenosis than in normal vessels. At this point it is necessary to stress that while NO can affect the production of the coronary type (CYP-dependent) EDHF, this is not necessarily the case for CYP-independent EDHFs, such as those detectable in the rat hepatic artery.

Although an EDHF can be detected in the perfused heart, mesentery, and hind limb of mice, it is currently unclear whether or not this factor is related to a CYP-dependent metabolite of arachidonic acid. Thus any investigation into the interaction between EDHF and NO in mice lacking the eNOS gene must necessarily await the detailed characterization of the murine EDHF(s).

Additional Reading

Baron, A., Frieden, M., and Bény, J.-L. (1997). Epoxyeicosatrienoic acids activate a high-conductance, Ca^{2+}-dependent K^+ channel on pig coronary artery endothelial cells. *J. Physiol.* **504.3,** 537–543.

Bolz, S. S., de Wit, C., and Pohl, U. (1999). Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca^{2+} during acetylcholine-induced dilation of microvessels. *Br. J. Pharmacol.* **128,** 124–134.

Campbell, W. B., Gebremedhin, D., Pratt, P. F., and Harder, D. R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ. Res.* **78,** 415–423.

Capdevila, J. H., Zeldin, D. C., Makita, K., Karara, A., and Flack, J. R. (1995). Cytochrome P450 and the metabolism of arachidonic acid and oxygenated eisocanoids. *In* ''Cytochrome P450: Structure, Mechanism, and Biochemistry'' (P. R. Ortiz de Montellano, ed.), 2nd Ed., pp. 443–472. Plenum, New York.

Edwards, G., Dora, K. A., Gardener, M. J., Garland, C. J., and Weston, A. H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* **396,** 269–272.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., and Busse, R. (1999). Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* **401,** 493–497.

Li, P. L., and Campbell, W. B. (1997). Epoxyeicosatrienoic acids activate K^+ channels in coronary smooth muscle through a guanine nucleotide binding protein. *Circ. Res.* **80,** 877–884.

Popp, R., Bauersachs, J., Hecker, M., Fleming, I., and Busse, R. (1996). A transferable, β-naphthoflavone-inducible, hyperpolarizing factor is synthesised by native and cultured porcine coronary endothelial cells. *J. Physiol.* **497.3,** 699–709.

Quilley, J., Fulton, D., and McGiff, J. C. (1997) Hyperpolarizing factors. *Biochem. Pharmacol.* **54,** 1059–1070.

Vanhoutte, P. M. (ed.) (1999). ''Endothelium-Dependent Hyperpolarizations.'' Harwood, Amsterdam.

Weintraub, N. L., Fang, X., Kaduce, T. L., VanRollins, M., Chatterjee, P., and Spector, A. A. (1997). Potentiation of endothelium-dependent relaxation by epoxyeicosatrienoic acids. *Circ. Res.* **81,** 258–267.

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CHAPTER 36

Role of Nitric Oxide in Myocardial Function

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OVER THE PAST TWO DECADES, THE ROLE OF NITRIC OXIDE (NO) AND RELATED CONGENERS IN CARDIOVASCULAR PHARMACOLOGY HAVE LARGELY BEEN ELUCIDATED. IN THIS CHAPTER, WE FOCUS ON THE EXTRAVASCULAR PRODUCTION OF NO IN THE CONTROL OF CARDIAC CONTRACTILE FUNCTION. CARDIAC MUSCLE CELLS EXPRESS THE NO SYNTHASE ISOFORM ORIGINALLY DESCRIBED IN ENDOTHELIAL CELLS (ENOS OR NOS3), WHICH IS COUPLED TO BOTH β-ADRENERGIC AND M2 CHOLINERGIC RECEPTORS IN CARDIAC MYOCYTES, INCLUDING SPECIALIZED PACEMAKER AND CONDUCTION CELLS WITHIN THE HEART. THE MOLECULAR PHARMACOLOGY OF NO GENERATION IN CONTROL OF MYOCARDIAL CONTRACTILE FUNCTION BY THE AUTONOMIC NERVOUS SYSTEM IS DESCRIBED IN DETAIL, AS IS THE TARGETING OF ENOS TO CAVEOLAR MICRODOMAINS AND THE INTERACTIONS OF ENOS WITH CAVEOLIN-3. EVIDENCE POINTING TO THE PRESENCE OF ANOTHER NOS ISOFORM, POSSIBLY NNOS (NOS1), IN THE SARCOPLASMIC RETICULUM OF CARDIAC MYOCYTES, IS BRIEFLY REVIEWED. AS IN ALL TISSUES IN RESPONSE TO INFECTION, MANY CELL TYPES WITHIN THE MYOCARDIUM CAN EXPRESS INOS (NOS2), AND THE IMPLICATIONS OF A ROBUST GENERATION OF NO BY INOS IN RESPONSE TO SYSTEMIC SEPSIS OR LOCALIZED INFECTION ARE DESCRIBED. FINALLY, THE IMPLICATIONS OF THE OBSERVATION THAT INOS IS ALSO EXPRESSED WITHIN THE INJURED OR FAILING MYOCARDIUM, IN THE ABSENCE OF EVIDENCE OF FOREIGN PATHOGENS, ARE CONSIDERED IN DETAIL.

Introduction

Autonomic neurotransmitters and hormones physiologically regulate the force and frequency of myocardial contraction. Norepinephrine and epinephrine, released by sympathetic nerves in the heart and by adrenal glands into the circulation, respectively, activate both α- and β-adrenergic receptors in cardiac muscle to increase myocardial contractility. Acetylcholine (ACh) released from parasympathetic nerves reduces contractility by binding to muscarinic cholinergic receptors, thereby opposing the action of catecholamines (Fig. 1). The proposal that nitric oxide (NO) produced endogenously within cardiac muscle regulates these two autonomic nervous systems contributes a new dimension to our understanding of the molecular mechanism of action of neurotransmitters in the heart. It also offers potential new therapeutic approaches for disease states, such as heart failure in which autonomic nervous system function is abnormal.

We will briefly review the molecular pathways leading to the intracellular actions of β-adrenergic and muscarinic cholinergic agonists in the activation of endothelial nitric oxide synthase (eNOS), as well as cytokine activation of inducible NOS (iNOS), in the cardiac myocyte, integrating evidence that demonstrates that NO-dependent signaling can be shown both at the level of the isolated myocyte and in the intact animal.

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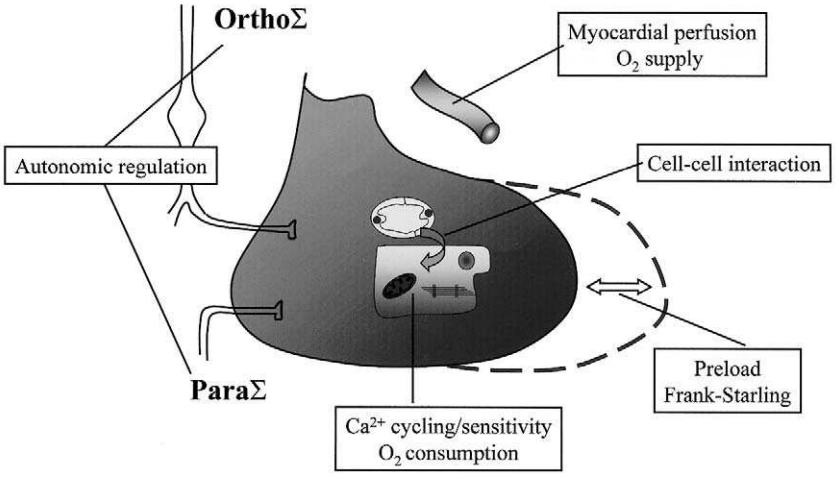


Figure 1 Physiological regulation of cardiac contraction.

The β-Adrenergic Pathway

β-Adrenergic agonists increase both the force and frequency of contraction and the rate of relaxation of cardiac muscle. Despite some differences between regions of the heart, the immediate increase in the force of contraction is generally related to an increase in transmembrane flux of calcium through phosphorylation of the L-type Ca channel followed by the calcium-induced calcium release from the sarcoplasmic reticulum. The sustained inotropic effect during subsequent contractions results from the increased influx and mobilization of internal calcium resulting from an increased replenishing of calcium in the sarcoplasmic reticulum (Gibbons, 1986; Morad and Cleeman, 1987).

A hallmark of the β-adrenergic effect on the heart is to increase the rate of relaxation as well as increasing the force of contraction. This involves more rapid termination of calcium influx, increased rate of calcium uptake and sequestration into the sarcoplasmic reticulum following phospholamban phosphorylation (Katz, 1977, 1979), increased expulsion of intracellular calcium via sodium/calcium exchange (Sheu and Blaustein, 1986), and desensitization of contractile myofilaments to calcium (Stull, 1980; Robertson *et al.,* 1982).

β-Adrenergic Receptors in the Heart

Although binding studies with selective ligands have identified two subtypes of β-adrenergic receptors in the heart (β_1 and β_2), the β_1 subtype usually predominates in the mammalian heart. In the rat heart, the percentage of β_1 subtype is 85%. The ratio of β_1 and β_2 differs in different regions of the human heart; for example, the percentage of β_1 is higher in ventricle than in atrium (Buxton *et al.,* 1987). Evidence was provided for the expression of β_3 adrenoreceptors (at the mRNA level) in biopsy specimens from human ventricles. Activation of this receptor with specific agonists (in the presence of β_1 and β_2 blockade) produced a unique negatively inotropic effect on the contraction of human myocardium (Gauthier *et al.,* 1996).

The Cyclic AMP Intracellular Pathway

Stimulation of β_1- and β_2-adrenergic receptors increases cyclic AMP (cAMP) synthesis by stimulating the catalytic activity of adenylyl cyclase through a process involving GTP-binding regulatory proteins containing the α_s subtype. Adenylyl cyclase belongs to a family of enzymes that convert ATP to cyclic AMP that activates several target molecules; for example, cyclic AMP-dependent protein kinases regulate the activity of numerous downstream proteins. Although adenylyl cyclase isoforms are widely distributed, the only isoforms that have been definitively identified in ventricular heart tissue are types V and VI. As with all the other isoforms, these two types are also stimulated by $G\alpha_s$ but are unaffected by either βγ subunits or calcium/calmodulin unlike other adenylyl cyclase isoforms (Ishikawa and Homcy, 1997).

Like other isoforms, type V and VI adenylyl cyclases are inhibited by G_i-linked receptors. In addition, type VI adenylyl cyclase can be at least as effectively inhibited by elevations in intracellular calcium, providing a mechanism for the cross talk between calcium- and cyclic AMP-dependent signaling pathways, both important regulators of cardiac contractility.

Cyclic AMP Phosphodiesterases

The levels of cyclic AMP are tightly regulated by a complex family of different phosphodiesterases (PDEs) widely distributed among various tissues, including the heart. Among the PDEs identified to date, members of at least seven families, PDE I, II, and III have been shown to be expressed in heart tissue. PDE I is regulated by calcium/calmodulin. PDE II contains a noncatalytic binding site with a high specificity for cyclic GMP. When cyclic GMP binds to this site, the affinity of the catalytic site is increased by allosteric interac-

tion. PDE II is therefore known as a cyclic GMP-stimulated phosphodiesterase. PDE II isoforms have been generally found to be expressed in tissues in which the effects of cyclic GMP are opposite to those of cyclic AMP. The expression of an isoform of PDE II in cardiac myocytes from several species, including rat (Bode *et al.,* 1991; Han *et al.,* 1998a), is an illustration of this principle.

In contrast, PDE III isoforms, also expressed in cardiac myocytes, are inhibited by cyclic GMP. In the heart, such inhibition by cyclic GMP would initially potentiate the increase in intracellular cyclic AMP with a resulting positive inotropic effect through an increase in L-type calcium current. The net result of an increase in cyclic GMP in heart muscle cells, however, is more difficult to predict given the coexpression of both PDE II and PDE III isoforms in the same myocytes (at least in the rat) (Bode *et al.,* 1991). The factors that determine whether there will be a potentiation or an attenuation of the effect of cAMP in heart muscle cells include the relative differences in affinity and V_{max} for this cyclic nucleotide between the two isoforms, and perhaps the subcellular localization of each PDE isoform, along with effector proteins for cAMP in the same subcellular compartment.

The Muscarinic Cholinergic Pathway

Molecular cloning has identified five muscarinic receptor subtypes named m1–m5 based on the order of their discovery (Bonner, 1989). Previous studies of the functional coupling of heterologously expressed receptors led to a distinction between isoforms that either mobilize intracellular calcium (m1, m3, and m5) or inhibit adenylyl cyclase (m2 and m4). However, this classification is somewhat arbitrary since there is ample evidence that m2 and m4 receptors do stimulate phospholipase C (through a pertussis toxin-sensitive G protein) and generate intracellular diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) with subsequent liberation of calcium from the endoplasmic reticulum (Ashkenazi *et al.,* 1987). Additional evidence established that G protein $\beta\gamma$ couples m2 receptor stimulation to phospholipase C $\beta 2$ (Katz *et al.,* 1992).

Intracellular Effectors for the Action of Acetylcholine in the Heart

Three main intracellular effectors account for most of the functional effects of acetylcholine in the heart: (1) activation of potassium channels, (2) inhibition of adenylyl cyclase, and (3) activation of soluble guanylyl cyclase leading to increases in intracellular cyclic GMP (which subsequently activates a number of downstream cyclic GMP-dependent effectors). A commonly held view is that the parasympathetic innervation predominantly influences the sinoatrial node and atrioventricular conducting systems in mammalian hearts, with little effect at the ventricular level. However, work using viral tracing experiments has also identified a rich distribution of vagal innervation throughout ventricular cavities (Standish *et al.,* 1994). Functionally, the effects of acetylcholine are small in ventricular tissue in the absence of β-adrenergic stimulation, but when the heart is first stimulated with catecholamines, the effects of acetylcholine are greatly potentiated, a phenomenon that has been termed accentuated antagonism (Levy, 1977).

$I_{K(Ach)}$ In the sinoatrial node and in atrial muscle, accentuated antagonism is mostly accounted for by the activation by acetylcholine of a unique species of potassium channels, designated as $I_{K(Ach)}$, although the resulting K current is also affected by acetylcholine in the absence of catecholamine stimulation. This channel is variably distributed within the heart, with little representation at the ventricular level. In addition, the proportion between heart chambers varies according to species, with a ventricular $I_{K(Ach)}$ density of about 25% that of the atrium in the frog (Hartzell and Simmons, 1987). In other species, where the channel is present at very low levels in the ventricular muscle, alternative pathways must be involved in the antiadrenergic effects of acetylcholine.

Inhibition of Adenylyl Cyclase Among these pathways, the attenuation of adenylyl cyclase activity by muscarinic cholinergic receptors has been well documented in the heart (Murad *et al.,* 1962). The sensitivity of this effect to pertussis toxin indicates its mediation through $G_{i/o}$ proteins. This leads to a decrease in the generation of intracellular cyclic AMP, which results in a decline in the rate and force of cardiac contraction. Experiments in isolated myocytes from homozygous mice deficient in $G\alpha_o$ identified this as the essential isoform for muscarinic receptor coupling to the attenuation of $I_{Ca\text{-}L}$, at least in the mouse heart (Valenzuela *et al.,* 1997).

NO and Cyclic GMP The observation of a dissociation between the functional effects of acetylcholine and cyclic AMP levels (Watanabe and Besh, 1975; Keeley *et al.,* 1978; Löffenholz and Pappano, 1985) prompted the search for other second messenger pathways linked to muscarinic receptor stimulation. These have included the activation of guanylyl cyclase by muscarinic signaling to produce increases in intracellular cyclic GMP. Although a critical review of the early controversial evidence for the role of cyclic GMP as regulator of cardiac contractility is beyond the scope of this chapter, more recent experiments using exogenous NO donors or cyclic GMP analogs have now established the importance of this cyclic nucleotide in the control of cardiac contractility. As will be developed later in the chapter, most of the initial controversy regarding the importance of NO-dependent signaling in the regulation of cardiac function may have stemmed from now well-recognized variations in the observed effects among different regions of the heart and among different animal species, with the specific cardiac muscle preparation employed, and on the concentration of the NO donor or cyclic GMP applied.

Despite clear evidence that acetylcholine increases cyclic GMP levels in heart tissue, the coupling of any muscarinic cholinergic receptor to activation of soluble guanylyl cyclase in ventricular myocytes has until relatively recently re-

mained poorly understood. Interestingly, Goldberg and Haddox had suggested in 1977 that the intracellular levels of calcium could regulate guanylyl cyclase activation. What was missing was the connection between elevation of intracellular calcium and activation of a guanylyl cyclase. Our demonstration of the constitutive expression of a calcium-sensitive isoform of nitric oxide synthase within human and rat ventricular myocytes supported the hypothesis that the parasympathetic regulation of cyclic GMP content in the heart is mediated by nitric oxide synthase (Balligand *et al.,* 1993a, 1995). Thus, muscarinic cholinergic receptor-mediated increases in intracellular IP_3 content, with an elevation in intracellular Ca^{2+}, would result in activation of a calcium-regulated NO synthase.

In the following sections, we will briefly review the data characterizing the identity of the isoforms of NO synthase expressed in heart muscle, which support a functional role for endogenously produced nitric oxide in regulating the contractile response to β-adrenergic and muscarinic cholinergic stimulation, both in whole heart and in isolated ventricular myocyte preparations.

NOS Isoforms in Heart Muscle

There is now ample evidence that all three isoforms of nitric oxide synthase [neuronal NOS (nNOS), iNOS, and eNOS each encoded by a different gene, *NOS1, NOS2,* and *NOS3,* respectively] are expressed within various cell types in the myocardium (Fig. 2).

nNOS

Both cholinergic and nonadrenergic, noncholinergic nerve terminals were shown to express nNOS, as well as specialized conduction tissue and sympathetic nerve terminals of the guinea pig heart (Schmidt *et al.,* 1992; Tanaka *et al.,* 1993). Cardiac myocytes, however, do not seem to express the canonical nNOS, at least in the rat (Balligand *et al.,* 1995; Belhassen *et al.,* 1996), or the muscle specific isoform of nNOS (nNOS-m; Silvagno *et al.,* 1996; Belhassen *et al.,* 1996). A protein with apparent immunoaffinity for anti-nNOS antibodies, but of a slightly different molecular weight, was identified in the sarcoplasmic reticulum of rabbit cardiomyocytes (Xu *et al.,* 1999). These observations await confirmation.

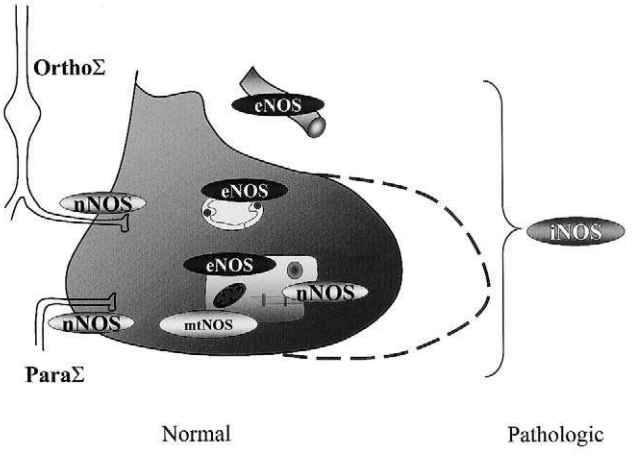


Figure 2 NOS isoforms in the heart.

eNOS

eNOS is expressed in endothelial cells from the endocardium and from arterial capillaries and veins of all mammalian species to date, including humans (Andries *et al.,* 1998; Gauthier *et al.,* 1998a; Balligand and Smith, 1997; Balligand and Cannon, 1997). In addition, there is now unequivocal evidence that eNOS is expressed in cardiomyocytes from atrial, atrioventricular nodal, and ventricular myocytes in most mammalian species (Balligand *et al.,* 1995; Han *et al.,* 1996; Seki *et al.,* 1996) including humans (Wei *et al.,* 1996; Gauthier *et al.,* 1998a; Balligand and Smith, 1997). In cardiac myocytes as in endothelial cells, palmitoylated and myristoylated eNOS is localized to detergent-insoluble glycosphingolipid-rich microdomains of the plasmalemma termed caveolae (Feron *et al.,* 1996). The presence of specific caveolin-binding domains within eNOS allows direct protein–protein interaction between the enzyme and the respective cell-specific caveolin isoform, namely, caveolin-3 in cardiomyocytes and caveolin-1 in endothelial cells (Michel *et al.,* 1997; Garcia-Cardena *et al.,* 1997; Ju *et al.,* 1997). Notably, caveolin-3 was shown to be expressed in the T-tubular system in cardiac muscle, which had been suggested for years to develop from the coalescence of clusters of caveolae at the sarcolemmal membrane (Ishikawa, 1968). Altogether, these observations add weight to the suggestion of a potential role of eNOS in the regulation of excitation–contraction coupling in the heart.

iNOS

Virtually all cell types within the myocardium can express iNOS on appropriate stimulation with specific combinations of inflammatory cytokines or of lipopolysaccharide (LPS) *in vivo.* These include cardiac microvascular endothelial cells, cardiac myocytes, neurons, vascular smooth muscle cells, and infiltrating inflammatory cells (for a review, see Balligand and Cannon, 1997). The relative abundance of iNOS expression within each cell type *in vivo* may be substantially different from the results observed after exposure of cultured cells to recombinant cytokines *in vitro.* In addition, immunostaining for iNOS in the myocardium is not only spatially heterogeneous but also discontinuous over time, which may explain some of the discrepancies between published reports, especially in human tissue. Finally, the relative expression of iNOS in parenchymal cells versus infiltrating monocytes varies significantly according to the etiology of the cardiomyopathy; for example, between septic, transplant rejection, ischemic, or dilated cardiomyopathies. In human dilated cardiomyopathic hearts, iNOS protein has

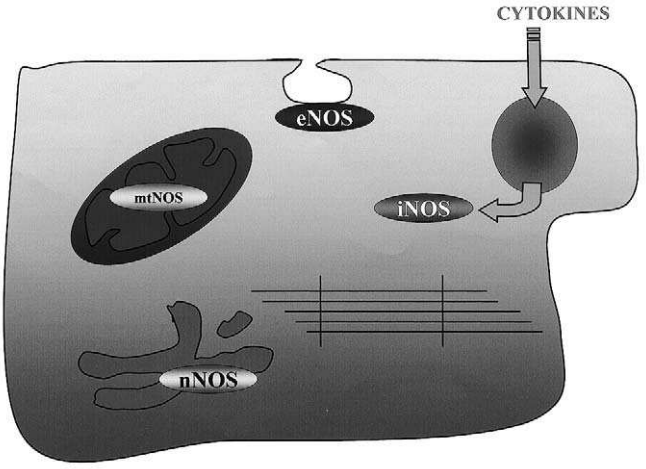


Figure 3 NOS in cardiac myocytes.

been colocalized with tumor necrosis factor (TNF-α), which along with other inflammatory mediators [i.e., interleukin (IL-1, IL-6, γ-interferon (IFN-γ) has been recognized as a component of the ''innate'' immune response. The fact that blood levels of all these inflammatory cytokines have been found to be elevated in heart failure raises the question of whether increased iNOS expression is an adaptive or maladaptive response of the innate immune system (Fig. 3).

Molecular Pharmacology of NOS-Mediated Regulation of Cardiac Myocyte Contraction

To determine whether an L-arginine–NO pathway was functionally active in neonatal and adult rat heart cell preparations and whether it played a role in the response of these preparations to inotropic and chronotropic interventions, the effects of NOS inhibitors were first studied on the amplitude of contractile shortening and changes in beating frequency of cardiac myocytes from adult and neonatal rat hearts, respectively, in response to β-adrenergic and cholinergic agonists.

The Isolated Cardiomyocyte Model

The choice of single contracting cells as an experimental model for functional experiments offers some practical advantages: above all, compared to intact tissue or whole organ preparations, they provide a contracting entity where neurohumoral and/or paracrine influences are largely inconsequential. They also allow a direct correlation between biochemical and molecular observations in single cells and in roughly similar culture preparations. Because of the complexity of the mechanical determinants of muscle cell contraction, however, one should bear in mind that even though isolated cells represent an apparent simplification of intact muscle or organ preparations, they may in fact be downscaling the problem from a megadimensional to merely a multidimensional ambiguity.

Among the most studied preparations, unattached cells (i.e., not physically attached to force transducers) are preferable because they exhibit less inhomogeneity in sarcomeric length variations. These unattached cells display a remarkable degree of regional sarcomeric uniformity both at rest and during active isotonic contraction. Still, the contractile behavior of unattached cells may be influenced by a number of variables (among these is the diastolic length that may influence the surface to volume ratio of the cells and perhaps the accessibility of ion exchange sites and actin/myosin cross-bridge cycling). In order to obtain maximal reproducibility, cells are routinely selected according to a number of criteria: stimulation threshold, shape, diastolic length, and absence of spontaneous contractions, as previously described (Berger *et al.,* 1994).

The edge-detection methods rely on some adhesion of the isolated cell to a stable surface. In these unattached myocytes, sarcomere length uniformity is well maintained during shortening. On the other hand, the exact load, both internal and external, against which the cells contract is not known. This emphasizes the need to carefully select the most straightforward, representative, and reproducible parameters with which to deduce the basis of changes in contractility in isolated cells, and to compare these with those observed in other models, for example, in multicellular preparations or intact tissue preparations.

A complete description of the mechanical component of the contractile process with which to relate the underlying biochemical processes would require measurements of force, stiffness, and shortening characteristics under a variety of perturbations. For technical reasons (mainly the difficulty of obtaining attachment of isolated myocytes to force transducers without perturbing sarcomeric homogeneity and sarcolemmal integrity), the establishment of the relation between active force development as a function of sarcomeric lengths remains an experimental challenge at the single cell level. The most extensive data, in this regard, have come from the analysis of shortening as an index of contractility.

Both the degree of shortening and the velocity of shortening (and relengthening) can be measured in a minimally loaded cell. The parallels that have been demonstrated between myocyte shortening responses and those of intact tissue confirm that the isolated myocyte is a useful model of cardiac tissue (e.g., Allen and Kentish, 1985). Since inotropic interventions may affect the maximum shortening and the rate of cell length change differently, a more complete assessment of contractility should include a description of both parameters. In the case of isoproterenol, the shortening and relaxation rates as well as the extent of cell shortening increase together, and previous studies have established the correlation between absolute changes in cell length and its first derivatives. Therefore, in most experiments with adult rat myocytes, only cell shortening amplitudes are presented. In the following sections we will first describe the paradigms originally proposed on the basis of single myocyte experi-

ments and correlate those with the results obtained in multicellular preparations or *in vivo* observations.

nNOS

Several lines of evidence indicate that nitric oxide produced endogenously by nerve terminals plays a role in the control of catecholamine release during electrical sympathetic nerve stimulation. This was confirmed in isolated perfused hearts (Schwarz *et al.,* 1995) but also *in vitro* in PC-12 cells (Kaye *et al.,* 1997) and in cocultures between peripheral cardiac neurons and cardiomyocytes from adult mammalian myocardium (Horackova *et al.,* 1995). In the latter experiments both exogenous NO release from *S*-nitroso-*N*-acetylpenicillamine (SNAP) and endogenous NO produced by cocultured intrinsic cardiac neurons acted to increase the spontaneous beating frequency of cocultured myocytes, whereas NO had no effect on monocultures of cardiomyocytes. These results are consistent with the interpretation that NO either increases the release or inhibits the reuptake of neuron-derived catecholamines at the presynaptic level. This interpretation was supported by independent experiments showing that *S*-nitrosothiols inhibit neuronal norepinephrine transport (Kaye *et al.,* 1997).

The physiological role of nNOS on cardiac function has been more directly examined *in vivo* in homozygous mice deficient for NOS1 (nNOS−/− mice). Under baseline conditions, nNOS−/− mice had a higher mean heart rate and lower heart rate variance than wild-type mice. Their chronotropic response to atropine was significantly blunted, suggesting a reduced baseline parasympathetic tone. However, the bradycardic response to a pressure challenge (i.e., phenylephrine injection after β-adrenergic blockade) in nNOS−/− mice was similar to that observed in wild-type controls. The response to pressure challenge was strikingly more sensitive to pertussis toxin (PTX) pretreatment in nNOS−/− mice compared to wild-type littermates, suggesting that the cardiac inhibitory G_i protein acts in parallel to neuronally derived NO to mediate autonomic slowing of heart rate in the mouse (Jumrussirikul *et al.,* 1998).

eNOS

Muscarinic Cholinergic Pathway

The first evidence for a regulatory role of NOS constitutively expressed in cardiac myocytes on the muscarinic cholinergic pathway was provided by the study of the effect of pharmacological NO or NOS inhibitors on myocyte responsiveness to carbamylcholine. These experiments were initially performed in cultures of neonatal rat ventricular myocytes, in which changes in spontaneous beating rate (not contractile amplitude) were monitored. Despite theoretical caveats associated with the neonatal phenotype, the conclusions from these initial studies were later confirmed in isolated ventricular myocytes from adult rat hearts, intact hearts, and whole preparations. In addition, the ability of neonatal myocytes to undergo a (limited) number of cell divisions in culture renders them more amenable to transfection for heterologous expression of plasmids encoding wild-type or mutated eNOS (e.g., in neonatal myocytes from eNOS−/− mice, see later) or regulatory proteins, such as caveolin, thereby enabling a more in-depth analysis of the molecular regulation of eNOS-dependent muscarinic cholinergic coupling in these cells (see later).

Chronotropic Response of Neonatal Myocytes to Carbachol: Effect of NO/NOS Inhibitors We studied primary cultures of neonatal cardiac myocytes 3 days after isolation and plating, at which time their spontaneous rate of contraction usually has stabilized. Superfusion of this multicellular preparation with carbachol abruptly reduced the beating rate in these cells. The onset of the effect of carbachol was rapid, persisted through the duration of superfusion, and was completely reversible after drug washout. Repetitive applications of carbachol caused similar marked declines in beating rate. Similar experiments were then repeated in the presence of oxyhemoglobin or methylene blue, both of which abolished the negative chronotropic effect of carbachol. The effects of graded increases in carbachol concentration (100 n*M* to 3 m*M*) were also studied in the same preparation in the presence or absence of the NOS inhibitor N^G-methyl-L-arginine (L-NMMA) (1 m*M*). L-NMMA markedly attenuated the ability of carbachol to reduce the beating rate of neonatal myocytes in primary culture, an effect that was partially reversed after addition of an excess of L-arginine, the substrate for NOS.

These data indicate that cardiac myocytes respond functionally to products of an endogenous NO synthase. The number of nonmyocyte cells in neonatal rat ventricular myocyte primary cultures is relatively low (typically 10% or less), and thus the origin of the NO was presumed to be cardiac myocytes. Even though the interpretation of the inhibitory effect of methylene blue may be confounded by its well-known atropine-like effect, first described in the frog ventricle as early as 1926 (Cook, 1926), the consistent effect of several pharmacological NO/NOS inhibitors [i.e., oxyhemoglobin, L-NMMA] supported this conclusion.

The L-arginine analogs N^G-nitro-L-arginine (L-NNA) and L-NMMA attenuated the production of NO in a variety of tissues by inhibiting the transformation of L-arginine to L-citrulline plus NO by members of the NOS protein family (Moore *et al.,* 1990; Rees *et al.,* 1998). Although this inhibition could be reversed with an excess of the natural substrate, L-arginine, in most tissues, some analogs, including L-NMMA, may irreversibly inactivate the enzyme (Dwyer *et al.,* 1991). Thus, the incomplete restoration of the negative chronotropic effect of carbachol by L-arginine in the presence of L-NMMA may reflect the relatively greater affinity of the NOS antagonist arginine analogs in neonatal cardiac cells, as well as possible irreversible inactivation of the enzyme. Although it seemed plausible that cardiac muscle cells themselves produced NO in these experiments, this chemical signal could still have originated from other cell types within

these primary cultures and surrounding the myocytes in the ventricular wall.

Role of NO in Accentuated Antagonism: Muscarinic Cholinergic Modulation of L-Type Calcium Current and Cell Shortening of Adult Cardiomyocytes Prestimulated with Adrenergic Agonists More insights into the functional role of eNOS constitutively expressed in cardiac myocytes were gained in subsequent studies of the effect of various inhibitors of NO or eNOS on both calcium current and the contraction of single, isolated cardiac myocytes from adult rat hearts. In contrast to neonatal cell preparations, adult rat cardiomyocytes can be studied by combined video-motion analysis and electrophysiological measurements for both L-type calcium current intensity and unloaded cell shortening. The use of the nystatin-perforated patch-clamp technique further allows internal dialysis of single cells with drugs, such as L-NMMA, combined with external application of various agonists. A major difference with neonatal cells is that adult cardiac myocytes in the first 24 to 48 hours of isolation do not beat spontaneously but do contract in response to electrical stimulation. Subsequent superfusion with agonists such as isoproterenol, a nonselective β-adrenergic activator allows the observation of increases in cell shortening amplitude as a reflection of changes in inotropic state (see earlier). When a stable increase in cell shortening is obtained with isoproterenol, the subsequent application of carbachol attenuates the β-adrenergic inotropic response (whereas carbachol has little or no effect in the absence of isoproterenol prestimulation), an equivalent at the single cell level of classic pharmacological accentuated antagonism. In experiments with adult myocytes, this antagonistic effect of carbachol is used as a measurement of cellular responsiveness to muscarinic cholinergic stimulation.

In a series of experiments using these protocols in isolated ventricular myocytes from adult rat hearts, we showed that carbamylcholine attenuated the isoproterenol-stimulated increase in $I_{Ca\text{-}L}$ and amplitude of unloaded cell shortening in paced ventricular myocytes. After extracellular perfusion or internal dialysis of the cell with the NO synthase inhibitor L-NMMA, the antagonistic effect of carbamylcholine on β-adrenergic stimulation of both $I_{Ca\text{-}L}$ and the amplitude of unloaded cell shortening was totally abolished. A similar reversal of the effect of carbamylcholine on contractility was also observed after NO blockade with hemoglobin. Qualitatively and quantitatively similar results were obtained with sinoatrial and atrioventricular nodal cells from adult rabbit hearts (Han *et al.,* 1996).

Muscarinic Cholinergic Coupling Is Absent in Adult Cardiomyocytes from Mice with Targeted Disruption of eNOS The conclusions based on the use of pharmacological inhibitors of NOS, as described earlier, were confirmed by a subsequent study of the accentuated antagonism exhibited by isolated ventricular myocytes from mice homozygous deficient for eNOS. The phenotype of these mice, originally developed and characterized by Paul Huang, Mark Fishman, and their colleagues is characterized by the progressive development of hypertension, mostly as a result of the loss of endothelial production of NO (Huang *et al.,* 1995).

The contractile response to maximal stimulation with Iso was unchanged in these eNOS−/− myocytes, as was maximal L-type calcium current. However, the antagonistic effect of carbachol on the adrenergic response, which was clearly observed in myocytes from wild-type animals (as described previously) was lost in myocytes from eNOS−/− mice (Han *et al.,* 1998b). Importantly, this was paralleled in the same cells by the inability of carbachol to increase intracellular cGMP levels, whereas in myocytes from wild-type (eNOS+/+) animals, cGMP levels clearly responded to carbachol stimulation and were sensitive to NOS inhibitors.

These results contrast with those from a subsequent study by Vandecasteele *et al.* (1999) who found no alteration of the muscarinic cholinergic response in cardiac cell muscle preparations from eNOS−/− mice. However, in our opinion, their contention that eNOS does not mediate muscarinic regulation of the heart is weakened by several methodological shortcomings. Foremost is the fact that their eNOS−/− mice were not matched with the appropriate controls: (1) homozygous founder mice were not backcrossed into their appropriate strains to obtain homogenous genetic backgrounds between eNOS−/− and control eNOS+/+ mice, as is advisable in such comparative studies; (2) the eNOS−/− mice used in the report by Vandecasteele *et al.* (1999) were studied between 3 and 6 months of age, when the chronic hypertension characteristic of this genetic model had resulted in a significant myocardial hypertrophy, a phenotype not independently accounted for in their comparison with normal controls (see comments by Hare and Stamler, 1999); (3) in their multicellular (papillary muscle and particularly, atrial) preparations, the persistence of the response to muscarinic cholinergic agonists in tissues from eNOS−/− mice may be explained by the activation of $I_{K(Ach)}$ which is known to be present in atrial and ventricular cells (Han *et al.,* 1998b) and can be upregulated in response to hypertrophic stimuli (Guo *et al.,* 1997); (4) such confounding effects of K^+ currents should have been controlled by Cs^+-containing intracellular and extracellular solutions in the experiments with single ventricular myocytes; however, the experiments described by Vandeasteele *et al.* (1999) were all performed at room temperature (19°–23°C), and the authors do not provide independent evidence that eNOS (in control myocytes) is active at such low temperatures, thereby preventing any valid comparison; and (5) there are precedents for misleading conclusions based on experiments designed to measure enzymatic activity when performed at temperatures well below 37°C (e.g., see the opposite conclusions regarding the functionality of a cyclic ADP ribose(cADPR) pathway in cardiomyocytes reached by Guo *et al.,* 1996 and Iino *et al.,* 1997, depending on the temperatures used to assay the activity of this pathway).

In contrast, in the study by Han *et al.* (1998b), eNOS−/− mice were appropriately backcrossed into their CR7/B6 and 129SvEv backgrounds, and studied at a younger age when significant cardiac hypertrophy had not yet occurred.

Electrophysiological experiments were also performed at 32.5°C. Whether these technical differences account for all of the discrepancy between these two studies will await further experimental confirmation.

Caveolar Targeting of the Muscarinic Cholinergic/eNOS Pathway

SUBCELLULAR LOCALIZATION OF eNOS The experiments described above suggest that the generation of nitric oxide is an obligate intermediate step in the signal transduction cascade involved in the muscarinic cholinergic (mAchR)-mediated inhibitory responses in the heart, at least in rodents. The colocalization within caveolae of eNOS and calmodulin with proteins known to regulate Ca^{2+} homeostasis, including a Ca^{2+}-ATPase and IP_3 receptor-like proteins, as well as with heterotrimeric G proteins (for references, see Couet *et al.,* 1997; Okamoto *et al.,* 1998), suggest that these plasmalemmal microdomains could constitute the platform for the recruitment and regulation of the signaling proteins involved in the NO-mediated muscarinic cholinergic pathway in heart muscle. Therefore, it was hypothesized that the dynamic targeting of agonist-stimulated muscarinic cholinergic receptors to caveolae in cardiac myocytes could facilitate the activation of eNOS.

As mentioned earlier, several studies have shown that, of the five mAchR subtypes identified to date, only the m1 and m2 subtypes are expressed in adult mammalian cardiac tissues (Gallo *et al.,* 1993; Sharma *et al.,* 1996). According to these reports, the m2 mAchR, which is expressed in the heart at a higher level than the m1 mAchR, triggers the inhibitory response, while m1 receptor activation, when stimulated by higher concentrations of agonist, elicits a compensatory excitatory effect on heart function. Therefore, distinct downstream signaling cascades must be involved following m1 and m2 mAchR activation. Both m1 and m2 receptor subtypes have also been reported to undergo translocation into specific subcompartments derived from plasma membrane (Harden *et al.,* 1985; Svoboda and Milligan, 1994; Goldman *et al.,* 1996), a characteristic of many G-protein-coupled receptors (GPR) following agonist binding. To date, two major pathways for GPR clustering and sequestration, which involve plasma membrane modifications that lead to the formation of either clathrin-coated or noncoated vesicles, have been reported. Whereas the human muscarinic cholinergic receptor Hm1 has been shown to internalize via clathrin-coated vesicles (Tolbert and Lameh, 1996), mAchRs have also been shown to be internalized through non-clathrin-coated vesicles in human fibroblasts, although the identity of these vesicular structures has not been defined (Raposo *et al.,* 1987).

A series of experiments was therefore designed to explore the hypothesis that m2 mAchRs are targeted to plasmalemmal caveolae on agonist stimulation in adult rat ventricular myocytes. Caveolin-enriched membranes have been historically isolated on the basis of their insolubility in nonionic detergents (such as Triton) due to their specialized lipid composition (Sargiacomo *et al.,* 1993). However, it has been reported that the inclusion of these detergents can result in the loss of proteins normally associated with caveolae (Smart *et al.,* 1995; Song *et al.,* 1996). Therefore, for isolating caveolae from cardiac myocytes, we have optimized a detergent-free purification method based on the resistance to extraction of caveolin complexes by sodium carbonate and on the fine disruption of cellular membrane by sonication, and followed by an overnight isopycnic centrifugation on sucrose gradient, as described for cardiac myocyte membranes (Feron *et al.,* 1997).

In these experiments, the majority of caveolin-3 and eNOS in ventricular myocyte preparations appeared in low density fractions which correspond to the 5–15% sucrose equilibrium densities (Feron *et al.,* 1997). This copurification of eNOS and caveolin-3 was in agreement with previous data on the coimmunoprecipitation of these two proteins from cardiac myocyte lysates (Feron *et al.,* 1996). As indicated by the pattern of distribution of various membrane markers across the gradient, the bulk of cellular protein that equilibrates at the high sucrose density corresponds to Golgi and sarcolemmal membranes (Feron *et al.,* 1997). Along with the immunohistochemical data mentioned above, these coimmunoprecipitation experiments also provided additional evidence for an association of eNOS with caveolin-3 in cardiac myocytes.

M2 ACHR IN MYOCYTES TRANSLOCATE TO CAVEOLAE ON AGONIST STIMULATION Interestingly, when exploring the effects of carbachol, a muscarinic cholinergic agonist, on the distribution of mAchR using the protocol described above, changes in receptor subcellular localization were observed (Feron *et al.,* 1997). Using the muscarinic cholinergic radioligand [^{3}H]QNB as a marker, mAchRs were almost exclusively found in high-density fractions issued from untreated myocyte lysates. In contrast, following carbachol treatment, one-third of the [^{3}H]QNB binding was recovered in low-density fractions, which corresponded to the caveolin-enriched membranes. The rest of the [^{3}H]QNB binding remained concentrated in a heavy membrane fraction and thus likely represented binding to noncaveolar sarcolemmal muscarinic receptors. Importantly, antibodies directed against the m2 mAchR and caveolin-3 were used to immunoprecipitate caveolar membranes and confirmed the dynamic targeting of muscarinic receptors to caveolae in cardiac myocytes.

Taken together, these data establish that the m2 mAchR is targeted to plasmalemmal caveolae of cardiac myocytes following agonist binding. The dynamic targeting of the m2 mAchR to caveolae has important implications for muscarinic receptor biology as well as for the regulation of eNOS activation. The copurification and coimmunoprecipitation of caveolin, eNOS, and the agonist-stimulated m2 mAchR in isopycnic centrifugation fractions (Feron *et al.,* 1996, 1997), which together represent less than 5% of total cellular protein, indicate that caveolae are a common structural platform for these proteins. The caveolar compartmentation described here for the muscarinic cholinergic pathway may serve as a

paradigm for other G protein receptor-mediated signaling cascades that are targeted to caveolae.

While numerous studies present the sequestration of G-protein-coupled receptors after agonist stimulation as a key event for initiating a process of desensitization (see Lefkowitz, 1998), our data support the hypothesis that, following stimulation by agonist, cardiac m2 mAchR translocation to caveolae may also be required to initiate specific downstream signaling cascades. Interestingly, several studies have shown that internalization of the m2 and m4 mAchR is mediated by mechanisms distinct from the phosphorylation by the G-protein-coupled receptor kinase (GRK) family known to lead to receptor desensitization (Pals-Rylaarsdam *et al.,* 1995; Bogatkewithsch *et al.,* 1996). The translocation of muscarinic receptors within caveolae would allow their interaction with heterotrimeric G-protein complexes known to be concentrated within these plasmalemmal microdomains and lead, after recruitment of cofactors and intermediate effector proteins, to the activation of eNOS, a resident caveolar protein in cardiac myocytes.

POSTTRANSLATIONAL MODIFICATIONS OF eNOS AFFECT ITS CAVEOLAR TARGETING Quantitative changes in NOS expression have been causally associated with cardiovascular disease states (Loscalzo and Welch, 1995). Likewise, the abrogation of NOS expression by targeted gene inactivation, or, conversely the enhancement of NOS pathways by gene overexpression, have permitted insights to be gained by generating quantitative changes in NOS abundance (Moncada and Higgs, 1995). However, it seems equally likely that qualitative changes in the NOS pathway may underlie many important aspects of NO physiology and pathophysiology. At least some of these qualitative changes may be affected by alterations in the posttranslational modifications of the enzyme, subcellular targeting, or protein–protein interactions. eNOS in endothelial cells and in cardiac myocytes undergoes cotranslational myristoylation and posttranslational palmitoylation. Myristoylation, in particular, is necessary for eNOS targeting to plasmalemmal caveolae (Michel and Feron, 1997). Moreover, as mentioned earlier, eNOS appears to be quantitatively associated with caveolins (caveolin-1 in endothelial cells and caveolin-3 in cardiac myocytes) through a stable interaction taking place at consensus sequences present in both partners which appears to contribute to downregulation of enzyme activity (Michel *et al.,* 1997). In endothelial cells, following agonist stimulation, Ca^{2+}-bound calmodulin is known to activate eNOS by disrupting the heteromeric complex formed between eNOS and caveolin (Feron *et al.,* 1998a,b). A regulatory cycle is then initiated wherein activated, caveolin-free eNOS, translocates from caveolae, and is followed by the enzyme reassociation with caveolin when Ca^{2+} returns to basal levels (a similar regulatory cycle is presumed to occur in cardiac myocytes) (Michel and Feron, 1997; Feron *et al.,* 1998b; Feron, 1999).

The targeting of eNOS in plasmalemmal caveolae probably facilitates paracrine signaling by NO, a pathway most clearly delineated in endothelial cells within the vascular wall. Furthermore, since an increase in shear stress appears as one of the most effective activators of NO production in endothelial cells, it seems logical to expect that at least for the flow responsive pool of eNOS, it would be more efficient for the enzyme to reside at the endothelial cell surface (Rizzo *et al.,* 1998). In cardiac myocytes, eNOS is also located within plasmalemmal caveolae (Feron *et al.,* 1996), but to date, the NO generated has only been shown to act in an autocrine fashion by modulating myocyte responsiveness to neurohumoral or mechanical stimuli. NO produced by myocytes has various endogenous targets (see later sections), and the location of eNOS within plasmalemmal caveolae of these cells is probably unrelated to a requirement for extracellular diffusion of NO, as documented for the endothelial cells. Instead, the functional importance of the subcellular location of eNOS in myocyte plasmalemmal caveolae appears to be the close proximity with regulatory proteins such as caveolin and calmodulin as well as with receptors and G protein-dependent signaling cascades.

MOLECULAR DETERMINANTS OF MYOCYTE eNOS TARGETING TO CAVEOLAE eNOS is unique among the NOS isoforms because it is acylated by the fatty acids myristate and palmitate (Sase and Michel, 1997). Whereas myristoylation is an essentially irreversible protein modification, thiopalmitoylation is a reversible posttranslational modification which may undergo dynamic regulation (Sase and Michel, 1997). Depalmitoylation was indeed proposed to occur following agonist stimulation, thereby promoting the enzyme translocation (Sase and Michel, 1997), and inversely, palmitoylation was shown to play an obligatory role for the retargeting of translocated eNOS to caveolae (Feron *et al.,* 1998b). Although it has been proposed that agonist-induced translocation of eNOS is part of a desensitization process, the use of acylation-deficient mutants was restricted to conventional transfectable cells, and this presented inherent limitations in the study of the effects of eNOS mistargeting on signal transduction in cardiac myocytes. On the other hand, the confounding effect of endogenous eNOS expressed in specialized cells, such as cardiac myocytes, hampered the interpretation of the data resulting from the expression of acylation-deficient eNOS mutants in these nontractable cells.

We were able to overcome this experimental limitation, however, by the isolation of ventricular myocytes from neonatal mice with targeted disruption of eNOS (i.e., eNOS$-/-$, as described earlier). Myocytes from eNOS$-/-$ mice were subsequently transfected with cDNA constructs encoding for either wild-type eNOS or a myristoylation-deficient (myr^-) eNOS mutant. Inactivation of the eNOS myristoylation site ($glycine_2$ mutated to alanine) not only blocks myristoylation but also prevents subsequent enzyme palmitoylation yielding an acylation-deficient enzyme. Accordingly, in transfected neonatal eNOS$-/-$ mice myocytes, expression of the acylation-deficient myr^- eNOS mutant appeared restricted to the cell cytosol, and the mutant enzyme could not be coimmunoprecipitated by caveolin antibodies (Feron *et al.,* 1998c). In contrast, the distribution of recombinant

wild-type (WT) eNOS expressed in transfected neonatal eNOS−/− mice myocytes was similar to that of native myocyte eNOS, and was found almost exclusively located in the particulate fraction associated with caveolin. Therefore, this approach provided an experimental model for eNOS signaling within cardiac myocytes with eNOS subcellular localization restricted to either caveolae or the cytosol. This permitted us to specifically address the role of eNOS caveolar targeting and the functionality of its coupling to muscarinic cholinergic stimulation.

Importantly, the normal response of myocytes to autonomic nervous system agonists could be rescued in eNOS−/− myocytes by transfection with a wild-type but not the acylation deficient myr⁻ mutant eNOS (Feron *et al.,* 1998c). Indeed, in eNOS−/− myocytes expressing recombinant WT eNOS, as with myocytes from backcrossed wild-type animals, the muscarinic cholinergic agonist carbachol elicited a strong negative chronotropic response and markedly activated cGMP production. In contrast, in myr⁻ mutant eNOS transfected myocytes, the response to muscarinic cholinergic stimulation was absent as a consequence of the single amino acid substitution preventing eNOS acylation and targeting to caveolae. Importantly, the calcium ionophore A23187, which increases intracellular Ca^{2+} in a receptor-independent fashion, increased cellular cGMP levels in myocytes expressing the myr⁻ eNOS mutant to a level equivalent to that seen with the WT enzyme. Since the absolute capacity to generate NO was not different for both wild-type and mutant eNOS constructs transfected into eNOS−/− myocytes, it can be postulated that the myr⁻ eNOS mutant is uncoupled from the muscarinic receptor because of the aberrant subcellular localization of the mutant and failure to target to caveolae. This result clearly established the key role of eNOS location in plasmalemmal caveolae for its activation and regulation of cardiac myocyte function.

THE CAVEOLIN-3–eNOS INHIBITORY INTERACTION IN CARDIAC MYOCYTES As mentioned above, the caveolin isoform expressed in myocytes, caveolin-3, exhibits only an ~60% amino acid identity with the caveolin-1 isoform expressed in endothelium, the C-terminal juxtamembranous region of 20 amino acids within both caveolin isoforms that have been shown to specifically interact with the α subunit of G proteins and modulate their GTPase activity (Song *et al.,* 1996; Tang *et al.,* 1996). The functionality of this caveolin-3 scaffolding domain, however, has only been more recently resolved by the identification of this modular protein domain as an endogenous inhibitor of eNOS activity in cardiac myocytes (Feron *et al.,* 1998c).

To address the specificity of the interaction between eNOS and caveolin-3, an approach that allows specific peptides to enter cultured myocytes by reversible cell permeabilization was used. Neonatal rat myocytes that endogenously express eNOS and caveolin-3 were permeabilized to introduce synthetic oligopeptides corresponding to the scaffolding domain of caveolin-3, using a method developed by Morgan and Morgan (1982) for loading aequorin into vascular smooth muscle cells. Reversible permeabilization of myocytes was obtained by their exposure to an ice-cold solution containing a Ca^{2+} chelator and the peptide, followed by the progressive rewarming of the medium bathing the myocytes and the restoration of physiological calcium concentration. This method does not require the use of detergents such as saponin but still allows the introduction of control polydextran (5–100 kDa) into the cells. Importantly, neonatal myocytes spontaneously began beating following the permeabilization protocol, thus allowing us to study the functional consequences of the modulation of the caveolin-3–eNOS interaction on the beating rate. The paradigm of muscarinic cholinergic NO-mediated regulation of heart rate has been established in this model of cultured neonatal myocytes (see earlier), offering a model system to study the posttranslational modification of an eNOS-mediated pathway in cardiac myocytes. Using neonatal rat ventricular myocytes, which beat spontaneously beginning 3 days after isolation, we performed measurements of beating rate which was determined on a temperature-controlled chamber on the stage of a microscope connected to a video-motion analyzer.

Two different peptides were used in these experiments: peptides containing the caveolin scaffolding domain corresponding to residues 55–74 of the rat caveolin-3 sequence (termed Cav-3); and a scrambled peptide (termed Cav-3X), wherein residues were exchanged pairwise at eight highly conserved positions within the 20 amino acid sequence of the Cav-3 peptide. The use of this conservatively designed peptide as control, which shares 100% identical amino acid composition and 60% amino acid sequence identity with the Cav-3 peptide, helped to ascertain the specificity of the effects seen with the Cav-3 peptide.

The permeabilization protocol did not significantly alter the basal beating rate which amounted to 70 ± 3 and 72 ± 4 min^{-1} ($n = 5$), before and after the peptide loading. When sham-permeabilized myocytes were exposed to 1 μ*M* carbachol, myocyte spontaneous contractions were completely abrogated; several minutes of wash out were required to restore the initial beating rate. In contrast, in neonatal myocytes loaded with the Cav-3 peptide, carbachol did not induce any change in beating rate. The specificity of this effect was demonstrated by using myocytes loaded with the control peptide Cav-3X. This scrambled control peptide did not alter the inhibitory effect of carbachol on the rate of myocyte contraction. Furthermore, cGMP determinations performed on parallel myocyte cultures revealed that the Cav-3 peptide inhibited the carbachol-evoked increase in cGMP levels to about the same extent as observed with the NOS inhibitor L-NNA applied on sham-permeabilized myocytes. Importantly, the Cav-3X peptide failed to reverse the fourfold increase in cGMP observed in control native myocytes.

These experiments validate the hypothesis according to which the compartmentation of eNOS within caveolae is intimately linked to the regulation of its activity by the interaction with caveolin. The close control of eNOS activity by caveolin is probably required to maintain a low basal production of NO and to protect the cell from undesired, po-

tentially cytotoxic, or nonphysiological bursts of NO in response to subtle increases in intracellular calcium. Enzyme kinetic analyses revealed that caveolin serves as a competitive inhibitor of calmodulin-dependent eNOS activation (Michel *et al.,* 1997). The calmodulin binding consensus sequence is located at the N terminus of the eNOS reductase domain, and calmodulin binding to this site activates NO synthesis by enabling the reductase domain to transfer electrons to the oxygenase domain (Ghosh *et al.,* 1998). Calmodulin is therefore likely to rescue the caveolin-inhibited eNOS by antagonizing the blockade (or slowing) of electron transfer due to the binding of caveolin to either of the domain of the enzyme. Therefore, the close control of enzyme activity may be particularly important for eNOS in caveolae, where calmodulin is enriched (Shaul *et al.,* 1996), potentially leading to undesired enzyme activation if the interaction of caveolin with eNOS were not keeping the system in check.

eNOS-Mediated Muscarinic Cholinergic Coupling in Other Mammalian Species Negative studies from others have emphasized that coupling of muscarinic cholinergic GPRs to NO production may not be operative in all animals species or even in all regions of the heart. The pathway seems to be functionally absent in frog atrial myocytes (Méry *et al.,* 1996), ventricular myocytes from adult guinea pigs (Stein *et al.,* 1993), or in papillary muscle from failing or nonfailing human hearts (Kilter *et al.,* 1995). In a study on the regulation of L-type calcium current of human atrial myocytes that is sensitive to application of exogenous NO donors, Vandecasteele and colleagues again found no effect of NO synthase inhibitors on the muscarinic cholinergic regulation of either baseline or adrenergically stimulated calcium current (Vandecasteele *et al.,* 1998). As noted earlier, in addition to other explanations for these discrepancies with our data, there was no assessment of NO production from these preparations with an independent technique in a particular experimental condition used (e.g., at temperatures lower than 37°C).

On the other hand, the involvement of endogenous NO in the parasympathetic regulation of heart function has been confirmed in a number of tissue preparations and *in vivo* models. In the anesthetized ferret adrenergically blocked with propranolol, NO synthase inhibitors significantly reduced the bradycardia induced by vagal stimulation, an effect that could be reversed by infusion of an excess of L-arginine (Conlon *et al.,* 1996). Similarly, in the anesthetized dog, infusion of L-NMMA into the sinus and atrioventricular nodal arteries attenuated the negative chronotropic and dromotropic responses to vagal nerve stimulation in the absence or presence of adrenergic activation (Elvan *et al.,* 1997). NOS inhibition, however, may not always affect the direct cholinergic actions of infused acetylcholine. In a series of experiments in anesthetized rabbits and in isolated guinea pig atria with preserved vagal innervation, Sears and colleagues outlined the existence of regulatory roles of exogenous or endogenous NO at various levels of cholinergic neurotransmission (Sears *et al.,* 1998, 1999). In isolated guinea pig atria, inhibition of nitric oxide synthase did not affect the maximal response rate to vagal nerve stimulation in adrenergically pretreated preparations, but significantly slowed down the time course of the vagal negative chronotropic effect. On the basis of the subsequent observation of an acceleration of the rate response to vagal stimulation in the presence of inhibitors of the hyperpolarization-activated current, or I_f, which is known to be stimulated by NO (Musialek *et al.,* 1997), they proposed that the modulatory role of endogenous NO on the vagal response may integrate both a potentiating effect, presumably mediated through inhibition of $I_{Ca\text{-}L}$, and an antagonistic effect activating I_f, both of which are superimposed on the direct activation by acetylcholine of $I_{K(Ach)}$, at least at the atrial level. Moreover, these authors observed that application of exogenous NO donors or 8-bromo-cyclic GMP potentiated the chronotropic effect of vagal nerve stimulation but not of directly applied acetylcholine, thereby implicating an additional role of NO at the presynaptic level, a conclusion in agreement with previous results in the anesthetized dog (Elvan *et al.,* 1997). The existence of functionally opposed effects of NO on the control of heart rate due to interaction with multiple currents (i.e., $I_{Ca\text{-}L}$, I_f, but not $I_{K(Ach)}$) in the atria probably explains the contradictory results provided by different laboratories, depending on the preparation and protocol used (i.e., using intact vagal nerve stimulation versus direct application of acetylcholine, in the presence or absence of initial adrenergic stimulation), and the species employed (which may affect the relative contribution of each current to the control of heart rate). In humans, we observed a partial abrogation of the accentuated antagonism by NOS inhibitors in electrically paced atrial strips exposed to acetylcholine after submaximal stimulation with isoproterenol (Gauthier *et al.,* 1998b).

In this regard, the contribution of eNOS to accentuated antagonism relative to other intracellular pathways coupled to muscarinic cholinergic receptors may be more identifiable at the ventricular level, given the absence of expression and/or significant functional role of $I_{K(Ach)}$ and I_f in ventricular cells. As for other muscarinic cholinergic responses, $G_{i/o}$ proteins appear to be critical in the coupling of the NO-mediated accentuated antagonism. Pretreatment of rats with pertussis toxin not only abolished the muscarinic cholinergic accentuated antagonism, which was shown to be NO-mediated, but also controlled the abundance of eNOS proteins in the isolated hearts from the same animals (Hare *et al.,* 1998a).

Finally, endogenous nitric oxide was also shown to mediate the indirect effects of adenosine on calcium current in rabbit heart pacemaker cells (Shimoni *et al.,* 1996), thereby recapitulating the paradigm proposed for muscarinic cholinergic coupling in the heart.

β-ADRENERGIC PATHWAY

The concept of a tight regulation of eNOS activation following muscarinic cholinergic receptor stimulation could theoretically be extended to other GPR associated with increases in intracellular calcium, the key activator of the

enzyme. As such, β-adrenoreceptors are well known to increase cytosolic calcium through stimulation of calcium influx through L-type calcium channels. According to the paradigms developed earlier, any subsequent activation of eNOS would exert a negative inotropic influence opposing the classic positive inotropism of the adrenergic pathway, thereby acting as a negative feedback mechanism.

Effects of NOS Inhibitors on the Positive Inotropic Effect of Isoproterenol in Adult Ventricular Myocytes Experiments were designed therefore to examine whether an NO-mediated signaling system could regulate the contractile (inotropic) response of ventricular myocytes to the β-adrenergic agonist isoproterenol. Adult rat ventricular myocytes were exposed to 2 n*M* isoproterenol, a concentration determined to yield about a half-maximal increase in contractile amplitude, in the presence or absence of NOS inhibitors. L-NMMA had no effect on baseline contractile function, but significantly potentiated the inotropic response to isoproterenol compared with that in myocytes superfused with control, L-arginine-containing medium.

The increase in the contractile response of adult rat ventricular myocytes to isoproterenol by NOS inhibitors suggested the existence of a countervailing, negatively inotropic, NO-mediated mechanism limiting the effect of β-adrenergic stimulation in adult myocytes. In this regard, the role of NO in cardiac muscle would be analogous to the control of the vascular tone by endothelium-derived NO (Egleme *et al.,* 1984; Furchgott and Zawadzki, 1980; Martin *et al.,* 1986; Godfraind *et al.,* 1985).

eNOS Is Activated by β_3-Adrenergic Stimulation in Cardiac Muscle The activation by catecholamines of negative inotropic effects can be rationalized in terms of the concurrent activation of a family of adrenergic receptors coupled to different intracellular pathways. In 1996, Gauthier *et al.* had described the functional expression of adrenoreceptors of the β_3 subtype in human ventricular muscle. β_3-adrenoceptors had been extensively studied for their role in mediating catecholamine-induced lipolysis in fat tissue, and had so far been considered to exclusively play a metabolic role. Their expression in cardiac tissue was demonstrated at the mRNA level, and confirmed more recently at the protein level (S. Moniotte and J. L. Balligand, unpublished results 2000), with significant levels of expression in cardiomyocytes. In contrast with β_1- or β_2-receptors, activation of β_3-adrenoceptors with isoform-selective agonists (e.g., BRL37344) or isoproterenol in the presence of full β_1 and β_2 blockade produced a striking negative inotropic effect, as measured by peak tension development of human ventricular biopsies in organ baths (Gauthier *et al.,* 1996). This effect was also completely abrogated by pretreatment with pertussis toxin, suggesting coupling through G proteins of the $\alpha_{i\text{-}o}$ subtypes.

Subsequent experiments demonstrated that the β_3 negative inotropic effect was mediated through activation of eNOS in human ventricular tissue (Gauthier *et al.,* 1998a), thereby lending support to our interpretation of the activation of countervailing inotropic pathways through different adrenoreceptors. As mentioned earlier, this paradigm somewhat recapitulates in the heart a similar coactivation of contracting and relaxing effects by catecholamines in the vasculature, where the resulting tone is controlled by a balance between direct vasoconstriction of smooth muscle and production of endothelium-derived relaxing factors, including NO. In the heart, the presence of both pathways (i.e., positively inotropic and NO-dependent negatively inotropic pathways) within each cardiac myocyte yields a similar negative feedback mechanism but in an autocrine fashion. Predictably, qualitative or quantitative alterations in either limb would alter the responsiveness to adrenergic stimulation and potentially contribute to the development of myocardial dysfunction, as reviewed later.

eNOS and Adrenergic Responsiveness in Other Cardiac Preparations Subsequent studies have emphasized that the functional consequence of endogenous NO production by eNOS on cardiac contraction is complex, with effects on peak contraction, duration of contraction, and onset of relaxation, the direction and magnitude of which vary according to the preparation used and the species considered. In addition, some confusion has arisen when results obtained with endogenous NOS inhibitors were directly compared with those of exogenous NO donor drugs. Experiments with the latter have identified a potentiation or a downregulation of the contractile response to β-adrenergic agonists in isolated hearts or cardiomyocytes, depending mainly on the concentration of the NO donor applied. Most studies evaluating the contractile effect of NO endogenously produced by eNOS do not identify an enhancement but rather an attenuation of the inotropic effect of catecholamines on heart muscle, as shown in our initial experiments detailed earlier. A similar observation was later reproduced in rat isolated atria (Sterin-Borda *et al.,* 1998) and atrial strips from human hearts (Gauthier *et al.,* 1998b). Furthermore, in rat atrial and ventricular myocytes, as well as in human ventricular tissue, exposure to isoproterenol induced an increase in intracellular cyclic GMP that was significantly reduced with NOS inhibitors (Gauthier *et al.,* 1998a; Sterin-Borda *et al.,* 1998; Joe *et al.,* 1998).

Several subsequent studies have since validated this paradigm in different animal preparations *in vivo,* where endogenous NO production blunts the positive inotropic effect of either infused catecholamines (Keaney *et al.,* 1996) or submaximal electrical stimulation of the left stellate ganglia in dogs (Takita *et al.,* 1998). In the latter study, intracoronary infusion of the NOS inhibitor, L-nitroarginine methyl ester (L-NAME), also significantly increased plasma norepinephrine concentration. The concept of eNOS-mediated attenuation of catecholamine effects received further confirmation from experiments in myocytes isolated from eNOS−/− myocytes, which exhibited an enhanced contractile responsiveness to positive inotropic agents in comparison to wild-type controls. This finding, independently obtained by two different groups (Gyurko *et al.,* 1997; Godecke *et al.,* 1998) was

not confirmed in a third study using isolated ventricular myocytes from these eNOS−/− mice, where the absence of potentiation of the effect of isoproterenol could be accounted for by the use of maximal stimulating concentrations of isoproterenol (1 μ*M* and greater) (Han *et al.,* 1998a).

LUSITROPIC EFFECTS OF eNOS

In the absence of pretreatment with catecholamines, stimulation of endocardial endothelial cells of ferret papillary muscles with substance P was shown to produce a shortening of the duration of contraction by inducing earlier onset of relaxation, with little effect on the peak force of contraction (Smith *et al.,* 1991). Qualitatively similar results were later obtained with bradykinin or substance P in isolated ejecting guinea pig hearts (Grocott-Mason *et al.,* 1994). In ventricular biopsy samples of human transplanted hearts which contain a mixture of endocardial and microvascular endothelial cells, and cardiac myocytes, activation of eNOS through β_3-adrenoceptor stimulation did not significantly alter the shape of electrically stimulated contractions [i.e., the shortening of the time to half-relaxation was mainly the result of a marked decrease in developed peak tension, without a significant change in diastolic relaxation (Gauthier *et al.,* 1998a)]. However, a study of the contractile performance of human transplanted hearts *in vivo* led to the observation that intracoronary administration of substance P reproduced the pattern of earlier onset of relaxation with a marginal decrease in peak systolic performance (Bartunek *et al.,* 1997), as has been previously identified in animal papillary muscle or isolated ejecting hearts. In addition, the interaction between β-adrenergic stimulation and the paracrine effects of substance P was examined by monitoring systolic and diastolic parameters in response to substance P infusion during the continuous infusion of dobutamine. Even though the results were mainly interpreted as an accentuation of the lusitropic effect of paracrine NO compared to that obtained with substance P alone, this study also showed a significant attenuation of the positive inotropic effect of dobutamine by NO that recapitulates the paradigm of the counterregulatory effect of eNOS activation on the response to catecholamines as detailed in the previous sections.

The mechanism underlying the potentiation of the lusitropic effect of substance P in the context of prestimulation with a β-adrenergic agonist remains elusive. It has been proposed to result from synergistic actions of NO-derived cyclic GMP and β-adrenergic-dependent activation of protein kinase A to desensitize contractile myofilaments to calcium. This interpretation appears to contradict the demonstration in isolated rat cardiomyocytes that the cyclic GMP and protein kinase G-mediated desensitization of cardiac myofilaments to calcium is abolished on concurrent stimulation with the β-adrenergic agonist isoproterenol (Shah *et al.,* 1994).

EFFECTS OF EXOGENOUS NO DONORS ON β-ADRENERGIC RESPONSIVENESS

Despite important biochemical differences between NO generated by exogenous NO donors or NO endogenously produced by nitric oxide synthase targeted to specific subcellular compartments, the use of nitrovasodilators or similar drugs has been valuable in understanding the potential intracellular mechanisms for the NO regulation of cardiac function. As previously observed with the regulation of L-type calcium current in isolated myocytes (Ono and Trautwein, 1991; Kirstein *et al.,* 1995), exogenous NO donors or cyclic GMP analogs exerted a biphasic effect on β-adrenergically induced positive inotropic effects depending on the concentration of the NO donor and the resulting increase in intracellular cyclic GMP (Fig. 4). This was shown both in isolated ventricular myocytes and papillary muscles from rats (Kojda *et al.,* 1996), open-chest dog hearts (Preckel *et al.,* 1997), and isolated feline papillary muscles (Mohan *et al.,* 1996). In the study by Mohan *et al.* (1996), a biphasic effect on developed tension was observed with either 8-bromo-cyclic GMP or treatment with zaprinast, an inhibitor of phosphodiesterase type V that increases endogenous cyclic GMP levels. Low concentrations of NO donor agents (and a resulting small increase in intracellular cGMP levels) increased developed tension, whereas higher concentrations resulted in a negative inotropic effect. Interestingly, the transition from positive to negative inotropic effect was shifted to lower concentrations of NO donor agents or zapri-

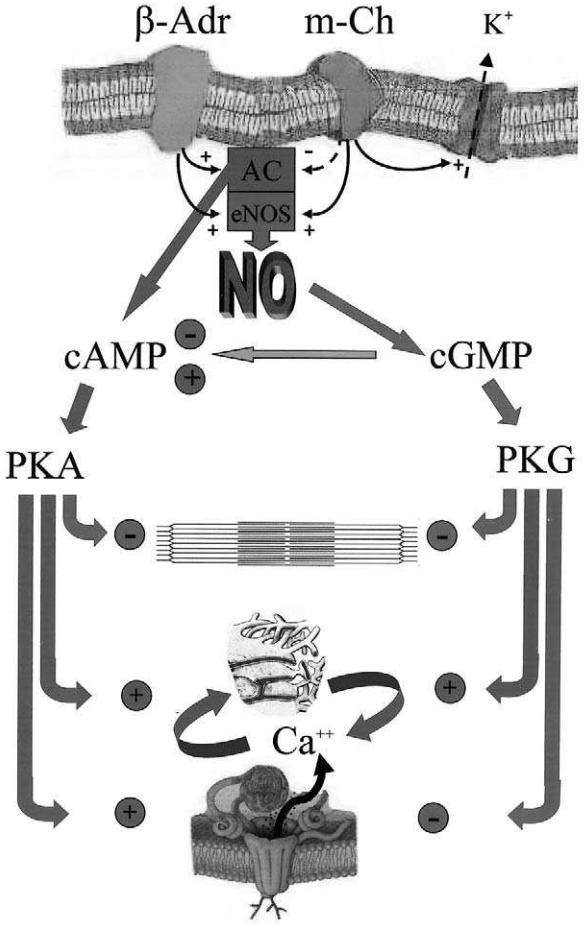


Figure 4 Intracellular pathways for the action of NO on excitation-contraction coupling.

nast when the muscles had been prestimulated with either muscarinic cholinergic agonists or with the β-adrenergic agonist isoproterenol. Even though the precise mechanism of the sensitization by previous β-adrenergic stimulation to the negative inotropic effect of cyclic GMP remained undetermined from these experiments, it is tempting to speculate that it may be related to the activation by isoproterenol of an endogenous nitric oxide synthase to further increase endogenous cyclic GMP, as observed in rat ventricular myocytes (Joe *et al.,* 1998) and human ventricular biopsies (Gauthier *et al.,* 1998a). The concentration-dependent shift from a positive to a negative inotropic effect of cyclic GMP elevation in the presence of β-adrenergic stimulation has not been observed in all species. For example, in a study on isolated ejecting guinea pig hearts, Prendergast and colleagues exclusively observed positive inotropic influences of either exogenous sodium nitroprusside or intracoronary substance P with β-adrenergic stimulation with dobutamine (Prendergast *et al.,* 1998). This positive inotropic influence was manifested only by a change in the decay of the peak developed pressure in response to long-term infusion of dobutamine, with no change in peak response to the catecholamine. Surprisingly, contrary to the previously mentioned observations in transplanted patients using similar protocols (Bartunek *et al.,* 1997), substance P had no significant effect on the lusitropic effect of dobutamine, emphasizing the need for caution when extrapolating functional results across species.

Regulation by iNOS

There is extensive evidence for the expression of iNOS protein in the multiple cell types comprising cardiac muscle, including in humans, in a variety of pathological conditions such as sepsis, transplant rejection, and in certain cases, heart failure. In these circumstances, the physiological/pathological role of NO extends beyond the fine tuning of cardiac contractile response to autonomic stimulation, as is the case for eNOS. NO produced by the ''high output'' iNOS enzyme has been implicated in many aspects of cardiomyocyte biology such as immune defense against intracellular microorganisms, including viruses, and apoptosis (for review see Balligand and Cannon, 1997). Although some or all of these phenomena may bear significantly on the contractile responsiveness of the intact heart, including of the heart *in vivo,* we will restrict our review on those effects of NO specifically produced by iNOS on the contractile responsiveness to β-adrenergic stimulation.

Cellular elements of the immune system have long been suspected to play a role in mediating the global myocardial dysfunction characteristic of septic shock, cardiac allograft rejection, and some forms of idiopathic cardiomyopathy (Lange and Schreiner, 1992). However, more recent work indicates that direct cell-mediated cytotoxicity is not required to induce myocardial depression in experimental models of sepsis in animals or contractile dysfunction in isolated ventricular myocytes exposed to inflammatory mediators, including sera from patients with septic shock (Gulick *et al.,* 1988, 1991; Reilly *et al.,* 1989; Natanson *et al.,* 1989). Cell-free supernatants obtained from activated lymphocyte or macrophage cultures reversibly inhibit the expected increase in cAMP and concomitant positive inotropic responses of ventricular myocytes to the β-adrenergic agonist isoproterenol, but they have no effect on basal cAMP levels or on baseline contractile function (Gulick *et al.,* 1988, 1991). This effect of activated immune-cell-conditioned medium on myocyte responsiveness to β agonists is not immediate, but requires hours to become apparent (Lange and Schreiner, 1992).

Several cytokines that are known to be present in medium conditioned by activated immune cells have been shown to induce the synthesis of the inducible isoform of nitric oxide (iNOS) in a number of cell types and tissues (Leone *et al.,* 1991; Scott-Burden *et al.,* 1992; Xie *et al.,* 1992) including in cardiac myocytes, as mentioned above.

iNOS and β-Adrenergic Stimulation

Since NO produced by constitutive eNOS in response to β-adrenergic agonists has been shown to exert a negatively inotropic, countervailing effect on cardiac myocyte contraction, subsequent experiments examined whether induction of iNOS with the resultant production of large quantities of NO would also modulate the contractile response to β-adrenergic stimulation in these cells.

Myocyte Contractile Function Following Exposure to Macrophage-Conditioned Medium Given the importance of an appropriate contextual ''network'' for the action of cytokines in many tissues, a first approach was to investigate the ability of physiological, species-specific combinations of inflammatory mediators to induce contractile dysfunction in adult cardiomyocytes. These were obtained from the supernatant of cultures of rat alveolar macrophages prestimulated with lipopolysaccharide (LPS).

In the absence of isoproterenol, exposure of primary isolates of adult rat ventricular myocytes to alveolar macrophage-conditioned medium for 24 hours affected neither contractile amplitude nor velocities of contraction or relaxation compared with myocytes incubated in a control, defined medium alone, whether or not the conditioned medium was obtained from macrophages preincubated with endotoxin. However, when myocytes were preincubated in medium containing supernatant from endotoxin-activated alveolar macrophage cultures, there was a marked decline in the amplitude of contraction in response to isoproterenol, as well as comparable declines in the velocities of shortening and relengthening. Importantly, addition of L-NMMA to myocytes preincubated in macrophage-conditioned medium fully restored the positive inotropic response of myocytes to isoproterenol.

This diminished inotropic response to isoproterenol only became apparent after a minimum of 12 to 16 hours of preincubation of myocytes with LPS-activated, macrophage-conditioned medium, consistent with increased synthesis of the inducible isoform of NO synthase in response to one

or more inflammatory mediators produced by activated macrophages (Knowles and Moncada, 1992; Balligand *et al.,* 1993b; Schulz *et al.,* 1992). This is in contrast to data previously reported by Finkel *et al.* (1992), who demonstrated that several recombinant cytokines, including TNF-α, IL-2, and IL-6, had negative inotropic effects within 2 to 3 min when added to superfusion medium bathing an isolated hamster papillary muscle preparation. This negative inotropic effect was apparent in the absence of any cardiotonic agents, including adrenergic agonists, and it could be prevented by addition of L-NMMA to the superfusion medium. As the rapid onset of the negative inotropic effect is inconsistent with increased transcription and synthesis of a calcium-insensitive, inducible isoform of NO synthase, it is possible, as these authors suggest (Finkel *et al.,* 1992), that these cytokines resulted in activation of a calcium/calmodulin-responsive constitutive isoform of NO synthase in papillary muscle.

Single Cell Analysis of iNOS Activity and Contractile Effect Although it is likely that induction of NO synthase within the myocytes themselves was the source of NO responsible for the negative inotropic response to isoproterenol, microvascular endothelial cells from adult rat ventricular tissue also respond to soluble inflammatory mediators in the endotoxin-activated alveolar macrophage-conditioned medium, suggesting that NO released by these cells may contribute to the contractile dysfunction of adjacent myocytes. To definitively characterize the functional significance of iNOS expression within cardiac myocytes themselves, the effect of various pharmacological modulators of NOS activity was examined on the contractile shortening and NO production in isolated single ventricular myocytes, as analyzed by both video-motion microscopy and a NO-specific porphyrinic microsensor.

The NO microsensor technology has been described and validated in both tissue and cell culture systems, with a detection limit of approximately 10 n*M*. The amperometric mode used in these experiments had been successfully used to measure the kinetics of NO release from endothelial cells in response to eNOS activation by agonists that increase intracellular calcium. In contrast with eNOS, the activity of the largely calcium-insensitive iNOS is not rapidly regulated by changes in intracellular calcium, but is usually sustained for several hours after it has been induced. In order to take advantage of the sensitivity and time resolution of the electrode, the cells were deprived of L-arginine, the substrate for NOS, in order to reduce their production of NO, and then the amino acid was abruptly reintroduced to observe an outburst of NO release with an acceptable signal-to-noise ratio.

Rapid changes of an analytical current could be obtained in the amperometric mode if cytokine-treated myocytes were first incubated for 4 hours in L-arginine-depleted medium and the recording initiated in buffer before and after L-arginine, the substrate for NO synthase, was reintroduced. Experimental conditions were then established that permitted recordings to be obtained of NO release from a single L-arginine-depleted cardiac myocyte. For these experiments, L-arginine was reintroduced through a micropipette with the injection parameters designed to deliver the substrate to a single cell. Following exposure to recombinant cytokines and a 4-hour incubation in L-arginine-depleted medium, the microinjection pipette and NO microsensor were aligned adjacent to an isolated rod-shaped cardiomyocyte. The preparation was then left until a stable baseline was observed for at least 10 min. The subsequent microinjection of L-arginine resulted in an increase of the NO signal that gradually returned to baseline over 15 to 20 min. The mean estimated concentration of NO at the plasma membrane was 104 ± 15.8 n*M*. In comparison, control experiments performed using the same technique on single rat alveolar macrophages induced *in vitro* with LPS, as described above (Balligand *et al.,* 1993b), resulted in readings between 155 and 800 n*M* ($n = 3$). Other controls were performed with cardiac myocytes, prepared as described above, after microinjection of D-arginine or L-arginine following a 4-hour incubation with the L-arginine analog L-NMMA, both conditions in which no signal could be recorded.

Finally, a typical decline in inotropic responsiveness to isoproterenol was also observed under experimental conditions in which NO release was detected from single cardiac myocytes with the NO microsensor. In other words, decline in responsiveness followed readdition of L-arginine, but not D-arginine, thereby reproducing the paradigm observed in cardiomyocytes exposed to LPS-activated macrophage-conditioned medium (Balligand *et al.,* 1993b, see earlier).

*Myocyte Contractile Function in Short-Term Heterotypic Cardiac Myocyte–Microvascular Endothelial Cell Cultures: Effect of IL-1*β To determine whether coronary microvascular endothelial cells (CMEC), after exposure to a specific immunologic stimulus, could generate sufficient nitric oxide to affect the function of adjacent cardiac myocytes, a coculture system was designed in which myocyte contractile function was used as a bioassay. Freshly isolated adult rat ventricular myocytes were plated directly on confluent, serum-starved low passage CMEC primary cultures that had been established either in control medium or in the presence of IL-1β (4 ng/ml). This concentration of the cytokine had clearly been shown to induce iNOS activity in microvascular endothelial cells (Ungureanu-Longrois *et al.,* 1995a). Coverslips containing the myocyte–CMEC cultures were then studied by videomicroscopy both at baseline and after perfusion with isoproterenol.

IL-1β alone did not have any effect on baseline or on isoproterenol-stimulated contractile function in cardiac myocytes after a 24-hour exposure (Ungureanu-Longrois *et al.,* 1995a; see earlier). However, cardiac myocytes plated on IL-1β-pretreated endothelial cells had a significantly reduced inotropic response to isoproterenol compared with myocytes plated on control endothelial cell cultures (Ungureanu-

Longrois *et al.,* 1995b). Furthermore, L-NMMA restored the responsiveness to isoproterenol of myocytes plated on IL-1β-pretreated endothelial cells, demonstrating that NO production from the endothelial cells was responsible for the decreased contractile response of cocultured myocytes.

POSTTRANSLATIONAL REGULATION OF iNOS ACTIVITY AFFECTS NO-DEPENDENT REGULATION OF CARDIAC CONTRACTION: L-ARGININE TRANSPORT

Among the constituents of the defined medium routinely used for the culture of adult rat ventricular myocytes, it was noted that insulin was required for NO production, as detected by nitrite release in cytokine-pretreated myocytes, although insulin had no effect on the extent of induction of iNOS mRNA or on maximal enzyme activity in myocyte cell lysates. Insulin was also required for the decrease in contractile responsiveness to isoproterenol to be manifest (Ungureanu-Longrois *et al.,* 1995a). Further experiments by W. Simmons and colleagues demonstrated that insulin significantly increased transmembrane L-arginine transport in myocytes and microvascular endothelial cells. This increase in NOS substrate availability could explain the increase in enzyme activity in intact cells, as well as the dependence on insulin for NO-mediated contractile dysfunction in myocytes (Simmons *et al.,* 1996).

Intracellular Mechanisms of Action of NO in Cardiac Muscle Cells[1]

The effects of NO can be distinguished between those dependent on cyclic GMP formation following the activation of guanylyl cyclase and those that are independent of cyclic GMP. This does not preclude from the possibility that NO acts simultaneously through both mechanisms, depending on the intra- or extracellular source, the intracellular compartmentation of NOS isoforms, the amount of NO produced, and local redox conditions, all of which are likely to impact on NO reactivity with its intracellular targets (Fig. 5). These restrictions aside, exogenous NO donor drugs as well as NOS inhibitors have been very useful to advance the understanding of the functional interaction between the NO synthase pathway and β-adrenergic signaling, which will only be considered briefly here.

Cyclic GMP-Dependent Mechanisms

ENHANCEMENT OF β-ADRENERGIC RESPONSE

Cardiomyocytes from most mammalian species express the type III isoform of cyclic nucleotide phosphodiesterases, or PDE3, which is allosterically inhibited by cyclic GMP. PDE3 has been implicated in mediating NO-induced increases in cyclic AMP and the subsequent activation of L-type calcium current and inotropy in isolated cardiac myocytes in rodents and in human atria (Kirstein *et al.,* 1995; Méry *et al.,* 1993). Low concentrations of NO donors also induced a moderate positively inotropic effect in adult rat ventricular myocytes (Kojda *et al.,* 1996; Preckel *et al.,* 1997) and in open chest dog hearts (Preckel *et al.,* 1997) as mentioned previously. In feline cardiac myocytes, the same pathway may mediate a rebound increase in L-type calcium current, calcium transients, and contraction after the abrupt removal of acetylcholine (Wang and Lipsius, 1995; Wang *et al.,* 1998).

Intracellular cyclic AMP levels might also be regulated ''upstream'' from the phosphodiesterases, for example, by direct regulation of adenylyl cyclase activity or its coupling to β-adrenoceptors through the stimulatory G protein α_s. Previous studies in lymphocytes also have shown that NO can modulate the GTPase activity of the small G protein p21-Ras (Lander *et al.,* 1995, 1997). NO-dependent increases in cyclic GMP may also potentiate cardiac contraction through mechanisms independent of cyclic AMP. Accordingly, Galione and colleagues (1991) provided evidence that intracellular cyclic GMP could activate calcium release from the ryanodine channel through activation of ADP ribosyl cyclase and subsequent increases in cyclic ADP ribose. More recently, these authors demonstrated that the pathway may be operative to increase calcium release from the sarcoplasmic reticulum in intact guinea pig cardiomyocytes (Iino *et al.,* 1997).

DECREASE IN β-ADRENERGIC RESPONSE

Cardiac myocytes from a variety of species also express a cyclic GMP-activated phosphodiesterase, or PDE2. A rat isoform of PDE2 was identified as a main cyclic nucleotide phosphodiesterase isoform in sinoatrial node cells from rabbits (Han *et al.,* 1995), where the muscarinic cholinergic accentuated antagonism on L-type calcium current was also completely abolished by a specific PDE2 inhibitor, EHNA. A similar pathway was found to be operative in atrioventricular and ventricular myocytes from rabbits and rats (Balligand *et al.,* 1995; Han *et al.,* 1995, 1996).

Combined with the notion that cardiomyocytes may express both PDE2 and PDE3, the coexistence of these functionally opposed pathways provides a explanation for the bidirectional effect of NO or cyclic GMP in response to β-adrenergic stimulation of L-type calcium current and contraction (Ono and Trautwein, 1991; Kojda *et al.,* 1996), as mentioned earlier. In circumstances where high levels of intracellular cyclic GMP are produced, such as those generated on NO production by iNOS, the resultant activation of PDE2 leads to an attenuation of isoproterenol-stimulated increase in cyclic AMP as well as the shortening of adult rat myocytes in culture (Joe *et al.,* 1998; Balligand *et al.,* 1993b). As mentioned before, a similar mechanism may be operative for the attenuation of β-adrenergic responsiveness by NO produced endogenously by eNOS through its ability to increase intracellular cyclic GMP following adrenergic stimulation, as

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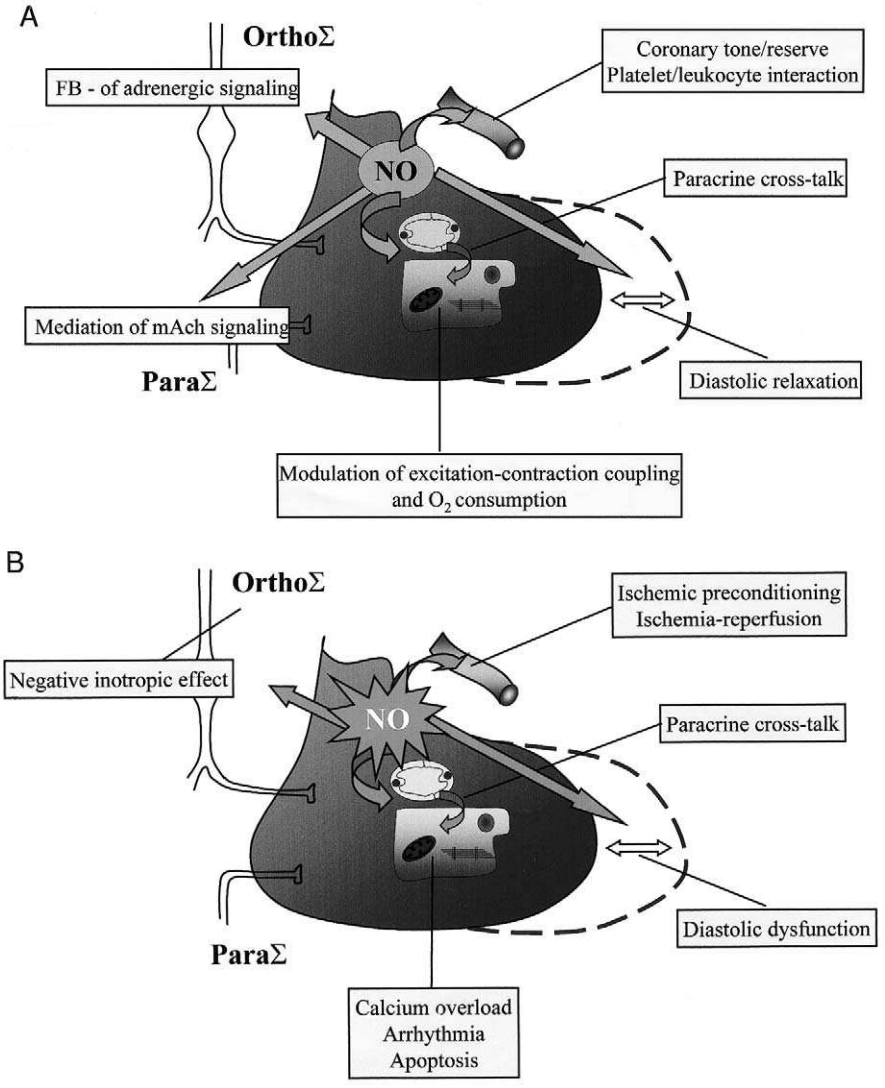


Figure 5 Modulation of cardiac contraction by NO in (A) normal heart and (B) diseased heart.

demonstrated in rat and human ventricular tissue (Gauthier *et al.,* 1998a; Joe *et al.,* 1998). The latter phenomenon could provide an explanation for the fact that NOS inhibitors affect myocyte contractile shortening only when they have been prestimulated with β-adrenoceptor agonists (Balligand *et al.,* 1993a,b).

In addition to activating PDE2, cyclic GMP can attenuate cardiomyocyte contraction by activating a cyclic GMP-dependent protein kinase (PKG) isoform. This protein may in turn decrease the L-type calcium current stimulated by cyclic AMP-dependent PKA (Méry *et al.,* 1991; Wahler and Dollinger, 1995), or downregulate the contractile responses of myofilament proteins independently of changes in calcium transients (Shah *et al.,* 1994; Yasuda and Lew, 1997; Goldhaber *et al.,* 1996). The latter mechanism, however, is unlikely to account for the attenuation of β-adrenergic responsiveness since it was shown to be abrogated on concomitant treatment of ventricular myocytes with isoproterenol (Shah *et al.,* 1994), as mentioned earlier.

Finally, using the model of *in vivo* endotoxin treatment, Sulakhe and colleagues studied in parallel the contractile responses to isoprenaline in isolated papillary strips and a variety of intracellular target proteins in cardiomyocytes isolated from endotoxin-injected rats. Attenuated contraction in response to isoprenaline in isolated muscles was paralleled by increased iNOS activity in myocytes, while the effect of phosphorylation of phospholamban and troponin-inhibitory subunit was decreased compared to extracts from control rats (Sulakhe *et al.,* 1996). These results would argue against an effect of iNOS-derived NO on PKG-mediated phosphorylation of troponin I to desensitize cardiac myofilaments to calcium (Yasuda and Lew, 1997).

Cyclic GMP-Independent Mechanisms

The oxidation of critical thiol residues on regulatory proteins by NO or by other NO-derived radicals (such as peroxynitrite) may account for some of the actions of NO on

cardiac function. These effects are more likely to be produced in the presence of high concentrations of NO and superoxide anion, such as following induction of inflammatory cytokines and iNOS in the setting of increased oxidative stress. Among the contractile regulatory proteins sensitive to oxidation, the cardiac calcium release channel (ryanodine receptor) and the L-type calcium channel have been shown to be possible targets (Xu *et al.,* 1998; Campbell *et al.,* 1996; Hu *et al.,* 1997; Zahradnikova *et al.,* 1997). NO and other oxidative metabolites may also regulate key enzymes regulating oxygen consumption and ATP generation within heart muscle, including creatine kinase and cytochrome C oxidase (for reviews, see Balligand and Cannon, 1997; Kelly *et al.,* 1996). These observations emphasize the need to verify that any effect produced with pharmacological concentrations of NO donors is representative of the action of endogenously generated NO within the temporal and spatial constraints imposed by the subcellular localization of each specific NOS isoform.

NOS and Contractile Dysfunction

Numerous studies have examined the involvement of either eNOS or iNOS in the depressed myocardial responsiveness to catecholamines in heart dysfunction from different etiologies, including sepsis, transplant rejection, and ischemic or dilated cardiomyopathies.

All of these pathologies have been shown to be accompanied by increased expression of iNOS in the myocardium, albeit with considerable variability regarding the abundance of iNOS protein and the predominant cellular source, as mentioned earlier. In contrast, although changes in the abundance of eNOS mRNA or protein may not be unidirectional in heart failure of all etiologies, it was shown to be reduced in end-stage failing hearts in ischemic and dilated cardiomyopathies (Drexler *et al.,* 1998) consistent with findings at the protein level (Gauthier *et al.,* 1998b).

eNOS and Arrhythmia

The changes in eNOS abundance noted earlier may bear on other important aspects of β-adrenergic stimulation of the heart. Aside from changes in inotropic parameters, we have observed that treatment of electrically paced human atrial strips with the NO synthase inhibitor, L-NMMA enhanced the arrhythmogenic effect of submaximal concentrations (10 n*M*) of isoproterenol, as manifested by the occurrence of aftercontractions (Gauthier *et al.,* 1998b). A role for eNOS in controlling the threshold for adrenergically induced ventricular arrhythmia is also supported by the following observations. In open chest dogs with acute coronary artery occlusion, Fei and colleagues (1997) showed that intrapericardial perfusion with L-arginine to increase NOS activity (as reflected by an increased NO effluent in the coronary sinus) protected the myocardium against the occurrence of ventricular fibrillation. More recently, the role of myocyte-specific eNOS was directly assessed in the control of ouabain-induced arrhythmias by comparing the occurrence of aftercontractions and I_{ti} current between ventricular myocytes from eNOS−/− mice and from wild-type controls. Ouabain induced more arrhythmic contractions and I_{ti} currents in eNOS−/− myocytes; these were efficiently prevented by the exogenous administration of *S*-nitrosoacetylcysteine, a NO donor (I. Kubota, X. Han, and R. A. Kelly, 2000, unpublished results), thereby supporting a role for myocyte eNOS in controlling the arrhythmic threshold. These observations may also explain the higher incidence of ventricular arrhythmias in circumstances where eNOS is downregulated. We previously showed such downregulation after intramyocardial elevation of cyclic AMP with drugs such as milrinone commonly employed in the treatment of end-stage heart failure (Belhassen *et al.,* 1996). The notion that eNOS may be downregulated in the failing myocardium (see later) may further explain the arrhythmogenic potential of inotropic drugs in patients with heart failure.

eNOS in Hypertrophic Myocardium

In contrast to idiopathic or ischemic dilated cardiomyopathies, eNOS expression may not be downregulated until the late, failing stage of hypertensive cardiomyopathy (and may even be transiently upregulated at earlier stages). In a study on isolated hypertrophic hearts and myocytes from rats with aortic stenosis, Bartunek and colleagues found that eNOS mRNA and protein levels were unchanged compared to controls but that chronic treatment with L-arginine, the substrate for NOS, significantly increased myocardial cyclic GMP levels. Despite the absence of any significant change in the development of cardiac hypertrophy, this chronic treatment with L-arginine blunted left ventricular systolic pressure and the contractile response to isoproterenol in both isolated hypertrophic hearts and myocytes (Bartunek *et al.,* 1998). In another study on isolated ventricular strips from hypertrophic hearts of either spontaneously hypertensive (SHR) nephrectomized or suprarenal aortic banded rats, treatment with 10 μmol of SNAP failed to reproduce the rightward shift of the concentration response curve for isoprenaline characteristically observed in control animals (Kotchi *et al.,* 1998). Such discrepancies again emphasize the need for caution when extrapolating the effects of exogenous NO donor drugs to endogenously produced NO.

Based on the previous observations that NO inhibits vascular smooth muscle cell proliferation, several authors have examined the potential influence of nitric oxide on the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. In one of these studies, Calderone and his colleagues found that inhibition of NO synthase with L-NMMA potentiated norepinephrine-stimulated protein synthesis in cultured cardiomyocytes. Atrial natriuretic peptide and the NO donor SNAP also suppressed adrenergically stimulated growth of myocytes and fibroblasts, an effect attributed to a cyclic GMP-dependent inhibition of calcium influx secondary to α_1-adrenergic receptor activation by the

catecholamines (Calderone *et al.,* 1998). These findings have been corroborated by the observation that NOS inhibition *in vivo* in rats augmented indices of cardiomyocyte hypertrophy (Devlin *et al.,* 1998), even though the experiments did not distinguish direct effects of NOS inhibition on cardiomyocytes from indirect effects secondary to increases in blood pressure in the animals.

Potential Role of iNOS in the Myocardial Depression of Heart Failure

When the response to increasing concentrations of isoproterenol was examined in isolated, electrically stimulated human ventricular myocytes *in vitro,* the response to β-adrenoceptor stimulation normalized to the maximum shortening induced by high calcium was depressed in myocytes from failing heart compared to those from nonfailing hearts. However, treatment with the NOS inhibitor L-NMMA failed to increase the isoproterenol-mediated rise in calcium in myocytes from failing hearts under the particular experimental conditions of this study (Harding *et al.,* 1998). In contrast, in another study on the isometric contractions of human left ventricular trabeculae from failing hearts, muscle strips exhibited a decreased responsiveness to β-adrenergic stimulation manifested by a depressed peak tension and abbreviated early relaxation time. Importantly, these alterations not only were significantly correlated with the extent of iNOS activity and iNOS mRNA abundance but also were corrected on treatment of the left ventricular trabeculae with L-NMMA (Drexler *et al.,* 1998). As mentioned earlier, the inotropic effect of dobutamine was similarly attenuated on intracoronary infusion of substance P (which is expected to increase paracrine production of NO) both in nonfailing transplant recipients and in patients with dilated nonischemic cardiomyopathy (Prendergast *et al.,* 1998). In patients with dilated cardiomyopathy, Hare and colleagues observed a potentiation of the inotropic response to peripheral infusion of dobutamine after intracoronary administration of L-NMMA (Hare *et al.,* 1995, 1998b). The contrast with unaltered responsiveness to the catecholamine in normal patients was interpreted as an increased sensitivity of the *in situ* failing heart to NOS inhibition. Other studies on cardiomyocytes isolated from animal models of heart failure lend support to the same paradigm (Yamamoto *et al.,* 1997). Finally, in isolated atrial and ventricular strips from human failing and nonfailing hearts, the ability of exogenous NO donors to produce quantitatively and qualitatively similar effects adds further evidence for a significant role of NO as a regulator of β-adrenergic responsiveness in the heart (Flesch *et al.,* 1997).

References

Allen, D. H., and Kentish, J. C. (1985). The cellular basis of the length-tension relation in cardiac muscle. *J. Mol. Cell. Cardiol.* **310,** 821–840.

Andries, L. J., Brutsaert, D. L., and Sys, S. U. (1998). Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. *Circ. Res.* **82,** 195–203.

Arstall, M. A., Bailey, C., Gross, W. L., Bak, M., Balligand, J. L., and Kelly, R. A. (1998). Reversible S-nitrosation of creatinine kinase by nitric oxide in adult rat ventricular myocytes. *J. Mol. Cell. Cardiol.* **30,** 979–988.

Ashkenazi, A., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J., and Ramachandran, J. (1987). An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science* **238,** 672–675.

Balligand, J. L., and Cannon, P. J. (1997). Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1846–1858.

Balligand, J. L., and Smith, T. W. (1997). Molecular regulation of NO synthase in the heart. *In* ''Endothelial Modulation of Cardiac Contraction'' (A. M. Shah and M. J. Lewis, eds.), pp. 53–71. Harwood, London.

Balligand, J. L., Kelly, R. A., Marsden, P. A., Smith, T. W., and Michel, T. (1993a). Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 347–351.

Balligand, J. L., Ungureanu, D., Kelly, K. A., Kobzik, L., Pimental, D., Michel, T., *et al.* (1993b). Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. *J. Clin. Invest.* **91,** 2314–2319.

Balligand, J. L., Kobzik, L., Han, X., Kaye, D. M., Belhassen, L., O'Hara, D. S., *et al.* (1995). Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (type III) nitric oxide synthase in cardiac myocytes. *J. Biol. Chem.* **270,** 14582–14586.

Bartunek, J., Shah, A. M., Vanderheyden, M., and Paulus, W. J. (1997). Dobutamine enhances cardiodepressant effects of receptor-mediated coronary endothelial stimulation. *Circulation* **95,** 90–96.

Bartunek, J., Dempey, S., Weinberg, E. O., Ho, N., Tajima, M., Rohrbach, S., and Lorell, B. H. (1998). Chronic L-arginine treatment increases cardiac cyclic guanosine 5'-monophosphate in rats with aortic stenosis: Effects on left ventricular mass and beta-adrenergic contraction reserve. *J. Am. Coll. Cardiol.* **32,** 528–535.

Belhassen, L., Kelly, R. A., Smith, T. W., and Balligand, J. L. (1996). Nitric oxide synthase (NOS3) and contractile responsiveness to adrenergic and cholinergic agonists in the heart. Regulation of NOS3 transcription in vitro and in vivo by cAMP in rat cardiac myocytes. *J. Clin. Invest.* **97,** 1908–1915.

Belhassen, L., Feron, O., Kaye, D. M., Michel, T., and Kelly, R. A. (1997). Regulation by cAMP of post-translational processing and subcellular targeting of endothelial nitric oxide synthase (type 3) in cardiac myocytes. *J. Biol. Chem.* **272**(17), 11198–11204.

Berger, H. J., Prasad, S. K., Davidoff, A. J., Pimental, D., Ellingsen, O., Marsh, J. D., Smith, T. W., and Kelly, R. A. (1994). Continual electric field stimulation preserves contractile function of adult ventricular myocytes in primary culture. *Am. J. Physiol.* **166,** 341–349.

Bode, D. C., Kanter, J. R., and Brunton, L. L. (1991). Cellular distribution of phosphodiesterase isoforms in rat cardiac tissue. *Circ. Res.* **68,** 1070–1079.

Bogatkewitsch, G. S., Lenz, W., Jakobs, K. H., and van Koppen, C. J. (1996). Receptor internalization delays m4 muscarinic acetylcholine receptor resensitization at the plasma membrane. *Mol. Pharmacol.* **50,** 424–429.

Bonner, T. I. (1989). The molecular basis of muscarinic receptor diversity. *Trends Neurosci.* **12,** 148–151.

Buxton, B. F., Jones, C. R., Molenaar, P., and Summers, R. J. (1987). Characterization and autoradiographic localization of β-adrenoreceptor subtypes in human cardiac tissues. *Br. J. Pharmacol.* **92,** 299–310.

Calderone, A., Thaik, C. M., Takahashi, N., Chang, D. L. F., and Colucci, W. S. (1998). Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. *J. Clin. Invest.* **101,** 812–818.

Campbell, D. L., Stamler, J. S., and Strauss, H. C. (1996). Redox modulation of L-type calcium channel in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and *S*-nitrosothiols. *J. Gen. Physiol.* **108,** 277–293.

Conlon, K., Collins, T., and Kidd, C. (1996). Modulation of vagal actions on heart rate produced by inhibition of nitric oxide synthase in the anesthetized ferret. *Exp. Physiol.* **81,** 547–550.

Cook, R. P. (1926). *J. Physiol.* **62,** 160.

Couet, J., Li., S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997). Identification of peptide and protein ligands for the caveolin-scaffolding domain. *J. Biol. Chem.* **272,** 6525–6533.

Devlin, A. M., Brosnan, M. J., Graham, D., Morton, J. J., McPhaden, A. R., Mcintyre, M., Hamiton, C. A., Reid, J. L., and Dominiczak, A. F. (1998). Vascular smooth muscle cell polyploidy and cardiomyocyte hypertrophy due to chronic NOS inhibition in vivo. *Am. J. Physiol.* **274,** 52–59.

Drexler, H., Kästner, S., Strobel, A., Studer, R., Brodde, O. E., and Hasenfuss, G. (1998). Expression, activity and functional significance of inducible nitric oxide synthase in the failing human heart. *J. Am. Coll. Cardiol.* **32,** 955–963.

Dwyer, M. A., Bredt, D. S., and Snyder, S. H. (1991). Nitric oxide synthase: Irreversible inhibition by L-N^G-nitroarginine in brain in vitro and in vivo. *Biomed. Biophys. Res. Commun.* **176,** 1136–1141.

Egleme, C., Godfraind, T., and Miller, R. C. (1984). Enhanced responsiveness of rat isolated aorta to clonidine after removal of the endothelial cells. *Br. J. Pharmacol.* **81,** 16–18.

Elvan, A., Rubart, M., and Zipes, D. P. (1997). NO modulates autonomic effects on sinus discharge rate and AV nodal conduction in open-chest dogs. *Am. J. Physiol.* **272,** H263–H271.

Fei, L., Baron, A. D., Henry, D. P., and Zipes, D. P. (1997). Intrapericardial delivery of L-arginine reduces the increased severity of ventricular arrhythmias during sympathetic stimulation in dogs with acute coronary occlusion: Nitric oxide modulates sympathetic effects on ventricular electrophysiological properties. *Circulation* **96,** 4044–4049.

Feron, O. (1999). Intracellular localization and activation of endothelial nitric oxide synthase. *Curr. Opin. Nephrol. Hypertens.* **8,** 55–59.

Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996). Endothelial nitric oxide synthase targeting to caveolae. Specific interaction with caveolin isoforms in cardiac myocytes and endothelial cells. *J. Biol. Chem.* **271,** 22810–22814.

Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J. Biol. Chem.* **272,** 17744–17748.

Feron, O., Michel, J. B., Sase, K., and Michel, T. (1998a). Dynamic regulation of endothelial nitric oxide synthase: Complementary roles of dual acylation and caveolin interactions. *Biochemistry* **37,** 193–200.

Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998b). The endothelial nitric oxide synthase–caveolin regulatory cycle. *J. Biol. Chem.* **273,** 3125–3128.

Feron, O., Dessy, C., Opel, D. J., Arstall, M. A., Kelly, R. A., and Michel, T. (1998). Modulation of the endothelial nitric oxide synthase-caveolin interaction in cardiac myocytes: Implications for the autonomic regulation of heart rate. *J. Biol. Chem.* **273,** 27383–27388.

Finkel, M. S., Oddis, C. V., Jacob, T. D., Watkin, S. C., Hattler, B. G., and Simmons, R. L. (1992). Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* **257,** 387–389.

Flesch, M., Kilter, H., Cremers, B., Lenz, O., Sudkamp, M., Kuhn-Regnier, F., and Bohm, M. (1997). Acute effect of nitric oxide and cyclic GMP on human myocardial contractility. *J. Pharmacol. Exp. Ther.* **281,** 1340–1349.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Galione, A., Lee, H. C., and Busa, WB. (1991). Ca$^{(2+)}$-induced Ca^{2+} release in sea urchin egg homogenates: Modulation by cyclic ADP-ribose. *Science* **253,** 1143–1146.

Gallo, M. P., Alloatti, G., Eva, C., Oberto, A., and Levi, R. C. (1993). M1 muscarinic receptors increase calcium current and phosphoinositide turnover in guinea pig ventricular cardiocytes. *J. Physiol.* **471,** 41–60.

Garcia-Cardena, G., Martasek, P., Siler Masters, B. S., Skidd, P. M., Couet, J., Li, S., *et al.* (1997). Dissecting the interaction between nitric oxide synthase and caveolin. *J. Biol. Chem.* **272,** 25437–25440.

Gauthier, C., Tavernier, G., Charpentier, F., Langin, D., and Le Marec, H. (1996). Functional β3-adrenoceptor in the human heart. *J. Clin. Invest.* **98,** 556–562.

Gauthier, C., Leblais, V., Kobzik, L., Trochu, J. N., Khandoudi, N., Bril, A., Balligand, J. L., and Le Marec, H. (1998a). The negative inotropic effect of beta-3-adrenergic stimulation is mediated by activation of nitric oxide synthase pathway in human ventricle. **102,** 1377–1384.

Gauthier, C., Morteza, E., Baron, O., and Balligand, J. L. (1998b). Control of contractile and rhythmic properties of human atrial tissue by a NO pathway. *Circulation* (Suppl. 17), 838 (Abstract).

Ghosh, S., Gachhui, R., Crooks, C., Wu, C., Lisanti, M. P., and Stuehr, D. J. (1998). Interaction between caveolin-1 and the reductase domain of endothelial nitric oxide synthase. *J. Biol. Chem.* **273,** 22267–22271.

Gibbons, W. R. (1986). Cellular control of cardiac contraction. *In* ''The Heart and Cardiovascular System'' (H. A. Fozzard, eds.), (Scientific Foundation). Vol. 1, pp. 747–778. Raven, New York.

Godecke, A., Heinicke, T., Decking, U. K. M., Stumpe, T., and Schrader, J. (1998). β-adrenergic stimulation of eNOS-deficient mice hearts. *Circulation* **98**(Suppl.), 1–70, and 353.

Godfraind, T., Egleme, C., and Al-Osachie, I. (1985). Role of endothelium in the contractile response of rat aorta to α-adrenoceptor agonists. *Clin. Sci.* **68**(Suppl. 10), 65–71.

Goldberg, N. D., and Haddox, M. K. (1977). Cyclic GMp metabolism and involvement in biological regulation. *Annu. Rev. Biochem.* **46,** 823–896.

Goldhaber, J. I., Kim, K. H., Natterson, P. D., Lawrence, T., Yang, P., and Weiss, J. N. (1996). Effects of TNF-alpha on $[Ca^{2+}]_i$ and contractility in isolated adult rabbit ventricular myocytes. *Am. J. Physiol.* **271,** H1449–H1455.

Goldman, P. S., Schlador, M. L., Shapiro, R. A., and Nathanson, N. M. (1996). Identification of a region required for subtype-specific agonist-induced sequestration of the m2 muscarinic acetylcholine receptor. *J. Biol. Chem.* **271,** 4215–4222.

Grocott-Mason, R., Anning, P., Evans, H., Lewis, M. J., and Shah, A. M. (1994). Modulation of left ventricular relaxation in isolated ejecting heart by endogenous nitric oxide. *Am. J. Physiol.* **267,** H1804–H1813.

Gulick, T., Chung, M. K., Pieper, S. J., Schreiner, G. F., and Lange, L. G. (1988). Immune cytokine inhibition of beta-adrenergic agonist stimulated cyclic AMP generation in cardiac myocytes. *Biochem. Biophys. Res. Comm.* **150**(7), 7–9.

Gulick, T., Pieper, S. J., Murphy, M. A., Lange, L. G., and Schreiner, G. F. (1991). A new method for assessment of cultured cardiac myocyte contractility detects immune factor-mediated inhibition of β-adrenergic responses. *Circulation* **84,** 313–321.

Guo, W., Kada, K., Kamiya, K., and Toyama, J. (1997). IGF-I regulates K$^{(+)}$-channel expression of cultured neonatal rat ventricular myocytes. *Am. J. Physiol.* **272,** H2599–H2606.

Guo, X., Laflamme, M. A., and Becker, P. L. (1996). Cyclic ADP-ribose does not regulate sarcoplasmic reticulum Ca^{2+} release in intact cardiac myocytes. *Circ. Res.* **79,** 147–151.

Gyurko, R., Fishman, M. C., and Huang, P. L. (1997). ''Enhanced Systolic Contractility and Preserved Diastolic Relaxation in Mice Deficient in Endothelial Nitric Oxide Synthase.'' Presented at the 4th International Meeting on the Biology of Nitric Oxide, Kyoto.

Han, X., Shimoni, Y., and Giles, W. R. (1995). A cellular mechanism for nitric oxide-mediated cholinergic control of mammalian heart rate. *J. Gen. Physiol.* **106,** 45–65.

Han, X., Kobzik, L., Balligand, J. L., Kelly, R. A., and Smith, T. W. (1996). Nitric oxide synthase (NOS3)-mediated cholinergic modulation of Ca^{2+} current in adult rabbit atrioventricular nodal cells. *Circ. Res.* **78,** 998–1008.

Han, X., Kubota, I., Feron, O., Opel, D. J., Arstall, M. A., Zhao, *et al.* (1998b). Muscarinic cholinergic regulation of cardiac myocyte ICa-L is absent in mice with targeted disruption of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6510–6515.

Han, X., Kobzik, L., Sverson, D., and Shimoni, Y. (1998a). Characteristics of nitric oxide-mediated cholinergic modulation of calcium current in rabbit sino-atrial node. *J. Physiol.* **15,** 741–754.

Harden, T. K., Petch, L. A., Traynelis, S. F., and Waldo, G. L. (1985). Agonist-induced alteration in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.* **260,** 13060–13066.

Harding, S. E., Davies, C. H., Money-Kyrle, A. M., and Poole-Wilson, P. A. (1998). An inhibitor of nitric oxide synthase does not increase contraction or β-adrenoceptor sensitivity in ventricular myocytes from failing human heart. *Cardiovasc. Res.* **40,** 523–529.

Hare, J. M., and Stamler, J. S. (1999). NOS: Modulator, not mediator of cardiac performance. *Nat. Med.* **5,** 273–274.

Hare, J. M., Loh, E., Creager, M. A., and Colucci, W. S. (1995). Nitric oxide inhibits the positive inotropic response to beta-adrenergic stimulation in humans with left ventricular dysfunction. *Circulation* **92,** 2198–2203.

Hare, J. M., Kim, B., Flavahan, N. A., Ricker, K. M., Peng, X., Colman, L., Weiss, R. G., and Kass, D. A. (1998a). Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart. *J. Clin. Invest.* **101,** 1424–1431.

Hare, J. M., Givertz, M. M., Creager, M. A., and Colucci, W. S. (1998b). Increased sensitivity to nitric oxide synthase inhibition in patients with heart failure. Potentiation of β-adrenergic inotropic responsiveness. *Circulation* **97,** 161–166.

Hartzell, N. C., and Simmons, M. A. (1987). Comparison of effects of acetylcholine on calcium and potassium currents in frog atrium and ventricle. *J. Physiol.* **389,** 411–422.

Horackova, M., Armour, J. A., Hopkins, D. A., and Huang, M. H. (1995). Nitric oxide modulates signaling between cultured adult peripheral cardiac neurons and cardiomyocytes. *Am. J. Physiol.* **269,** 504–510.

Hu, H., Chiamvimonvat, N., Yamaghishi, T., and Marban, E. (1997). Direct inhibition of expressed cardiac L-type Ca^{2+} channels by *S*-nitrosothiol nitric oxide donors. *Circ. Res.* **81,** 742–752.

Huang, P. L., Huang, Z., Moshimo, H., Block, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **377,** 239–242.

Iino, S., Cui, Y., Galione, A., and Terrar, D. A. (1997). Actions of cADP-ribose and its antagonists on contraction in guinea pig isolated ventricular myocytes. Influence of temperature. *Circ. Res.* **81,** 879–884.

Ishikawa, H. (1968). Formation of elaborate networks of T-system tubules in cultured skeletal muscle with special reference to the T-system formation. *J. Cell Biol.* **38,** 51–66.

Ishikawa, Y., and Homcy, C. J. (1997). The adenylyl cyclases as integrators of transmembrane signal transduction. *Circ. Res.* **80,** 297–304.

Joe, E. K., Schussheim, A. E., Longrois, D., Mäki, T., Kelly, R. A., Smith, T. W., and Balligand, J. L. (1998). Regulation of cardiac myocyte contractile function by inducible nitric oxide synthase (iNOS): Mechanisms of contractile depression by nitric oxide. *J. Mol. Cell. Cardiol.* **30,** 303–315.

Ju, H., Zou, R., Venema, V. J., and Venema, R. C. (1997). Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J. Biol. Chem.* **272,** 18522–18525.

Jumrussirikul, P., Dinerman, J., Dawson, T. M., Dawson, V. L., Ekelund, U., Georgakopoulos, D., Schramm, L. P., Calkins, H., Snyder, S. H., Hare, J. M., and Berger, R. D. (1998). Interaction between neuronal nitric. *J. Clin. Invest.* **102,** 1279–1285.

Katz, A., Wu, D., and Simon, M. I. (1992). Subunits βg of heterotrimeric G-protein activate β2 isoform of phospholipase C. *Nature* **360,** 686–689.

Katz, A. M. (1977). ''Physiology of the Heart,'' pp. 137–159. Raven, New York.

Katz, A. M. (1979). Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: An historical review. *Adv. Cyclic. Nucleotide Res.* **5,** 453–472.

Kaye, D. M., Wiviott, D., Kobzik, L., Kelly, R. A., and Smith, T. W. (1997). *S*-nitrosothiols inhibit neuronal norepinephrine transport. *Am. J. Physiol.* **272,** H875–H883.

Keaney, J. F., Jr., Hare, J. M., Balligand, J. L., Loscalzo, J., Smith, T. W., and Colucci, W. S. (1996). Inhibition of nitric oxide synthase augments myocardial contractile responses to beta-adrenergic stimulation. *Am. J. Physiol.* **271,** H2646–H2652.

Keeley, S. L., Lincoln, T. M., and Corbin, J. D. (1978). Interaction of acetylcholine and epinephrine on heart cyclic AMP-dependent protein kinase. *Am. J. Physiol.* **234,** H432–H438.

Kelly, R. A., Balligand, J. L., and Smith, T. W. (1996). Nitric oxide and cardiac function. *Circ. Res.* **79**(3), 363–380.

Kilter, H., Lenz, O., La Rosée, K., Flesch, M., Schwinger, R. H., Mädge, M., *et al.* (1995). Evidence against a role of nitric oxide in the indirect negative inotropic effect of M-cholinoreceptor stimulation in human ventricular myocardium. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352,** 308–312.

Kirstein, M., Rivet-Bastide, M., Hatem, S., Benardeau, A., Mercadier, J. J., and Fischmeister, R. (1995). Nitric oxide regulates the calcium current in isolated human atrial myocytes. *J. Clin. Invest.* **95,** 794–802.

Knowles, R. G., and Moncada S. (1992). Nitric oxide as a signal in blood vessels. *Trends Biochem. Sci.* **17,** 399–402.

Kojda, G., Kottenberg, K., Nix, P., Schluter, K. D., Piper, H. M., and Noack, E. (1996). Low increase of cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. *Circ. Res.* **78,** 91–101.

Kotchi, E., Weisselberg, T., Rohnert, P., Preiss, M., Heinroth-Hoffmann, I., Osten, B., and Brodde, O. E. (1998). Nitric oxide inhibits isoprenaline-induced positive inotropic effects in normal, but not in hypertrophied rat heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **357,** 579–583.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6438–6452.

Lander, H. M., Ogiste, J. S., Pearce, S. F., Levi, R., and Novogrodsky, A. (1995). Nitric oxide-stimulated guanine nucleotide exchange on p21ras. *J. Biol. Chem.* **270,** 7017–7020.

Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., and Quilliam, L. A. (1997). A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J. Biol. Chem.* **272,** 4323–4326.

Lange, L. G., and Schreiner, G. F. (1992). Immune cytokines and cardiac disease. *Trends Cardiovasc. Med.* **2,** 145–151.

Lefkowitz, R. J. (1998). G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* **273,** 18677–18680.

Leone, A., Palmer, R. J. J., Knowles, R. G., Francis, P. L., Ashton, D. S., and Moncada, S. (1991). Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. *J. Biol. Chem.* **266,** 23790–23795.

Levy, M. N. (1977). Parasympathetic control of the heart. *In* ''Neural Regulation of the Heart'' (W. C. Randal ed.), pp. 95–129. Oxford Univ. Press, New York.

Löffenholz, K., and Pappano, A. J. (1985). The parasympathetic neuroeffector junction of the heart. *Pharm. Rev.* **37,** 1–24.

Loscalzo, J., and Welch, G. (1995). Nitric oxide and its role in the cardiovascular system. *Prog. Cardiovasc. Dis.* **38,** 87–104.

Martin, W., Furchgott, R. F., Villani, G. M., and Jothianandan, D. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.* **237**(2), 529–538.

Méry, P. F., Lohmann, S. M., Walter, U., and Fischmeister, R. (1991). Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **288,** 1197–1201.

Méry, P. F., Mavoine, C., Belhassen, L., Pecker, F., and Fischmeister, R. (1993). Nitric oxide regulates cardiac Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J. Biol. Chem.* **268,** 26286–26295.

Méry, P. F., Hove-Madsen, L., Chesnais, J. M., Hartzell, H. C., and Fischmeister, R. (1996). Nitric oxide synthase does not participate in negative inotropic effect of acetylcholine in frog heart. *Am. J. Physiol.* **270,** H1178–H1188.

Michel, T., and Feron, O. (1997). Nitric oxide synthases: Which, where, how and why? *J. Clin. Invest.* **100,** 2146–2152.

Michel, J. B., Feron, O., Sase, K., Prabhakar, P., and Michel, T. (1997). Caveolin versus calmodulin. Counterbalancing allosteric modulators of nitric oxide synthase. *J. Biol. Chem.* **272,** 25907–25912.

Mohan, P., Brutsaert, D., Paulus, W., and Sys, S. U. (1996). Myocardial contractile response to nitric oxide and cGMP. *Circulation* **93,** 1223–1229.

Moncada, S., and Higgs, E. A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* **9,** 1319–1330.

Moore, P. K., al-Swayeh, D. A., Chong, N. W. S., Evans, R. A., and Gibson, A. (1990). L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. *Br. J. Pharmacol.* **99,** 408–412.

Morad, M., and Cleeman, L. (1987). Role of Ca^{2+} channel in development of tension in heart muscle. *J. Mol. Cell. Cardiol.* **19,** 527–553.

Morgan, J. P., and Morgan, K. G. (1982). Vascular smooth muscle: The first recorded Ca^{2+} transients. Pfluegers *Arch.* **395,** 75–77.

Murad, F., Chi, Y. M., Rall, T. W., and Sutherland, E. M. (1962). Adenylylcyclase. The effect of catecholamines and choline esters on the formation of adenosine 3',5'-phosphate by preparations from cardiac muscle and liver. *J. Biol. Chem.* **237,** 1233–1238.

Musialek, P., Lei, M., Brown, H. F., Paterson, D. J., and Casadei, B. (1997). Nitric oxide can increase heart rate by stimulating the hyperpolarization-activated inward current, I(f). *Circ. Res.* **81,** 60–68.

Natanson, C., Eichenholz, P. W., Danner, R. L., Eichacker, P. Q., Hoffman, W. D., Kuo, G. C., Banks, S. M., MacVittie, T. J., and Parrillo, J. E. (1989). Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *J. Exp. Med.* **169**(3), 823–832.

Okamoto, T., Schlegel, A., Scherer, P., and Lisanti, M. P. (1998). Caveolin, a family of scaffolding proteins for organizing ''preassembled signaling complexes'' at the plasma membrane. *J. Biol. Chem.* **273,** 5419–5422.

Ono, K., and Trautwein, W. (1991). Potentiation by cyclic GMP of beta-adrenergic effect on Ca^{2+} current in guinea-pig ventricular cells. *J. Physiol.* **443,** 387–404.

Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J. L., and Hosey, M. M. (1995). Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. *J. Biol. Chem.* **270,** 29004–29011.

Preckel, B., Kojda, G., Schlack, W., Ebel, D., Kottenberg, K., Noack, E., and Thamer, V. (1997). Inotropic effects of glyceryl trinitrate and spontaneous NO donors in the dog heart. *Circulation* **96,** 2675–2682.

Prendergast, B. D., MacCarthy, P., Wilson, J. F., and Shah, A. M. (1998). Nitric oxide enhances the inotropic response to β-adrenergic stimulation in the isolated guinea-pig heart. *Basic Res. Cardiol.* **93,** 276–284.

Raposo, G., Dunia, I., Marullo, S., André, Guillet, J.-G., Strosberg, A. D., Benedetti, E. L., and Hoebeke, J. (1987). Redistribution of muscarinic acetylcholine receptors on human fibroblasts induced by regulatory ligands. *Biol. Cell* **60,** 117–124.

Rees, D. D., Palmer, R. M., Hoolson, H. F., and Moncada, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.* **96**(2), 418–424.

Reilly, J. M., Cunnion, R. E., Burch-Whitman, C., Parker, M. M., Shelhamer, J. H., and Parrillo, J. E. (1989). A circulating myocardial depressant substance is associated with cardiac dysfunction and peripheral hypoperfusion (lactic acidemia) in patients with septic shock. *Chest* **95**(5), 1072–1080.

Rizzo, V., McIntosh, D. P., Oh, P., and Schnitzer, J. E. (1998). In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. *J. Biol. Chem.* **273,** 34724–34729.

Robertson, S. P., Johnson, J. D., Holroyde, M. J., Kranias, E. G., Potter, J. D., and Solaro, R. J. (1982). The effect of troponin I phosphorylation on the Ca^{2+} binding properties of the Ca^{2+}-regulatory site of bovine cardiac troponin. *J. Biol. Chem.* **257,** 260–263.

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993). Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* **122,** 789–807.

Sase, K., and Michel, T. (1997). Expression and regulation of endothelial nitric oxide synthase. *Trends Cardiovasc. Med.* **7,** 25–34.

Schmidt, H. H., Gagne, G. D., Nakane, M., Pollock, J. S., Miller, M. F., and Murad, F. (1992). Mapping of neural nitric oxide synthase in the rat suggests frequent colocalization with NADPH diaphorase but not with soluble guanylyl cyclase and novel paraneural functions for nitrinergic signal transduction. *J. Histochem. Cytochem.* **40,** 139–156.

Schultz, R., Nava, E., and Moncada, S. (1992). Induction and potential biological relevance of a Ca^{2+}-dependent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.* **105,** 575–580.

Schwarz, P., Diem, R., Dun, N. J., and Förstermann, U. (1995). Endogenous and exogenous nitric oxide inhibits norepinephrine release from rat heart sympathetic nerves. *Circ. Res.* **77,** 841–848.

Scott-Burden, T., Schini, V. B., Elizondo, E., Junquero, D. C., and Vanhoutte, P. M. (1992). Platelet-derived growth factor suppresses and fibroblast growth factor enhances cytokine-induced production of nitric oxide by cultured smooth muscle cells. Effects on cell proliferation. *Circ. Res.* **71,** 1088–1100.

Sears, C. E., Choate, J. K., and Paterson, D. J. (1998). Inhibition of nitric oxide synthase slows heart rate recovery from cholinergic activation. *J. Appl. Physiol.* **84,** 1596–1603.

Sears, C. E., Choate, J. K., and Paterson, D. J. (1999). NO–cGMP pathway accentuates the decrease in heart rate caused by cardiac vagal nerve stimulation. *J. Appl. Physiol.* **86,** 510–234.

Seki, T., Hagiwara, H., Naruse, K., Kadowaki, M., Kashiwagi, M., Demura, H., *et al.* In situ identification of messenger RNA of endothelial type nitric oxide synthase in rat cardiac myocytes. (1996). *Biochem. Biophys. Res. Commun.* **218,** 601–605.

Shah, A. M., Spurgeon, H. A., Sollott, S. J., Talo, A., and Lakatta, E. G. (1994). 8-Bromo-cGMP reduces the myofilament response to Ca^{2+} in intact cardiac myocytes. *Circ. Res.* **74,** 970–978.

Sharma, V. K., Colecraft, H. M., Wang, D. X., Levey, A. I., Grigorenko, E. V., Yeh, H. H., and Sheu, S.-S. (1996). Molecular and functional identification of m1 muscarinic acetylcholine receptors in rat ventricular myocytes. *Circ. Res.* **79,** 86–93.

Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., *et al.* (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271,** 6518–6522.

Sheu, S. S., and Blaustein, P. M. (1986). Sodium/calcium exchange and regulation of cell calcium and contractility in cardiac muscle, with a note about vascular smooth muscle. *In* ''The Heart and Cardiovascular System'' (H. A. Fozzard, *et al.,* eds.), pp. 509–535. Raven, New York.

Shimoni, Y., Han, X., Severson, D., and Giles, W. R. (1996). Mediation by nitric oxide of the indirect effects of adenosine on calcium current in rabbit heart pacemaker cells. *Br. J. Pharmacol.* **119,** 1463–1469.

Silvagno, F., Xia, H., and Bredt, D. S. (1996). Neuronal nitric oxide synthase mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J. Biol. Chem.* **271,** 11204–11208.

Simmons, W. W., Closs, E. L., Cunningham, J. M., Smith, T. W., and Kelly, R. A (1996). Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes. Regulation of L-arginine transport and NO production by CAT-1, CAT-2A, and CAT-2B. *J. Biol. Chem.* **271,** 11694–11702.

Smart, E. J., Ying, Y.-S., Mineo, C., and Anderson, R. G. W. (1995). A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10104–10108.

Smith, J. A., Shah, A. M., and Lewis, M. J. (1991). Factors released from endocardium of the ferret and pig modulate myocardial contraction. *J. Physiol.* **439,** 1–14.

Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996). Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J. Biol. Chem.* **271,** 9690–9697.

Standish, A., Enquist, L. W., and Schaber, J. S. (1994). Innervation of the heart and its central medullary origin defined by viral tracing. *Science* **263,** 232–234.

Stein, B., Drogmuller, A., Mulsch, A., Schmitz, W., and Scholz, H. (1993). Ca (++)-dependent constitutive nitric oxide synthase is not involved in

the cyclic GMP-increasing effects of carbachol in ventricular cardiomyocytes. *J. Pharmacol. Exp. Ther.* **266,** 919–925.

Sterin-Borda, L., Genaro, A., Perez Leiros, C., Cremaschi, G., Echague, A. V., and Borda, E. (1998). Role of nitric oxide in cardiac beta-adrenoceptor-inotropic response. *Cell Signal.* **10,** 253–257.

Stull, J. T. (1980). Phosphorylation of contractile proteins in relation to muscle function. *Adv. Cyclic Nucleotide Res.* **13,** 39–93.

Sulakhe, P. V., Sandirasegarane, L., Davis, J. P., Vo, X. T., Costain, W. J., and Mainra, R. R. (1996). Alterations in inotropy, nitric oxide and cyclic GMP synthesis, protein phosphorylation and ADP-ribosylation in the endoxin-treated rat myocardium and cardiomyocytes. *Mol. Cell. Biochem.* **163–164;** 305–318.

Svoboda, P., and Milligan, G. (1994). Agonist-induced transfer of the alpha subunits of the guanine-nucleotide-binding regulatory proteins Gq and G11 and of muscarinic m1 acetylcholine receptors from plasma membranes to a light-vesicular membrane fraction. *Eur. J. Biochem.* **224,** 455–462.

Takita, T., Ikeda, J., Sekiguchi, Y., Demachi, J., Li, SL., and Shirato, K. (1998). Nitric oxide modulates sympathetic control of left ventricular contraction in vivo in the dog. *J. Auton. Nerv. Syst.* **71,** 69–74.

Tanaka, K., Hassall, C. J., and Burnstock, G. (1993). Distribution of intracardiac neurons and nerve terminals that contain a marker for nitric oxide, NADPH-diaphorase, in the guinea-pig heart. *Cell. Tissue Res.* **273,** 293–300.

Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996). Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J. Biol. Chem.* **271,** 2255–2261.

Tolbert, L. M., and Lameh, J. (1996). Human muscarinic cholinergic receptor Hm1 internalizes via clathrin-coated vesicles. *J. Biol. Chem.* **271,** 17335–17342.

Ungureanu-Longrois, D., Balligand, J. L., Okada, I., Simmons, W. W., Kobzik, L., Lowenstein, C. J., *et al.* (1995a). Contractile responsiveness of ventricular myocytes to isoproterenol is regulated by induction of nitric oxide synthase activity in cardiac microvascular endothelial cells in heterotypic primary culture. *Circ. Res.* **77,** 486–493.

Ungureanu-Longrois, D., Balligand, J. L., Kelly, R. A., and Smith, T. W. (1995b). Myocardial contractile dysfunction in the systemic inflammatory response syndrome: Role of a cytokine-inducible nitric oxide synthase in cardiac myocytes. *J. Mol. Cell. Cardiol.* **27,** 155–167.

Valenzuela, D., Han, X., Mende, U., Fankhauser, C., Mashimo, H., Huang, P., Pfeffer, J., Neer, E. J., and Fishman, M. C. (1997). G alpha(0) is necessary for muscarinic regulation of Ca^{2+} channels in mouse heart. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 1727–1732.

Vandecasteele, G., Eschenhagen, T., and Fischmeister, R. (1998). Role of the NO–cGMP pathway in the muscarinic regulation of the L-type Ca^{2+} current in human atrial myocytes. *J. Physiol.* **506,** 653–663.

Vandecasteele, G., Eschenhagen, T., Scholz, H., Stein, B., Verde, I., and Fischmeister, R. (1999). Muscarinic and beta-adrenergic regulation of heart-rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase. *Nat. Med.* **5**(3), 331–334.

Wahler, G. M., and Dollinger, S. J. (1995). Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am. J. Physiol.* **268,** C45–C54.

Wang, Y. G., and Lipsius, S. L. (1995). Acetylcholine elicits a rebound stimulation of Ca^{2+} current mediated by pertussis toxin-sensitive G protein and cAMP-dependent protein kinase A in atrial myocytes. *Circ. Res.* **76,** 634–644.

Wang, Y. G., Rechenmacher, C. E., and Lipsius, S. L. (1998). Nitric oxide signaling mediates stimulation of L-Type Ca^{2+} current elicited by withdrawal of acetylcholine in cat atrial myocytes. *J. Gen. Physiol.* **111,** 113–125.

Watanabe, A. M., and Besh, H. (1975). Interaction between cyclic adenosine monophosphate and cyclic guanosine monophosphate in guinea pig ventricular myocardium. *Circ. Res.* **37,** 309–317.

Wei, C. M., Jiang, S. W., Lust, J. A., Daly, R. C., and MacGregor, C. G. (1996). Genetic expression of endothelial nitric oxide synthase in human atrial myocardium. *Mayo Clin. Proc.* **71,** 346–350.

Xie, Q. W., Cho, H. J., Calaycay, J., Mumforol, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**(5054), 225–228.

Xu, K. Y., Huso, D. L., Dawson, T. M., Bredt, D. S., and Becker, L. C. (1999). Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 657–662.

Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998). Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* **279,** 234–237.

Yamamoto, S., Tsutsui, H., Tagawa, H., Saito, K., Takahashi, M., Tada, H., Yamamoto, M., Katoh, M., Egashira, K., and Takeshita, A. (1997). Role of myocyte nitric oxide in beta-adrenergic hyporesponsiveness in heart failure. *Circulation* **95,** 1111–1114.

Yasuda, S., and Lew, W. Y. (1997). Lipopolysaccharide depresses cardiac contractility and beta-adrenergic contractile response by decreasing myofilament response to Ca^{2+} in cardiac myocytes. *Circ. Res.* **81,** 1011–1020.

Zahradnikova, A., Minarovic, I., Venema, R. C., and Meszaros, L. G. (1997). Inactivation of the cardiac ryanodine receptor calcium release channel by nitric oxide. *Cell. Calcium* **22,** 447–454.

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CHAPTER 37

Role of Nitric Oxide in the Microcirculation

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SMALL ARTERIES PLAY AN IMPORTANT ROLE IN THE REGULATION OF PERIPHERAL VASCULAR RESISTANCE. THE ENDOTHELIUM OF RESISTANCE ARTERIES REGULATES VASCULAR FUNCTION BY WAY OF ITS BARRIER ROLE, BY INTERACTION WITH CIRCULATING CELLS SUCH AS PLATELETS, WHICH MAY RELEASE VASOACTIVE OR GROWTH REGULATING AGENTS, AND BY PRODUCTION OF SUBSTANCES THAT MODULATE VASCULAR TONE AND SMOOTH MUSCLE CELL GROWTH. IMPORTANT MEDIATORS ARE NITRIC OXIDE (NO) AND, ESPECIALLY IN SMALL ARTERIES, ENDOTHELIUM-DEPENDENT HYPERPOLARIZING FACTOR (EDHF) AND ENDOTHELIN-1. THE ENDOTHELIUM IS AN OBVIOUS TARGET ORGAN OF CARDIOVASCULAR RISK FACTORS. ACCORDINGLY, FUNCTIONAL ALTERATIONS DO OCCUR WITH AGING, HYPERTENSION, AND HYPERCHOLESTEROLEMIA. ALL CONDITIONS ARE ASSOCIATED WITH A DECREASED BASAL AND SIMULATED RELEASE OF ENDOTHELIUM-DERIVED NO. ON THE OTHER HAND, THE PRODUCTION OF CYCLOOXYGENASE-DEPENDENT ENDOTHELIUM-DERIVED CONTRACTING FACTOR AND ENDOTHELIN-1 APPEARS TO BE INCREASED, WHICH OFFSETS THE EFFECTS OF NO AND EDHF. THUS, THE ENDOTHELIUM IS AN IMPORTANT REGULATOR OF THE MICROCIRCULATION. ALTERATIONS OF ENDOTHELIAL FUNCTION MAY HAVE IMPORTANT CLINICAL IMPLICATIONS FOR THE PATHOGENESIS OF CARDIOVASCULAR DISEASE.

Introduction

The vascular endothelium is strategically positioned between the bloodstream and the vascular smooth muscle cells (VSMC) and is a source of vasoactive factors such as nitric oxide (NO), endothelium-dependent hyperpolarizing factor (EDHF), prostacyclin, prostaglandins, and endothelin (Furchgott and Zawadzki, 1980; Lüscher and Vanhoutte, 1990). NO is an important vasodilator agent that counteracts tonic vascular constriction of neuronal, endocrine, or local origin. Furthermore, NO also contributes inhibition of platelet function and thus plays an important functional role in maintaining vascular homeostasis. This review focuses on the role of this potent vasodilator, which regulates vascular tone and structure of the microcirculation under physiological conditions as well as in aging, hypertension, and hypercholesterolemia. Furthermore, modulation of endothelium-derived NO by pharmacotherapy will also be discussed.

Endothelium-Derived NO

Nitric oxide is a diffusible substance with a short half-life of a few seconds (Fig. 1, right) (Ignarro *et al.,* 1987; Palmer *et al.,* 1987). It is formed from L-arginine by oxidation of its guanidine–nitrogen terminus (Palmer *et al.,* 1988), requiring cofactors such as oxygen, NADPH, 5,6,7,8-tetrahydrobiop-

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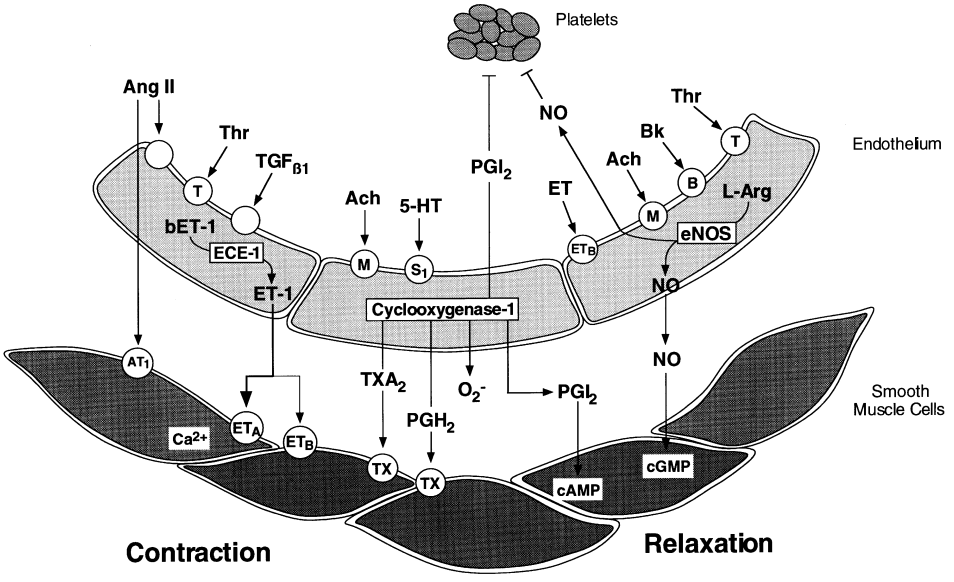


Figure 1 Endothelium-derived relaxing factors (right) and endothelium-derived contracting factors (left). Endothelial cells form nitric oxide (NO) from L-arginine via the activity of the endothelial NO synthase (eNOS), and it causes increases in cyclic 3',5'-guanosine monophosphate (cGMP) in VSMC and platelets, which in turn mediates relaxation and platelet inhibition, respectively. In addition, prostacyclin (PGI_2) causes relaxation via a cyclic adenosine monophosphate (cAMP)-dependent mechanism. Stimulation of the cyclooxygenase-1 pathway by receptor-operated agonists such as acetylcholine (Ach) and serotonin (5-HT), or by physical forces, can lead to the formation of prostaglandin H_2 (PGH_2), thromboxane A_2 (TXA_2), or superoxide anions (O_2^-), which in turn elicit direct vasoconstrictions and also inactivate NO. In addition, ET-1 and ET-3 can activate ET_B receptors on the endothelial cell membrane, which are linked to the production of NO and PGI_2. ECE-1, endothelin converting enzyme-1; TX, thromboxane; M, muscarinerg; S, serotonin; circle, receptors. Adapted from Lüscher *et al., Hypertension* **19,** 117–130 (1992); with permission.

terin, and Ca^{2+}/calmodulin (Bredt *et al.,* 1990; Ignarro, 1990; Moncada *et al.,* 1991). The formation of NO occurs via nitric oxide synthase (NOS), which either is expressed constitutively or can be induced by cytokines and other stimuli (Bredt *et al.,* 1990; Förstermann *et al.,* 1991; Pollock *et al.,* 1991). So far, three different isoforms of NOS have been described, namely, neurohumoral (nNOS or NOS I), inducible (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) (Moncada *et al.,* 1997).

Most of eNOS appears to be bound to the endothelial cell membrane, whereas only a small fraction is of cytosolic origin (Sessa *et al.,* 1992). Receptor-dependent agonists (i.e., acetylcholine, bradykinin, substance P, and platelet-derived products such as thrombin and adenosine diphosphate) increase intracellular free Ca^{2+}, which in turn activates eNOS and elicts endothelium-dependent relaxations (Fig. 1, right) (Lüscher and Vanhoutte, 1990). The endothelial L-arginine–NO pathway is further activated by shear forces exerted by the circulating blood cells, thereby causing flow-dependent vasodilation (Pohl *et al.,* 1986).

Relaxations in response to the abluminal release of endothelium-derived NO are associated with stimulation of soluble guanylyl cyclase and in turn formation of cyclic guanosine 3',5'-monophosphate (cGMP) in VSMC; this in turn reduces intracellular calcium and dephosphorylates myosin light chains (Rapoport *et al.,* 1983). Soluble guanylyl cyclase, also present in platelets, is activated by the luminal release of endothelium-derived NO (Busse *et al.,* 1987), and this limits adhesion and aggregation (Fig. 1) (Radomski *et al.,* 1990). Therefore, endothelium-derived NO causes both vasodilation and platelet deactivation, and it thereby represents an important antithrombotic feature of the endothelium.

Importance of Endothelium-Derived NO in the Microcirculation

Nitric oxide also regulates vascular tone in small arteries. Indeed, in intramyocardial porcine coronary arteries, L-N^G-monomethylarginine (L-NMMA) causes endothelium-dependent contraction (Tschudi *et al.,* 1991a). In the perfused porcine ophthalmic circulation, L-NMMA reduces flow by about 40% (Meyer *et al.,* 1993). Accordingly, in the human forearm circulation (Joannides *et al.,* 1995a) and coronary microcirculation (Quyyumi *et al.,* 1995), L-NMMA reduces flow considerably (Fig. 2). After prolonged ischemia in the human forearm, flow increases rapidly. The maximal increase in flow after ischemia is not affected by L-NMMA, indicating that it is metabolically mediated (Joannides *et al.,* 1995b; Meredith *et al.,* 1996). However, the half-life of the hyperemic response is markedly reduced by L-NMMA. Thus, NO markedly contributes to the maintance of flow-dependent vasodilation in the microvasculature.

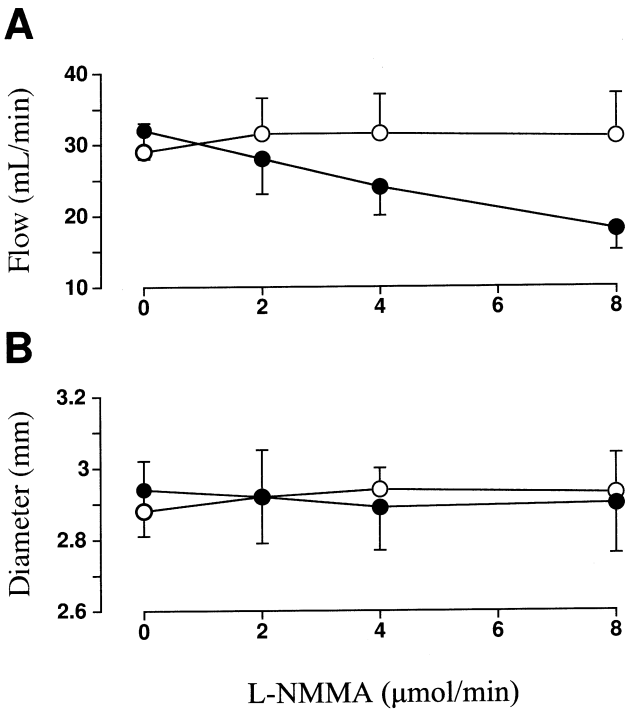


Figure 2 Mean radial blood flow (A) and mean internal diameter (B) during control period (open circles) and after infusion of increasing doses of L-N^G-monomethyl arginine (L-NMMA; filled circles) in healthy volunteers. L-NMMA dose-dependently decreases radial blood flow after 8 μmol/min L-NMMA ($p < 0.01$) but does not affect radial artery internal diameter. Values are means ± SEM of 11 subjects. Adapted from Joannides *et al.,* (1995a), Role of basal and stimulated release of nitric oxide in the regulation of radial artery caliber in humans. *Hypertension* **26,** 327–331, with permission.

Furthermore, NO also plays a crucial role in the regulation of blood pressure (Gardiner *et al.,* 1990). Indeed, when infused intravenously, inhibitors of NOS such as L-NMMA or N^ω-nitro-L-arginine methylester (L-NAME) induce long-lasting increases in blood pressure and vascular resistance in the rabbit and in humans (Haynes *et al.,* 1993). This demonstrates that the resistance circulation is in a constant state of vasodilation due to the continuous basal release of picomolar quantities of NO by the vascular endothelium.

Although NO is an important modulator of vascular tone, not all endothelium-dependent relaxations are prevented by inhibitors of the L-arginine pathway. In epicardial coronary arteries, L-NMMA, hemoglobin, or methylene blue only partially prevent endothelium-depedent relaxations to bradykinin. These NO-independent relaxations are even more prominent in intramyocardial arteries (Tschudi *et al.,* 1991a). In mesenteric resistance arteries, L-NMMA, methylene blue, and hemoglobin only partially inhibit endothelium-dependent relaxations to acetylcholine (Dohi *et al.,* 1990; Nagao *et al.,* 1992), an indication that NO only in part accounts for endothelium-dependent responses. Precontraction with depolarizing solution, on the other hand, markedly reduces NO-independent relaxations. Because these conditions cause VSMC to become transiently hyperpolarized via the activation of an adenosine triphosphate-sensitive K^+ channel (Standen *et al.,* 1989) and/or Na^+, K^+-adenosine triphosphatase (Feletou and Vanhoutte, 1988), an endothelium-dependent hyperpolarizing factor (EDHF) has been proposed. Thus, the importance of endothelium-dependent hyperpolarizing factor for endothelium-dependent relaxations increases as the vessel size decreases in both the coronary and mesenteric circulations (Nagao *et al.,* 1992; Hwa *et al.,* 1994; Shimokawa *et al.,* 1996).

In addition to NO, prostacyclin is released by endothelial cells in response to several mediators, shear stress, and hypoxia (Fig. 1) (Yang *et al.,* 1992; Chida and Voelkel, 1996). The platelet inhibitory effects of prostacyclin may be more important than its contribution to endothelium-dependent relaxation (Moncada *et al.,* 1977). Indeed, in mesenteric resistance arteries, indomethacin does not affect endothelium-dependent relaxations to acetylcholine (Dohi *et al.,* 1990). In the human forearm circulation, acetyl-salicylic acid also does not alter the increase in blood flow exerted by acetylcholine (Linder *et al.,* 1990). However, certain investigators found some reduction in flow-dependent vasodilation in the forearm with cyclooxygenase inhibition (Duffy *et al.,* 1998). In platelets, NO and prostacyclin synergistically inhibit the aggregation, suggesting that the activity of both mediators is required to exert full antiplatelet activity (Radomski *et al.,* 1987).

Interaction of NO with Endothelium-Derived Contracting Factors

Endothelium-derived NO not only causes vasodilation and inhibition of platelet aggregation but also can interact with endothelium-derived contracting factors at the level of both the endothelium and the VSMC.

Interaction with Endothelin

Endothelin-1 (ET-1) is a potent vascoconstrictor. Expression of messenger RNA and release of the peptide is stimulated by vasoconstrictor hormones, coagulation products, and cytokines (Fig. 1, left) (Yanagisawa *et al.,* 1988; Hahn *et al.,* 1990; Dohi *et al.,* 1992). On the other hand, NO as well as prostacyclin inhibit ET-1 production via a cGMP-dependent mechanism (Boulanger and Lüscher, 1990) and, furthermore, reduce ET-1-induced vasoconstriction in VSMC of mesenteric resistance arteries. This indicates that NO protects the microcirculation against contractions to ET-1 under physiological conditions (Dohi and Lüscher, 1990; Lüscher *et al.,* 1990a). ET-1 exerts its biological effects via activation of distinct specific ET receptors (Arai *et al.,* 1990; Sakurai *et al.,* 1990). One receptor type shows high selectivity to ET-1 and most likely represents the ET_A receptor on VSMC (Alberts *et al.,* 1994). The other type, the ET_B receptors, which are equally activated by ET-1 and ET-3, are present on endothelial cells linked to the formation of endothelium-derived NO and prostacyclin (de Nucci *et al.,* 1988; Warner

et al., 1989; Hirata *et al.,* 1993). Accordingly, in isolated rat perfused mesenteries, ET-1 produces concentration-dependent vasodilation that is unaffected by selective blockade of ET_A receptors but is abolished when both ET_A and ET_B receptors are blocked (Warner *et al.,* 1993; Takase *et al.,* 1995). Similarly, in the human forearm circulation, intraarterial ET-1 causes an initial vasodilation forwarded by vasoconstriction that is particularly pronounced at higher concentrations of the peptide (Kiowski, 1991). In isolated mesenteric resistance arteries of the rat, NO added directly or via acetylcholine from the endothelium can reserve ET-1-induced contraction (Dohi and Lüscher, 1990). In contrast, in the human forearm circulation, neither acetylcholine nor sodium nitroprusside blocks ET-1-induced vasoconstriction, but calcium antagonists do (Kiowski *et al.,* 1991).

Interaction with Cyclooxygenase-Derived Products

Most agonists stimulating the L-arginine–NO pathway in the endothelium also activate phospholipase A_2 and thereby lead to the formation of thromboxane A_2 (TXA_2) and prostaglandin endoperoxide H_2 (PGH_2) (Fig. 1) (Moncada and Higgs, 1986). Indeed, exogenous arachidonic acid induces endothelium-dependent contractions in different isolated rat vascular beds, which are prevented by indomethacin, an cyclooxygenase inhibitor (Toda *et al.,* 1988; Lüscher *et al.,* 1990b). TXA_2 and PGH_2 affect not only VSMC via the activation of TXA_2 receptors (Lang *et al.,* 1995) but also platelets, which aggregate and hence counteract the beneficial effects of NO and prostacyclin in both cell types (Gerritsen, 1996).

In addition, the cyclooxygenase pathway is a source of superoxide anions (O_2^-) which can evoke endothelium-dependent contractions either by inactivating endothelium-derived NO (Gryglewski *et al.,* 1986) or by directly stimulating VSMC (Kukreja *et al.,* 1986; Katusic and Vanhoutte, 1989).

NO Derived from Vascular Smooth Muscle in Inflammation and Septic Shock

Vascular smooth muscle cells can generate large quantities of NO via induction of iNOS. iNOS is activated by cytokines or bacterial products such as endotoxin, interleukin-1, interferon, and tumor necrosis factor (Bernhardt *et al.,* 1991; Nava *et al.,* 1991; Schneider *et al.,* 1992; Hishikawa and Lüscher, 1998). The induction of iNOS by inflammatory mediators also explains the hyperenic response at sites of inflammation and of generalized septic shock in patients (Vallance *et al.,* 1989). Indeed, preliminary studies suggest a beneficial effect of NO inhibition under these conditions. After induction, contractions to norepinephrine are reduced dramatically *in vitro* (Fleming *et al.,* 1990), and blood pressure is markedly lowered *in vivo* (Nava *et al.,* 1992).

Role of NO in Cardiovascular Disease

Aging and the Microcirculation

Aging is a physiological process that is independent of cardiovascular risk factor. Direct measurements of NO with a porphyrinic sensor revealed marked reductions in the aorta, and to a lesser degree in resistance arteries, but no change in the pulmonary circulation (Tschudi *et al.,* 1996a). In perfused mesenteric resistance arteries of Wystar-Kyoto (WKY) rats, aging is associated with a reduced response to L-NMMA and acetylcholine (Dohi *et al.,* 1990; Moreau *et al.,* 1998a), although the changes appear less pronounced than in large conduit arteries. The inhibitory effect of the endothelium against contraction induced by norepinephrine decreases with age in rat mesenteric resistance arteries, suggesting reduced basal release of NO (Atkinson *et al.,* 1994). However, *in vivo* studies in Sprague-Dawley rats revealed that oral treatment with L-NAME increases blood pressure and renal vascular resistance (Reckelhoff and Manning, 1993). Furthermore, the vasoconstricting effects of L-NAME increase with age, suggesting, at least in this model, that in renal and systemic circulations basal release of NO may increase with age.

Since the endothelium-independent relaxation induced by the NO donor agent linsidomine does not change with age, the reduced response to the muscarinic agonist must be due to a decreased production and/or release of relaxing factor(s). Similarly, endothelium-dependent increase in coronary flow in response to acetylcholine decreases with age in the rat (Katano *et al.,* 1993) and in the human coronary (Zeiher *et al.,* 1993) and forearm circulation (Taddei *et al.,* 1995). Impaired vasodilation in cerebral arterioles of aged rats does not appear to be related to an increased production of a cyclooxygenase-derived constrictor substances (Mayhan *et al.,* 1990).

Alterations of the Microcirculation in Hypertension

The hallmark of hypertension is an increase in peripheral vascular resistance that is considered to be due to an increased vascular tone of small arteries. This could be related to (1) an increased release of constricting factors and/or (2) a decreased release of relaxing factors, as well as (3) changes in vascular structure (eutrophic and hypertrophic remodeling). In most experimental models of hypertension, high blood pressure is associated with impaired endothelium-dependent relaxations and appears to occur as blood pressure rises, hence, it is a consequence rather than a cause of hypertension (Lüscher, 1990). However, in the offspring of hypertensive patients, impaired endothelium-dependent vasodilation to acetylcholine even before the onset of high blood pressure has been reported (Taddei *et al.,* 1996).

The basal formation of NO, as assessed by the contractile effects of inhibitors of NO production, is reduced in established hypertension in spontaneously hypertensive rats (SHRs), renovascular hypertensive rats, and ren-2 transgenic rats (Dohi *et al.,* 1990, 1991; Tschudi *et al.,* 1994a). Indeed,

in the mesenteric resistance circulation, L-NMMA increases vasoconstrictor responses to norepinephrine more in normotensive WKY rats than in SHRs (Dohi *et al.,* 1990). In ren-2 transgenic rats, endothelium-dependent contractions to L-NAME are markedly blunted as the duration of hypertension increases (Tschudi *et al.,* 1994a). In addition, the decrease in forearm arteriolar blood flow induced by L-NMMA is smaller in patients with untreated essential hypertension than in normotensive patients (Calver *et al.,* 1992).

In isolated perfused mesenteric arteries of genetic models of hypertension such as SHRs or Dahl salt-sensitive rats and of experimental models such as renovascular or L-NAME-induced hypertension, endothelium-dependent relaxations are reduced (Dohi *et al.,* 1990, 1991; Moreau *et al.,* 1995; d'Uscio *et al.,* 1997). However, the mechanism of endothelial dysfunction differs in different models of hypertension. In the resistance circulation of SHRs, the NO pathway is upregulated by a mechanism involving increased expression and activity of constitutive NO synthase and overproduction of NO (Fig. 3A) (Nava *et al.,* 1998). Moreover, the concentration of nitrate, the oxidative product of NO, is higher in SHR as compared to age-matched WKY rats (Fig. 3B). However, further study suggests that the level of cyclic GMP in mesenteric arteries is similar in SHR and in WKY, suggesting that NO is not sufficiently bioactive to stimulate the formation of cyclic GMP on VSMC and to maintain an adequate NO-dependent vasodilatory tone in spontaneous hypertension (Fig. 3C) (Nava *et al.,* 1998). This is likely to be due to an increased NO decomposition by superoxide anions. Indeed, experimental evidence using a porphyrinic microsensor for direct measurement of NO has demonstrated that in the presence of superoxide dismutase, a superoxide anion scavenger, NO release from isolated mesenteric resistance arteries is improved in stroke-prone SHR (Tschudi *et al.,* 1996b). One possible explanation is an imbalance between production of superoxide anions and activity of superoxide dismutase in the vessel wall, or a dysfunction of NO synthase may be involved (Cosentino *et al.,* 1998). Thus, endothelial dysfunction is linked to an overproduction of endothelium-derived radicals that lead to the formation of peroxynitrite. In contrast, impaired endothelium-dependent relaxations seen in Dahl rats is due to the reduced activity of endothelial NO synthase (Hayakawa *et al.,* 1997; Hayakawa and Raij, 1997).

Although it is commonly assumed that impaired endothelium-dependent relaxations are related to a reduced formation or release of NO under certain conditions, this may also be due to an enhanced production of endothelium-derived contracting factors (Fig. 4). Indeed, the production of a cyclooxygenase-dependent endothelium-derived contracting factor such as PGH_2/TXA_2 is increased in the microcirculation of SHRs and ren-2 transgenic rats and offsets the effects of NO in VSMC and platelets (Lüscher *et al.,* 1986, 1990b; Diederich *et al.,* 1990; Noll *et al.,* 1997). Similarly, in the human forearm circulation indomethacin increases but does not normalize the reduced vasodilator response of hypertensive patients to acetylcholine (Taddei *et al.,* 1993).

On the other hand, as NO antagonizes the release of ET-1 (Boulanger and Lüscher, 1990) and regulates the production of the peptide *in vivo* (Sventek *et al.,* 1996; Filep, 1997), NO deficiency leads to enhanced ET-1 formation. This imbalance between NO and ET-1 production could contribute to endothelial dysfunction in hypertension. Accordingly, chronic blockade of ET_A receptors normalizes blunted endothelium-dependent relaxations of small mesenteric arteries in hypertension (d'Uscio *et al.,* 1997).

Of note is the fact that not all hypertensive blood vessels and not all forms of hypertension exhibit alterations of

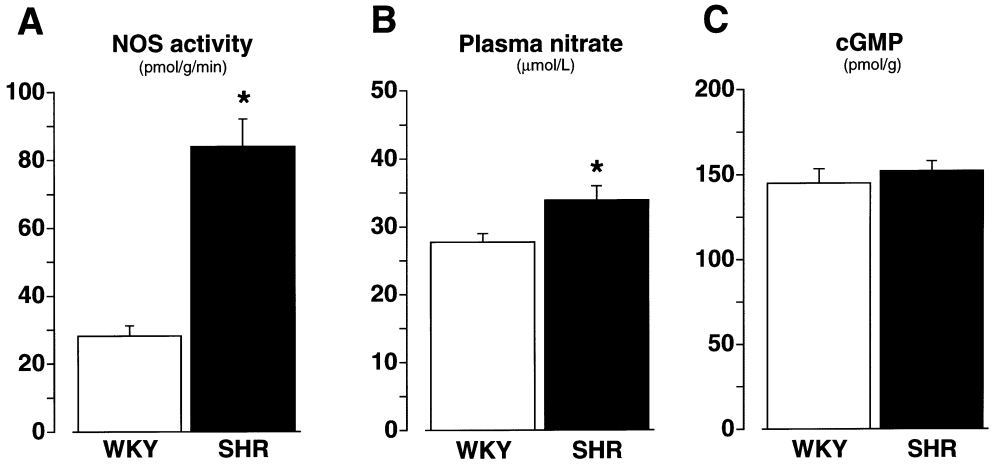


Figure 3 (A) Activity of the constitutive NO synthase (NOS) in mesenteric resistance arteries from Wystar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). (B) Concentration of nitrate in plasma of WKY rats and SHR. Both vascular activity of NOS and overall release of NO, assessed by measuring plasma nitrate levels, appear to be significantly higher in tissues from SHR. (C) Levels of cyclic GMP (cGMP) in mesenteric resistance arteries from WKY rats and SHR. $*p < 0.05$ versus WKY rats. Adapted from Nava *et al.,* (1998), Alterations to the nitric oxide pathway in the spontaneously hypertensive rat. *Journal of Hypertension* **16,** 609–615, with permission.

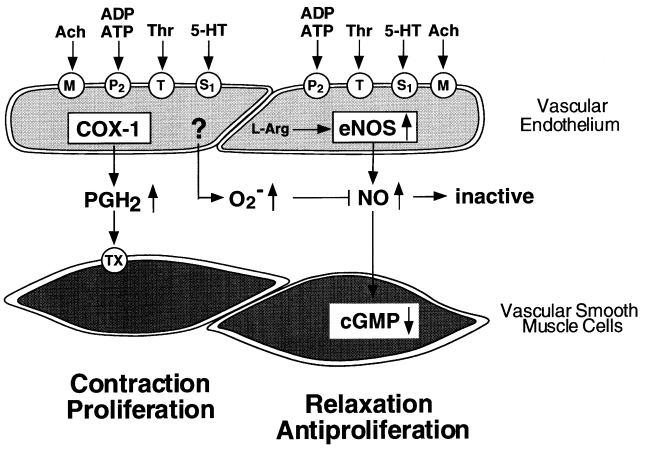


Figure 4 Proposed mechanisms of endothelial dysfunction in hypertension: Endothelium-dependent relaxations in genetic hypertension. In spontaneously hypertensive rats, nitric oxide synthase (NOS) activity is increased, but the biological activity of nitric oxide (NO) is reduced, possibly due to inactivation by superoxide anion (O_2^-). In addition, the production of thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) via cyclooxygenase (COX-1) is increased. Adapted from Lüscher *et al.,* Endothelium-derived contracting factors. *Hypertension* **19,** 117–130, with permission. (1992).

the L-arginine–NO pathway. Indeed, in the coronary circulation of the SHR, a small amount of endothelial dysfunction is observed except in very old rats (Tschudi *et al.,* 1991b).

Effects of Hypercholesterolemia in the Microcirculation

Hypercholesterolemia and atherosclerosis interfere with the formation of NO in the endothelium and the response to it in VSMC. In hypercholesterolemia, a major risk factor predisposing to atherosclerosis, endothelial dysfunction is present before structural vascular changes occur (Freiman *et al.,* 1986; Celermajer *et al.,* 1992). Interestingly, oxidized low density lipoprotein (LDL), but not native LDL, reduces endothelium-dependent relaxations in coronary arteries (Tanner *et al.,* 1991; Zeiher *et al.,* 1991a). On the other hand, blood vessels exhibit enhanced vasoconstriction and increased adhesion of platelets and monocytes (Heistad *et al.,* 1984; Mathew *et al.,* 1997).

Although atherosclerosis primarily affects conduit arteries, endothelium-dependent relaxations are impaired not only in large arteries but also in the microcirculation of atherosclerotic animals and humans. In cremasteric vessels of the cholesterol-fed rabbit, cholinergic arteriolar vasodilation is defective (Yamamoto *et al.,* 1988). In coronary resistance arteries, hypercholesterolemia is associated with impaired endothelium-dependent relaxations to the agonists serotonin, bradykinin, and histamine (Chilian *et al.,* 1990; Sellke *et al.,* 1990a; Zeiher *et al.,* 1991b; Kuo *et al.,* 1992), indicating that the pathophysiological consequences of atherosclerosis may also extend into the microcirculation in humans.

Explanations for impaired endothelium-dependent vascular relaxations in hypercholesterolemia and atherosclerosis include (1) alterations in endothelial cell signal transduction [G_i-mediated pathways in particular (Shimokawa *et al.,* 1989)], (2) deficiencies in the substrate (L-arginine) for the enzyme NO synthase (Drexler *et al.,* 1991; Tanner *et al.,* 1991; Kuo *et al.,* 1992), (3) alterations in NO synthase activity or one of its cofactors (e.g., tetrahydrobiopterin deficiency) (Wever *et al.,* 1998), (4) excess destruction of NO by the superoxide anion (Münzel *et al.,* 1997; Hein and Kuo, 1998), and (5) reduced expression of the enzyme (Oemar *et al.,* 1998).

Increased local ET-1 levels, on the other hand, may also be an early marker for endothelial dysfunction. In this context, a relationship between plasma ET-1 levels and endothelial dysfunction in coronary circulation in early atherosclerosis in humans with atherosclerotic vascular disease has been reported (Lerman *et al.,* 1995). This study suggested that increased activation of the ET system—as demonstrated by increased plasma ET-1 levels—could mediate endothelial dysfunction and in turn enhance vasoconstrictor responses of blood vessels.

Other Vascular Diseases

In resistance arteries from diabetic animals and humans, several studies have shown impairment of NO-mediated endothelium-dependent relaxations as well as increased release of vasoconstrictor factors (Diederich *et al.,* 1994; Tribe *et al.,* 1998). This impairment is restored by PGH_2/TXA_2 receptor blockade or superoxide dismutase, indicating that the PGH_2 and/or superoxide anion generation contribute to endothelial dysfunction (Tesfamariam, 1994).

In Alzheimer's disease, the cerebral microcirculation undergoes specific biochemical changes. NO synthase activity is increased in the microvessels from affected brains and may be a potential neurotoxic mediator for the central nervous system (CNS). This could contribute to the susceptibility of neurons to injury and cell death in Alzheimer's disease (Dorheim *et al.,* 1994).

Hypoxia alters vascular tone in coronary resistance arterioles during prolonged ischemia, potentially through the modulation of endothelial cell metabolism as well as endothelial function. Indeed, hypoxia activates endothelial cNOS in coronary resistance arterioles and in turn increases the production of NO (Xu *et al.,* 1995).

NO and Vascular Structure

NO plays an important role in modulating vascular structure under physiological and pathophysiological conditions. In hypertension, resistance arteries adapt to the increased wall tension by changing their geometry. Accordingly, a reduced lumen diameter, an increased wall thickness, or both can normalize the excessive tension applied on the vessel wall and may then protect the microcirculation against the

blood pressure rise. Alterations of small artery structure may be mediated by eutrophic remodeling, a rearrangement of the same amount of vascular material around a smaller lumen, or hypertrophic remodeling (increase in cross-sectional area) of the vascular wall, or by a combination of both processes (Heagerty *et al.,* 1993). The vascular endothelium, by its anatomical position and by releasing several factors (see earlier), may influence the local vascular environment and modulate the changes of vascular geometry observed in the context of hypertension. Chronic inhibition of NO with L-NAME produces an elevation of blood pressure and is not associated with hypertrophic remodeling of cerebral and mesenteric arteries (Moreau *et al.,* 1995; Takase *et al.,* 1996), which would be expected if NO has a tonic inhibition on VSMC growth (Moreau *et al.,* 1998b), but is associated with eutrophic inward remodeling in proportion to the increase in blood pressure. Furthermore, prevention of hypertension in the L-NAME model by antihypertensive agents prevents inward remodeling, suggesting that NO blockade per se does not induce any vascular structural change *in vivo.*

Modulation of NO by Pharmacotherapy

Endothelial dysfunction seems to be a common feature of several pathological processes. Several drugs that could improve endothelial function or substitute factors needed for the NO pathway may have a potential advantage for protection and treatment of cardiovascular diseases.

Nitrovasodilators

Nitrovasodilators such as nitrogylcerin, sodium nitroprusside, and linsidomine cause vasodilation by releasing NO from their molecules (Feelisch and Noack, 1987) and therefore acting through a mechanism identical with that of endogenous NO. Interestingly, vascular production of NO reduces the sensitivity of blood vessels to nitrovasodilators both in large conduit arteries (Pohl *et al.,* 1986; Lüscher *et al.,* 1990c) and in intramyocardial coronary resistance arteries (Tschudi *et al.,* 1991a). Similarly, in the human forearm circulation the response to sodium nitroprusside is enhanced after infusion of L-NMMA (Joannides *et al.,* 1995a). This suggests that the basal formation of NO in the endothelium reduces the sensitivity to nitrovasodilators also in the microcirculation.

The biotransformation of nitroglycerin is diminished in small coronary microvessels (60–100 μm in diameter) compared with larger coronary microarteries with 300 μm diameters (Sellke *et al.,* 1990b). This is likely to be due to a relative deficiency of available sulfhydryl groups or a lack of enzymes necessary for conversion of nitroglycerin to its active metabolites in small coronary resistance vessels (Moreau and Lüscher, 1997). This may explain why nitrogylcerin is more effective in large arteries and veins than in the microcirculation.

Calcium Antagonists

Although the production of endothelium-derived NO in endothelial cells is associated with an increase in intracellular Ca^{2+}, calcium antagonists do not affect the release of the factor under acute conditions (Himmel *et al.,* 1993). Indeed, endothelial cells do not appear to possess voltage-operated calcium channels. There are reports, however, that dihydropyridine calcium antagonists stimulate NO formation in cells in culture (Ding and Vaziri, 1998). In hypertensive rats, chronic treatment with calcium antagonists, however, does improve endothelial function in resistance arteries (Dohi *et al.,* 1994; Tschudi *et al.,* 1994b; Moreau *et al.,* 1995; Takase *et al.,* 1996). Because this beneficial effect is also present in L-NAME-induced hypertension, where the synthesis of NO is still blocked, an alternative NO pathway must be involved (Takase *et al.,* 1996), most likely an enhanced production of EDHF.

Angiotensin-Converting Enzyme Inhibitors

The renin angiotensin system and endothelium-derived substances are important regulators of the microcirculation. Angiotensin-converting enzyme (ACE), which transforms angiotensin I into angiotensin II, a potent vasoconstrictor with proliferative properties, is expressed on endothelial cell membrane (Fig. 5) (Ng and Vane, 1967). ACE is identical to kinase II, which inactivates bradykinin, a potent stimulator of the L-arginine and cyclooxygenase pathways (Vanhoutte *et al.,* 1993). Therefore, ACE inhibitors not only prevent the formation of angiotensin II but also increase the local concentration of bradykinin and in turn increase the production of NO, EDHF, and prostacyclin through B_2 receptors (Wiemer *et al.,* 1991; Yang *et al.,* 1993; Meyer *et al.,* 1995).

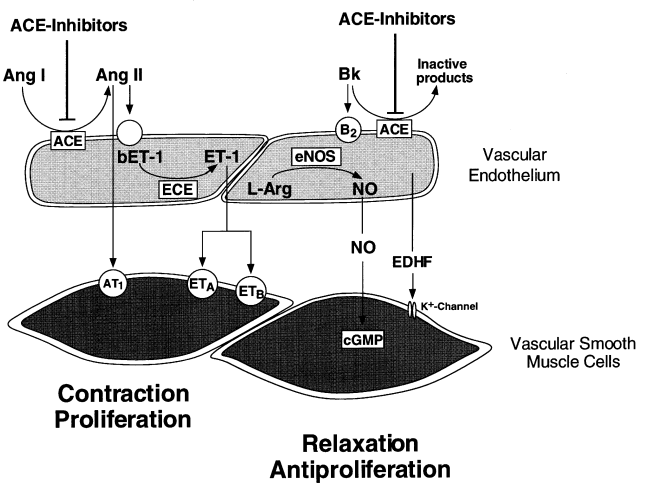


Figure 5 A renin–angiotensin system exists on the endothelial cell membrane, which provides locally produced Ang II from Ang I via angiotensin-converting enzyme (ACE). Ang I, angiotensin I; Bk, bradykinin. The relaxing factors (right) include nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF). NO and PGI_2 cause not only relaxation but also inhibition of platelet function.

The decreased breakdown of bradykinin could therefore explain improved endothelial function (Vanhoutte *et al.,* 1993; Tschudi *et al.,* 1994b; Takase *et al.,* 1996) and also normalization of vascular remodeling with ACE inhibitors in small resistance arteries of hypertensive rats (Deng and Schiffrin, 1993; Moreau *et al.,* 1995) as well as in subcutaneous resistance arteries of hypertensive patients (Schiffrin and Deng, 1995).

Endothelin Antagonists

Endothelial dysfunction seen in salt-sensitive hypertension is normalized by ET_A receptor blockade (Fig. 6) (d'Uscio *et al.,* 1997). This suggests that improvement of endothelium-dependent relaxation is mediated in large part via selective activation of ET_B receptors, which in turn stimulates the release of NO and/or prostacyclin, while combined blockade of ET_A/ET_B receptors does not affect endothelial function (Li *et al.,* 1994). This is in line with a study showing that selective blockade of the ET_A receptors increases endogenous NO generation (Verhaar *et al.,* 1998). ET_A receptor antagonists are also able to lower systolic blood pressure and to prevent hypertrophic remodeling (d'Uscio *et al.,* 1997; Matsumura *et al.,* 1999). Thus, functional and structural vascular changes in hypertension are mediated, at least in part, by endogenous ET-1 through activation of ET_A receptors.

As described earlier, the production of ET-1 is enhanced in hypercholesterolemia and atherosclerosis. Previous observations showed that selective blockade of ET_A receptors reduces the formation of fatty streak formation in cholesterol-fed hamsters (Kowala *et al.,* 1995). Interestingly, concomitant administration of an ET_A receptor antagonist inhibits vascular lesion formation and normalizes NO-mediated endothelium-dependent relaxations of the aorta in apolipoprotein E-deficient mice, an animal model of human atherosclerosis (Barton *et al.,* 1998). In addition, epicardial atherosclerosis in humans is associated with impaired endothelium-dependent relaxations in the coronary resistance arteries, indicating that the pathophysiological consequences of atherosclerosis may extend into the microcirculation (Zeiher *et al.,* 1991b). Another study shows that blockade of ET_A receptors normalizes functional changes of small resistance arteries in atherosclerotic apoE-deficient mice (d'Uscio *et al.,* 1998).

Lipid-Lowering Drugs

Plasma cholesterol concentrations play a pivotal role in the development and therapy of hypercholesterolemia and atherosclerosis. Studies have demonstrated that lowering of plasma cholesterol levels has beneficial effects on endothelial function, suggesting that plasma cholesterol is indeed an important factor determining endothelial dysfunction (Anderson *et al.,* 1995; Goode and Heagerty, 1995). It is of most interest that reduced endothelium-dependent relaxations also occur in the microcirculation of patients with hyperlipidemia (Stroes *et al.,* 1995). The mechanisms by which cholesterol-lowering therapy influences vascular changes may involve

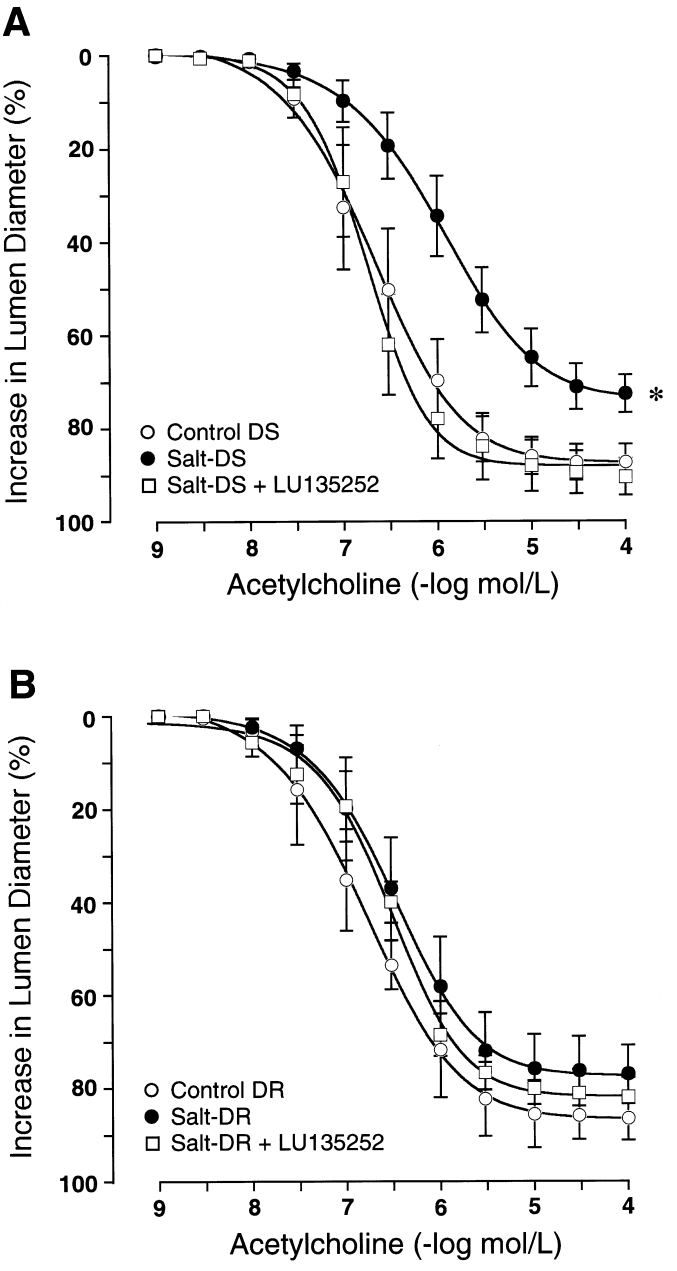


Figure 6 Endothelium-dependent relaxations to acetylcholine of mesenteric resistance arteries in Dahl salt-sensitive (A) and Dahl salt-resistant (B) rats. Results are shown as means ± SEM ($n = 6$ or 7 per group), and relaxations are expressed as percent of the increase in intraluminal diameter from precontraction with norepinephrine. LU135252: ET_A-receptor antagonist. *$p < 0.05$ versus control for maximal response (ANOVA + Bonferroni). From d'Uscio *et al.,* (1997), Structure and function of small arteries in salt-induced hypertension: Effects of chronic endothelin-subtype-A receptor blockade. *Hypertension* **30,** 905–911, with permission.

improvement of the NO pathway (Hernandez-Perera *et al.,* 1998). Indeed, statins are able to prevent the inhibitory action exerted by oxidized LDL on endothelial NOS mRNA and protein levels. Thus, these drugs may influence vascular tone of the microcirculation by modulating the expression of endothelium-derived NO. Impaired endothelial function in the microcirculation may contribute to ischemia occurring

under these conditions and in turn may also be important for prognosis of these patients.

Conclusion

NO contributes to the maintainance of vascular tone and structure, by itself and through interactions with cyclooxygenase-derived products and endothelin, in both the endothelium and VSMC. The relative activation and potency of these mediators and their interaction determine the vascular response. In aging, hypertension, hypercholesterolemia, and atherosclerosis, an endothelial dysfunction occurs that is related to an imbalance of endothelial mediators and their action. Some commonly used therapeutic agents such as ACE inhibitors, statins, and possibly ET antagonists seem to have favorable effects on the endothelial function by increasing release and bioavailability of NO.

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References

Alberts, G. F., Peifley, K. A., Johns, A., Kleha, J. F., and Winkles, J. A. (1994). Constitutive endothelin-1 overexpression promotes smooth muscle cell proliferation via an external autocrine loop. *J. Biol. Chem.* **269,** 10112–10118.

Anderson, T. J., Meredith, I. T., Yeung, A. C., Frei, B., Selwyn, A. P., and Ganz, P. (1995). The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. *N. Engl. J. Med.* **332,** 488–493.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., and Nakanishi, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348,** 730–732.

Atkinson, J., Tatchum-Talom, R., and Capdeville-Atkinson, C. (1994). Reduction of endothelial function with age in the mesenteric arterial bed of the normotensive rat. *Br. J. Pharmacol.* **111,** 1184–1188.

Barton, M., Haudenschild, C. C., d'Uscio, L. V., Shaw, S., Münter, K., and Lüscher, T. F. (1998). Endothelin ETA receptor blockade restores NO-mediated endothelial dysfunction and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14367–14372.

Bernhardt, J., Tschudi, M. R., Dohi, Y., Gut, I., Urwyler, B., Buhler, F. R., and Lüscher, T. F. (1991). Release of nitric oxide from human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **180,** 907–912.

Boulanger, C., and Lüscher, T. F. (1990). Release of endothelin from the porcine aorta: Inhibition by endothelium-derived nitric oxide. *J. Clin. Invest.* **85,** 587–590.

Bredt, D. S., Hwang, P. M., and Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neuronal role for nitric oxide. *Nature* **347,** 768.

Busse, R., Lückhoff, A., and Bassenge, E. (1987). Endothelium-derived relaxing factor inhibits platelet activation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336,** 566–562.

Calver, A., Collier, J., Moncada, S., and Vallance, P. (1992). Effect of local intra-arterial N^G-monomethyl-L-arginine in patients with hypertension: The nitric oxide dilator mechanism appears abnormal. *J. Hypertens.* **10,** 1025–1031.

Celermajer, D. S., Sorensen, K. E., Gooch, V. M., Spiegelhalter, D. J., Miller, O. I., Sullivan, I. D., Lloyd, J. K., and Deanfield, J. E. (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* **340,** 1111–1115.

Chida, M., and Voelkel, N. F. (1996). Effects of acute and chronic hypoxia on rat lung cyclooxygenase. *Am. J. Physiol.* **270,** L872–L878.

Chilian, W. M., Dellsperger, K. C., Layne, S. M., Eastham, C. L., Armstrong, M. A., Marcus, M. L., and Heistad, D. D. (1990). Effects of atherosclerosis on the coronary microcirculation. *Am. J. Physiol.* **258,** H529–H539.

Cosentino, F., Patton, S., d'Uscio, L. V., Werner, E. R., Werner-Felmayer, G., Moreau, P., Malinski, T., and Lüscher, T. F. (1998). Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *J. Clin. Invest.* **101,** 1530–1537.

d'Uscio, L. V., Barton, M., Shaw, S., Moreau, P., and Lüscher, T. F. (1997). Structure and function of small arteries in salt-induced hypertension: Effects of chronic endothelin-subtype-A receptor blockade. *Hypertension* **30,** 905–911.

d'Uscio, L. V., Barton, M., Shaw, S., and Lüscher, T. F. (1998). Endothelin causes endothelial dysfunction and hypertrophic remodeling of the microcirculation of apoE-knockout mice with atherosclerosis via ET_A-receptors. *Circulation* **98,** I–33 (Abstract).

Deng, L. Y., and Schiffrin, E. L. (1993). Effect of antihypertensive treatment on response to endothelin of resistance arteries of hypertensive rats. *J. Cardiovasc. Pharmacol.* **21,** 725–731.

de Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T. D., and Vane, J. R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 9797–9800.

Diederich, D., Yang, Z. H., Bühler, F. R., and Lüscher, T. F. (1990). Impaired endothelium-dependent relaxations in hypertensive resistance arteries involve cyclooxygenase pathway. *Am. J. Physiol.* **258,** H445–H451.

Diederich, D., Skopec, J., Diederich, A., and Dai, F. X. (1994). Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: Role of free radicals. *Am. J. Physiol.* **266,** H1153–H1161.

Ding, Y., and Vaziri, N. D. (1998). Calcium channel blockade enhances nitric oxide synthase expression by cultured endothelial cells. *Hypertension* **32,** 718–723.

Dohi, Y., and Lüscher, T. F. (1990). Aging differentially affects direct and indirect actions of endothelin-1 in perfused mesenteric arteries of the rat. *Br. J. Pharmacol.* **100,** 889–893.

Dohi, Y., Thiel, M. A., Bühler, F. R., and Lüscher, T. F. (1990). Activation of endothelial L-arginine pathway in resistance arteries. Effect of age and hypertension. *Hypertension* **16,** 170–179.

Dohi, Y., Criscione, L., and Lüscher, T. F. (1991). Renovascular hypertension impairs formation of endothelium-derived relaxing factors and sensitivity to endothelin-1 in resistance arteries. *Br. J. Pharmacol.* **104,** 349–354.

Dohi, Y., Hahn, A., Boulanger, C. M., Bühler, F. R., and Lüscher, T. F. (1992). Endothelin stimulated by angiotensin II augments contractility of spontaneously hypertensive rat resistance arteries. *Hypertension* **19,** 131–137.

Dohi, Y., Criscione, L., Pfeiffer, K., and Lüscher, T. F. (1994). Angiotensin blockade or calcium antagonists improve endothelial dysfunction in hypertension: Studies in perfused SHR mesenteric resistance arteries. *J. Cardiovasc. Pharmacol.* **24,** 372–379.

Dorheim, M. A., Tracey, W. R., Pollock, J. S., and Grammas, P. (1994). Nitric oxide synthase activity is elevated in brain microvessels in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **205,** 659–665.

Drexler, H., Zeiher, A. M., Meinzer, K., and Just, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* **338,** 1546–1550.

Duffy, S. J., Tran, B. T., New, G., Tudball, R. N., Esler, M. D., Harper, R. W., and Meredith, I. T. (1998). Continuous release of vasodilator prostanoids contributes to regulation of resting forearm blood flow in humans. *Am. J. Physiol.* **274,** H1174–H1183.

Feelisch, M., and Noack, E. A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.* **139,** 19–30.

Feletou, M., and Vanhoutte, P. M. (1988). Endothelium-dependant hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.* **93,** 515–524.

Filep, J. G. (1997). Endogenous endothelin modulates blood pressure, plasma volume and albumin escape after systemic nitric oxide blockade. *Hypertension* **30,** 22–28.

Fleming, I., Gray, G. A., Julou-Schaeffer, G., Parratt, J. R., and Stoclet, J. C. (1990). Incubation with endotoxin activates the L-arginine pathway in vascular tissue. *Biochem. Biophys. Res. Commun.* **171,** 562–568.

Förstermann, U., Schmitt, H. H. H. W., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M., and Murrad, F. (1991). Isoforms of nitric oxide synthase: Characterization and purification from different cell types. *Biochem. Pharmacol.* **42,** 1849–1857.

Freiman, P. C., Mitchell, G. G., Heistad, D. D., Armstrong, M. L., and Harrison, D. G. (1986). Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ. Res.* **58,** 783–789.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Gardiner, S. M., Compton, A. M., Bennett, T., Palmer, R. M. J., and Moncada, S. (1990). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension* **15,** 486–492.

Gerritsen, M. E. (1996). Physiological and pathophysiological roles of eicosanoids in the microcirculation. *Cardiovasc. Res.* **32,** 720–732.

Goode, G. K., and Heagerty, A. M. (1995). In vitro responses of human peripheral small arteries in hypercholesterolemia and effects of therapy. *Circulation* **91,** 2898–2903.

Gryglewski, R. J., Palmer, R. M. J., and Moncada, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* **320,** 454–456.

Hahn, A. W. A., Resink, T. J., Scott-Burden, T., Powell, J., Dohi, Y., and Buhler, F. R. (1990). Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: A novel autocrine function. *Cell Regul.* **1,** 649–659.

Hayakawa, H., and Raij, L. (1997). The link among nitric oxide synthase activity, endothelial function, and aortic and ventricular hypertrophy in hypertension. *Hypertension* **29,** 235–241.

Hayakawa, H., Coffee, K., and Raij, L. (1997). Endothelial dysfunction and cardiorenal injury in experimental salt-sensitive hypertension: Effects of antihypertensive therapy. *Circulation* **96,** 2407–2413.

Haynes, W. G., Noon, J. P., Walker, B. R., and Webb, D. J. (1993). Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J. Hypertens.* **11,** 1375–1380.

Heagerty, A. M., Aalkjaer, C., Bund, S. J., Korsgaard, N., and Mulvany, M. J. (1993). Small artery structure in hypertension: Dual processes of remodeling and growth. *Hypertension* **21,** 391–397.

Hein, T. W., and Kuo, L. (1998). LDLs impair vasomotor function of the coronary microcirculation: Role of superoxide anions. *Circ. Res.* **83,** 404–414.

Heistad, D. D., Armstrong, M. L., Marcus, M. L., Piegors, D. J., and Mark, A. L. (1984). Augmented responses to vasoconstrictor stimuli in hypercholesterolemic and atherosclerotic monkeys. *Circ. Res.* **54,** 711–718.

Hernandez-Perera, O., Perez-Sala, D., Navarro-Antolin, J., Sanchez-Pascuala, R., Hernandez, G., Diaz, C., and Lamas, S. (1998). Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J. Clin. Invest.* **101,** 2711–2719.

Himmel, H. M., Whorton, A. R., and Strauss, H. C. (1993). Intracellular calcium, currents, and stimulus-response coupling in endothelial cells. *Hypertension* **21,** 112–127.

Hirata, Y., Emori, T., Eguchi, S., Kanno, K., Imai, T., Ohta, K., and Marumo, F. (1993). Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J. Clin. Invest.* **91,** 1367–1373.

Hishikawa, K., and Lüscher, T. F. (1998). Felodipine inhibits free-radical production by cytokines and glucose in human smooth muscle cells. *Hypertension* **32,** 1011–1015.

Hwa, J. J., Ghibaudi, L., Williams, P., and Chatterjee, M. (1994). Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. *Am. J. Physiol.* **266,** H952–H958.

Ignarro, L. J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* **30,** 535–560.

Ignarro, L. J., Byrns, R. E., Buga, G. M., and Woods, K. S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacological and chemical properties identical to those of NO radical. *Circ. Res.* **61,** 866–879.

Joannides, R., Richard, V., Haefeli, W. E., Linder, L., Lüscher, T. F., and Thuillez, C. (1995a). Role of basal and stimulated release of nitric oxide in the regulation of radial artery caliber in humans. *Hypertension* **26,** 327–331.

Joannides, R., Haefeli, W. E., Linder, L., Richard, V., Bakkali, E. H., Thuillez, C., and Lüscher, T. F. (1995b). Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* **91,** 1314–1319.

Katano, Y., Ishihata, A., Morinobu, S., and Endoh, M. (1993). Modulation by aging of the coronary vascular response to endothelin-1 in the rat isolated perfused heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **348,** 82–87.

Katusic, Z. S., and Vanhoutte, P. M. (1989). Superoxide anion is an endothelium-derived contracting factor. *Am. J. Physiol.* **257,** H33–H37.

Kiowski, W. (1991). Endothelial function in humans. Studies of forearm resistance vessels. *Hypertension* **18,** 1184–1189.

Kiowski, W., Lüscher, T. F., Linder, L., and Bühler, F. R. (1991). Endothelin-1-induced vasoconstriction in humans. Reversal by calcium channel blockade but not by nitrovasodilators or endothelium-derived relaxing factor. *Circulation* **83,** 469–475.

Kowala, M. C., Rose, P. M., Stein, P. D., Goller, N., Recce, R., Beyer, S., Valentine, M., Barton, D., and Durham, S. K. (1995). Selective blockade of the endothelin subtype A receptor decreases early atherosclerosis in hamsters fed cholesterol. *Am. J. Pathol.* **146,** 819–826.

Kukreja, R. C., Kontos, H. A., Hess, M. L., and Ellis, E. F. (1986). PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circ. Res.* **59,** 612–619.

Kuo, L., Davis, M. J., Cannon, M. S., and Chilian, W. M. (1992). Pathophysiological consequences of atherosclerosis extend into the coronary microcirculation. Restoration of endothelium-dependent responses by L-arginine. *Circ. Res.* **70,** 465–476.

Lang, M. G., Noll, G., and Lüscher, T. F. (1995). Effect of aging and hypertension on contractility of resistance arteries: Modulation by endothelial factors. *Am. J. Physiol.* **269,** H837–H844.

Lerman, A., Holmes, D. R., Jr., Bell, M. R., Garratt, K. N., Nishimura, R. A., and Burnett, J. C., Jr. (1995). Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation* **92,** 2426–2431.

Li, J. S., Larivière, R., and Schiffrin, E. L. (1994). Effect of a nonselective endothelin antagonist on vascular remodeling in deoxycorticosterone acetate–salt hypertensive rats: Evidence for a role of endothelin in vascular hypertrophy. *Hypertension* **24,** 183–188.

Linder, L., Kiowski, W., Bühler, F. R., and Lüscher, T. F. (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. *Circulation* **81,** 1762–1767.

Lüscher, T. F. (1990). The endothelium. Target and promoter of hypertension? *Hypertension* **15,** 482–485.

Lüscher, T. F., and Vanhoutte, P. M. (1990). ''The Endothelium: Modulator of Cardiovascular Function.'' CRC Press, Boca Raton, Florida.

Lüscher, T. F., Rubanyi, G. M., Aarhus, L. L., Edoute, Y., and Vanhoutte, P. M. (1986). Serotonin reduces coronary flow in the isolated heart of the spontaneously hypertensive rat. *J. Hypertens.* **4**(Suppl.), S148–S150.

Lüscher, T. F., Yang, Z., Tschudi, M., R., V. S., Stulz, P., Boulanger, C., Siebenmann, R., Turina, M., and Bühler, F. R. (1990a). Interaction between endothelin-1 and endothelium-derived relaxing factor in human arteries and veins. *Circ. Res.* **66,** 1088–1094.

Lüscher, T. F., Aarhus, L. L., and Vanhoutte, P. M. (1990b). Indomethacin improves the impaired endothelium-dependent relaxations in small mesenteric arteries of the spontaneously hypertensive rat. *Am. J. Hypertens.* **3,** 55–58.

Lüscher, T. F., Richard, V., and Yang, Z. (1990c). Interaction between endothelium-derived nitric oxide and SIN-1 in human and porcine blood vessels. *J. Cardiovasc. Pharmacol.* **14**(Suppl 11), 76–80.

Lüscher, T. F. (1992). Endothelium-derived contracting factors and hypertension. *In* ''Endothelial Regulation of Vascular Tone'' (U. S. Ryan and G. M. Rubanyi, eds.), pp. 275–296. Dekker, New York, Basel, and Hong Kong.

Lüscher, T. F., Boulanger, C. M., Doki, Y., and Yang, Z. (1992). Endothelium-derived contracting factors. *Hypertension* **19,** 117–130.

Mathew, V., Cannan, C. R., Miller, V. M., Barber, D. A., Hasdai, D., Schwartz, R. S., Holmes, D. R., Jr., and Lerman, A. (1997). Enhanced endothelin-mediated coronary vasoconstriction and attenuated basal nitric oxide activity in experimental hypercholesterolemia. *Circulation* **96,** 1930–1936.

Matsumura, Y., Hashimoto, N., Taira, S., Kuro, T., Kitano, R., Ohkita, M., Opgenorth, J. T., and Takaoka, M. (1999). Different contributions of endothelin-A and endothelin-B receptors in the pathogenesis of deoxycorticosterone acetate–salt-induced hypertension in rats. *Hypertension* **33,** 759–765.

Mayhan, W. G., Faraci, F. M., Baumbach, G. L., and Heistad, D. D. (1990). Effects of aging on responses of cerebral arterioles. *Am. J. Physiol.* **258,** H1138–H1143.

Meredith, I. T., Currie, K. E., Anderson, T. J., Roddy, M. A., Ganz, P., and Creager, M. A. (1996). Postischemic vasodilation in human forearm is dependent on endothelium-derived nitric oxide. *Am. J. Physiol.* **270,** H1435–H1440.

Meyer, P., Flammer, J., and Lüscher, T. F. (1993). Endothelin-dependent regulation of the ophthalmic microcirculation in the perfused porcine eyes: Role of nitric oxide and endothelins. *Invest. Ophthalmol. Visual Sci.* **34,** 3614–3621.

Meyer, P., Flammer, J., and Lüscher, T. F. (1995). Local action of the renin angiotensin system in the porcine ophthalmic circulation: Effects of ACE-inhibitors and angiotensin receptor antagonists. *Invest. Ophthalmol. Visual Sci.* **36,** 555–562.

Moncada, S., and Higgs, E. A. (1986). Arachidonate metabolism in blood cells and the vessel wall. *Clin. Haematol.* **15,** 273–292.

Moncada, S., Herman, A. G., Higgs, E. A., and Vale, C. (1977). Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the antithrombotic propertiers of vascular endothelium. *Thromb. Res.* **11,** 323–344.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Moncada, S., Higgs, A., and Furchgott, R. F. (1997). XIV. International union of pharmacology nomenclature in nitric oxide research. *Pharmacol. Rev.* **49,** 137–142.

Moreau, P., and Lüscher, T. F. (1997). Hypertension. *In* ''The Endothelium in Clinical Practice'' (G. M. Rubanyi and V. J. Dzau, eds.), pp. 331–354. Dekker, New York.

Moreau, P., Takase, H., Küng, C. F., van Rooijen, M.-M., Schaffner, T., and Lüscher, T. F. (1995). Structure and function of the rat basilar artery during chronic nitric oxide synthase inhibition. *Stroke* **26,** 1922–1929.

Moreau, P., d'Uscio, L. V., and Lüscher, T. F. (1998a). Structure and reactivity of small arteries in aging. *Cardiovasc. Res.* **37,** 247–253.

Moreau, P., Takase, H., d'Uscio, L. V., and Lüscher, T. F. (1998b). Effect of chronic nitric oxide deficiency on angiotensin II-induced hypertrophy of rat basilar artery. *Stroke* **29,** 1031–1035.

Münzel, T., Heitzer, T., and Harrison, D. G. (1997). The physiology and pathophysiology of the nitric oxide/superoxide system. *Herz* **22,** 158–172.

Nagao, T., Illiano, S., and Vanhoutte, P. M. (1992). Heterogenous distribution of endothelium-dependent relaxations resistant to N^G-nitro-L-arginine in rats. *Am. J. Physiol.* **263,** H1090–H1094.

Nava, E., Plamer, R. M. J., and Moncada, S. (1991). Inhibition of nitric oxide synthesis in septic shock: How much is beneficial? *Lancet* **338,** 1555–1557.

Nava, E., Palmer, R. M. J., and Moncada, S. (1992). The role of nitric oxide in endotoxic shock: Effects of N^G-monomethyl-L-arginine. *J. Cardiovasc. Pharmacol.* **20**(Suppl. 12), S132–S134.

Nava, E., Farré, A. L., Moreno, C., Casado, S., Moreau, P., Cosentino, F., and Lüscher, T. F. (1998). Alterations to the nitric oxide pathway in the spontaneously hypertensive rat. *J. Hypertens.* **16,** 609–615.

Ng, K. K., and Vane, J. R. (1967). Conversion of angiotensin I to angiotensin II. *Nature* **216,** 762.

Noll, G., Lang, M., Tschudi, M. R., Ganten, D., and Lüscher, T. F. (1997). Endothelial vasoconstrictor prostanoids modulate contractions to acetylcholine and angiotensin II in Ren-2 rats. *Am. J. Physiol.* **272,** H493–H500.

Oemar, B. S., Tschudi, M. R., Godoy, N., Brovkovich, V., Malinski, T., and Lüscher, T. F. (1998). Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation* **97,** 2494–2498.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327,** 524–526.

Palmer, R. M. J., Ashton, D. S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333,** 664–666.

Pohl, U., Holtz, J., Busse, R., and Bassenge, E. (1986). Crucial role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension* **8,** 37–44.

Pollock, J. S., Förstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H., Nakane, M., and Murad, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10480–10484.

Quyyumi, A. A., Dakak, N., Andrews, N. P., Husain, S., Arora, S., Gilligan, D. M., Panza, J. A., and Cannon III, R. O. (1995). Nitric oxide activity in the human coronary circulation. Impact of risk factors for coronary atherosclerosis. *J. Clin. Invest.* **95,** 1747–1755.

Radomski, M. W., Palmer, R. M., and Moncada, S. (1987). The anti-aggregating properties of vascular endothelium: Interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.* **92,** 639–646.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1990). An L-arginine pathway in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 5193–5197.

Rapoport, R. M., Draznin, M. B., and Murad, F. (1983). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature* **306,** 174–176.

Reckelhoff, J. F., and Manning, R. D., Jr. (1993). Role of endothelium-derived nitric oxide in control of renal microvasculature in aging male rats. *Am. J. Physiol.* **265,** R1126–R1131.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348,** 732–735.

Schiffrin, E. L., and Deng, L. Y. (1995). Comparison of effects of angiotensin I-converting enzyme inhibition and beta-blockade for 2 years on function of small arteries from hypertensive patients. *Hypertension* **25,** 699–703.

Schneider, F., Schott, C., Stoclet, J. C., and Julou-Schaeffer, G. (1992). L-Arginine induces relaxation of small mesenteric arteries from endotoxin-treated rats. *Eur. J. Pharmacol.* **211,** 269–272.

Sellke, F. W., Armstrong, M. L., and Harrison, D. G. (1990a). Endothelium-dependent vascular relaxation is abnormal in the coronary microcirculation of atherosclerotic primates. *Circulation* **81,** 1586–1593.

Sellke, F. W., Myers, P. R., Bates, J. N., and Harrison, D. G. (1990b). Influence of vessel size on the sensitivity of porcine coronary microvessels to nitroglycerin. *Am. J. Physiol.* **258,** H515–H520.

Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D'Angelo, D. D., Lynch, K. R., and Peach, M. J. (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J. Biol. Chem.* **267,** 15274–15276.

Shimokawa, H., Flavahan, N. A., Shepherd, J. T., and Vanhoutte, P. M. (1989). Endothelium-dependent inhibition of ergonovine-induced contraction is impaired in porcine coronary arteries with regenerated endothelium. *Circulation* **80,** 643–650.

Shimokawa, H., Yasutake, H., Fujii, K., Owada, M. K., Nakaike, R., Fukumoto, Y., Takayanagi, T., Nagao, T., Egashira, K., Fujishima, M., and Takeshita, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.* **28,** 703–711.

Standen, N. B., Quayle, J. M., Davies, N. W., Brayden, J. E., Huang, Y., and Nelson, M. T. (1989). Hyperpolarizing vasodilators activate ATP sensitive K^+ channels in arterial smooth muscle. *Science* **245,** 177–180.

Stroes, E. S., Koomans, H. A., de Bruin, T. W., and Rabelink, T. J. (1995). Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* **346,** 467–471.

Sventek, P., Li, J. S., Grove, K., Deschepper, C. F., and Schiffrin, E. L. (1996). Vascular structure and expression of endothelin-1 gene in L-NAME-treated spontaneously hypertensive rats. *Hypertension* **27,** 49–55.

Taddei, S., Virdis, A., Mattei, P., and Salvetti, A. (1993). Vasodilation to acetylcholine in primary and secondary forms of human hypertension. *Hypertension* **21,** 929–933.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Gennari, A., Fasolo, C. B., Sudano, I., and Salvetti, A. (1995). Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation* **91,** 1981–1987.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Sudano, I., and Salvetti, A. (1996). Defective L-arginine–nitric oxide pathway in offspring of essential hypertensive patients. *Circulation* **94,** 1298–1303.

Takase, H., Moreau, P., and Lüscher, T. F. (1995). Endothelin receptors subtypes in small arteries: Studies with FR139317 and bosentan. *Hypertension* **25,** 739–743.

Takase, H., Moreau, P., Küng, C. F., Nava, E., and Lüscher, T. F. (1996). Antihypertensive therapy prevents endothelial dysfunction in chronic nitric oxide deficiency: Effect of verapamil and trandolapril. *Hypertension* **27,** 25–31.

Tanner, F. C., Noll, G., Boulanger, C. M., and Lüscher, T. F. (1991). Oxidized low-density lipoproteins inhibit relaxations of porcine coronary arteries: Role of scavenger receptor and endothelium-dereived nitric oxide. *Circulation* **83,** 2012–2020.

Tesfamariam, B. (1994). Free radicals in diabetic endothelial cell dysfunction. *Free Radicals Biol. Med.* **16,** 383–391.

Toda, N., Inoue, S., Bian, K., and Okamura, T. (1988). Endothelium-dependent and independent responses to prostaglandin H2 and arachidonic acid in isolated dog cerebral arteries. *J. Pharmacol. Exp. Ther.* **244,** 297–302.

Tribe, R. M., Thomas, C. R., and Poston, L. (1998). Flow-induced dilatation in isolated resistance arteries from control and streptozotocin-diabetic rats. *Diabetologia* **41,** 34–39.

Tschudi, M., Richard, V., Bühler, F. R., and Lüscher, T. F. (1991a). Importance of endothelium-derived nitric oxide in porcine coronary resistance arteries. *Am. J. Physiol.* **260,** H13–H20.

Tschudi, M. R., Criscione, L., and Lüscher, T. F. (1991b). Effect of aging and hypertension on endothelial function of rat coronary arteries. *J. Hypertens.* **9,** 164–165.

Tschudi, M. R., Noll, G., Arnet, U., Novosel, D., Ganten, D., and Lüscher, T. F. (1994a). Alterations in coronary artery vascular reactivity of hypertensive Ren-2 transgenic rats. *Circulation* **89,** 2780–2786.

Tschudi, M. R., Criscione, L., Novosel, D., Pfeiffer, K., and Lüscher, T. F. (1994b). Antihypertensive therapy augments endothelium-dependent relaxations in coronary arteries of spontaneously hypertensive rats. *Circulation* **89,** 2212–2218.

Tschudi, M. R., Barton, M., Bersinger, N. A., Moreau, P., Cosentino, F., Noll, G., Malinski, T., and Lüscher, T. F. (1996a). Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. *J. Clin. Invest.* **98,** 899–905.

Tschudi, M., Mesaros, S., Lüscher, T. F., and Malinski, T. (1996b). In situ measurement of nitric oxide in mesenteric resistance arteries. *Hypertension* **27,** 32–35.

Vallance, P., Collier, J., and Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* **2,** 997–1000.

Vanhoutte, P. M., Boulanger, C. M., Illiano, S. C., Nagao, T., Vidal, M., and Mombouli, J. V. (1993). Endothelium-dependent effects of converting-enzyme inhibitors. *J. Cardiovasc. Pharmacol.* **22,** S10–S16.

Verhaar, M. C., Strachan, F. E., Newby, D. E., Cruden, N. L., Koomans, H. A., Rabelink, T. J., and Webb, D. J. (1998). Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation* **97,** 752–756.

Warner, T. D., de Nucci, G., and Vane, J. R. (1989). Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. *Eur. J. Pharmacol.* **159,** 325–326.

Warner, T. D., Allcock, G. H., Corder, R., and Vane, J. R. (1993). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br. J. Pharmacol.* **110,** 777–782.

Wever, R. M., Lüscher, T. F., Cosentino, F., and Rabelink, T. J. (1998). Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* **97,** 108–112.

Wiemer, G., Schölkens, B. A., Becker, R., and Busse, R. (1991). Ramiprilat enhances endothelial autocoid formation by inhibiting breakdown of endothelium-derived bradykinin. *Hypertension* **18,** 558–563.

Xu, X. P., Pollock, J. S., Tanner, M. A., and Myers, P. R. (1995). Hypoxia activates nitric oxide synthase and stimulates nitric oxide production in porcine coronary resistance arteriolar endothelial cells. *Cardiovasc. Res.* **30,** 841–7.

Yamamoto, H., Bossaller, C., Cartwright, J., Jr., and Henry, P. D. (1988). Videomicroscopic demonstration of defective cholinergic arteriolar vasodilation in atherosclerotic rabbit. *J. Clin. Invest.* **81,** 1752–1758.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332,** 411–415.

Yang, B. C., Lawson, D. N., and Mehta, J. L. (1992). Role of eicosanoids in rat aortic ring response to agonists and acetylcholine with special reference to the biphasic effects of prostacyclin. *Eicosanoids* **5,** 135–139.

Yang, Z., Arnet, U., von Segesser, L., Siebenmann, R., Turina, M., and Lüscher, T. F. (1993). Different effects of angiotensin-converting enzyme inhibition in human arteries and veins. *J. Cardiovasc. Pharmacol.* **22,** S17–S22.

Zeiher, A. M., Drexler, H., Wollschlager, H., and Just, H. (1991a). Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* **83,** 391–401.

Zeiher, A. M., Drexler, H., Wollschlager, H., and Just, H. (1991b). Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. *Circulation* **84,** 1984–1992.

Zeiher, A. M., Drexler, H., Saurbier, B., and Just, H. (1993). Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J. Clin. Invest.* **92,** 652–662.

CHAPTER 38

Activation of NOS by Ca^{2+}-Dependent and Ca^{2+}-Independent Mechanisms

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THE NITRIC OXIDE SYNTHASES (NOS) ARE MULTIDOMAIN ENZYMES CONSISTING OF AN N-TERMINAL OXYGENASE DOMAIN THAT CONTAINS BINDING SITES FOR HEME, L-ARGININE, AND TETRAHYDROBIOPTERIN (H_4B), AND A REDUCTASE DOMAIN WITH BINDING SITES FOR NADPH, FMN, FAD, AND CALMODULIN (CAM) (FIG. 1). DURING THE SYNTHESIS OF NITRIC OXIDE (NO), NADPH-DERIVED ELECTRONS PASS TO FLAVINS IN THE REDUCTASE DOMAIN AND MUST THEN BE TRANSFERRED TO THE HEME LOCATED IN THE OXYGENASE DOMAIN, SO THAT THE HEME IRON CAN BIND O_2 AND CATALYZE THE STEPWISE SYNTHESIS OF NO FROM L-ARGININE. THE ASSOCIATION OF CAM WITH ITS BINDING SITE IS GENERALLY ACCEPTED TO ACTIVATE NO SYNTHESIS BY ENABLING THE REDUCTASE DOMAIN TO TRANSFER ELECTRONS TO THE OXYGENASE DOMAIN.

Neuronal NOS

Cellular Expression of nNOS

Neuronal NOS (nNOS) was first characterized in, and purified from, rat and porcine cerebellum. Since then, nNOS has been found to have a widespread distribution in specific neurons of the central and peripheral nervous systems. nNOS is likely to play an important role not only in physiological neuronal functions such as neurotransmitter release, neural development, regeneration, synaptic plasticity, and regulation of gene expression but also in a variety of neurological disorders in which excessive production of NO leads to neural injury. However, nNOS expression is not confined to neuronal cells. In various species, nNOS mRNA transcripts and protein have been detected in nonneuronal cell types including skeletal muscle, macula densa cells, myometrium, and certain vascular smooth muscle cells.

The subcellular localization of nNOS protein varies greatly among the cell types studied. In neurons, both soluble and particulate nNOS protein is found. In skeletal muscle, nNOS protein is mostly particulate, a phenomenon attributed to the PDZ/GLGF motif found within the NH_2-terminal sequence of the nNOS protein. This motif participates in protein–protein interactions with several other membrane-associated proteins. In neurons, synaptic association of nNOS is mediated by the binding of the PDZ/GLGF motif to the postsynaptic density protein PSD-95 and/or to the related PSD-93 protein. *N*-Methyl-D-aspartate (NMDA) receptors are also known to be associated with PSD-95. In fast-twitch skeletal muscle fibers, the muscle-specific isoform (θnNOS) is attached to the sarcolemma–dystrophin complex via the PDZ/GLGF motif and interacts mainly with α_1-syntrophin. Recent studies demonstrated a selective loss of sarcolemmal nNOS in mdx mice, which lack endogenous dystrophin. A similar loss of membrane-associated nNOS

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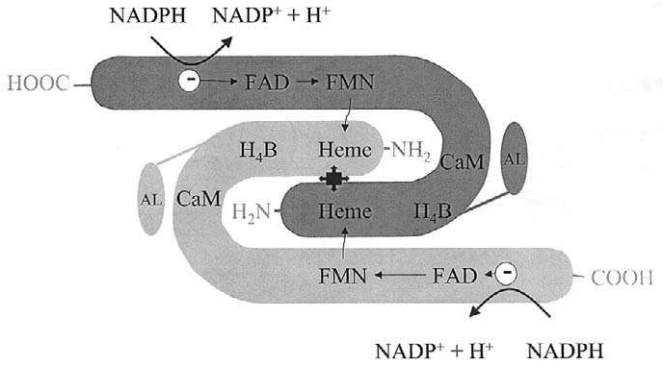


Figure 1 Proposed model for an nNOS dimer indicating domain swapping and electron transfer pathway. CaM, calmodulin; H_4B, tetrahydrobiopterin; AL, autoinhibitory loop; ◆ represents the ZnS_4 recently described to be positioned equidistant from the two heme groups and H_4B. (Modified from Fleming and Busse, 1999.)

was seen in Duchenne and in Becker muscular dystrophy, diseases in which the dystrophin gene is mutated.

Physiological and Pathophysiological Role of nNOS

An excessive production of NO can lead to neurotoxicity. NO plays a role in mediating neurotoxicity associated with a variety of neurologic disorders, including stroke, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease, and HIV dementia. These diseases are associated with partial neuronal depolarization and secondary activation of voltage-dependent NMDA receptors, which results in excitotoxic neuronal cell death (secondary excitotoxicity). Activation of NMDA receptors and the subsequent increase in intracellular Ca^{2+} leads to the activation of Ca^{2+}-dependent enzymes, including nNOS.

Ca^{2+}/CaM-Dependent NO Production by nNOS

Two phases (early and late phases) of NO production by nNOS have been reported in response to receptor activation. The kinetics of NO production by nNOS in various systems have been reported to mirror changes in intracellular Ca^{2+} level. The rapid phase of nNOS activation is dependent on the release of Ca^{2+} from intracellular stores, whereas the delayed phase is associated with the transmembranous influx of Ca^{2+} and is accompanied by the prolonged formation of NO at functionally effective levels.

How does Ca^{2+} activate NOS? The Ca^{2+}-dependent activation mechanism is generally accepted to involve the formation of a Ca^{2+}/CaM complex and the association of this complex with the CaM-binding domain situated between the oxygenase and reductase domains. The binding of Ca^{2+}/CaM to nNOS is thought to enhance NO production by promoting an intramolecular one-electron transfer between the flavins FAD and FMN.

One similarity with endothelial NOS (eNOS) is that nNOS contains an insert of 40–50 amino acids in the middle of the FMN-binding domain, which is thought to act as an autoinhibitory loop that disables the enzyme when the physiological concentration of Ca^{2+} within cells is low (Fig. 1). Indeed, activation of the Ca^{2+}-dependent NOS isoforms is thought to require the physical displacement of the autoinhibitory peptide so that CaM can access its binding domain. Consistent with the known effect of CaM on electron transfer between FAD and FMN, the autoinhibitory loop regulates nNOS activity by preventing electron transfer from FMN to heme. The situation in the eNOS and nNOS isoforms is not identical, as there is quite a large difference in the basal activity of the two enzymes (nNOS > eNOS). Although there is a high homology between certain sections of the autoinhibitory inserts, in other sections the sequence is markedly different.

Regulation of nNOS by Caveolin

Caveolins associate with and regulate the activity of NOS isoforms, and nNOS interacts with caveolin-3. Caveolin-3 is approximately 60% identical with caveolin-1 and is part of the dystrophin complex. Interactions between nNOS and caveolin-3 appear to be direct and to involve two distinct caveolin scaffolding or inhibitory domains. This interaction is thought to be competitive with CaM, and thus the interaction of nNOS with caveolin-3 inhibits Ca^{2+}-dependent enzyme activity. Caveolin may regulate NOS function at more than one level, and the induction of caveolin-3, which may lead to an increased number of caveolae, alters the nNOS–caveolin cycle.

Regulation of nNOS Activity by Lipids

The activity of nNOS has also been proposed to be regulated by interactions with membrane lipids. Phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA) both attenuate L-citrulline production by nNOS, apparently by binding in the vicinity of the CaM-binding domain and interfering with the ability of the enzyme to bind Ca^{2+}/CaM. Indeed, both PIP_2 and PA protect the CaM-binding site against trypsic digestion.

Regulation of nNOS by NO

High concentrations of NO inhibit nNOS activity by interacting with the heme prosthetic group. In rat forebrain slices oxygen–glucose deprivation results in a decrease in Ca^{2+}-dependent NOS activity and is paralleled by an increase in Ca^{2+}-independent cytokine-inducible NOS (iNOS) activity. Both dexamethasone and cycloheximide, which completely inhibit the induction of the Ca^{2+}-independent NOS activity, caused a 40–70% recovery in Ca^{2+}-dependent NOS activity. Since oxyhemoglobin also produced complete recovery of Ca^{2+}-dependent NOS activity, it appears that NO formed after oxygen–glucose deprivation is responsible for this downregulation. Consistently, exposure of nNOS to high concentrations of NO donor agents decreases its activity.

Regulation of nNOS by Phosphorylation

Both cyclic-GMP-dependent (PKG) and cyclic-AMP-dependent (PKA) protein kinases phosphorylate nNOS on a

single serine residue. The consequence of the phosphorylation of nNOS is a decrease in the catalytic activity of the enzyme. When phosphorylation occurs within the 26-amino acid residue CaM-binding domain of nNOS, the binding of CaM is blocked, suggesting that phosphorylation of the CaM-binding domain of nNOS could be a negative feedback loop to turn off nNOS activity. Indeed, the V_{max} of the Ser-847 phosphorylated recombinant wild-type nNOS is less than the nonphosphorylated form. Purified recombinant nNOS can also be phosphorylated *in vitro* on Ser-847 by Ca^{2+}/CaM-dependent protein kinases (CaM kinases), including CaM kinase Iα (CaM-KIα), CaM kinase IIα (CaM-KIIα), and CaM kinase IV (CaM-KIV).

Activation of protein kinase C (PKC) in rat cerebellar slices enhances the calcium sensitivity of nNOS. However, in the latter experiments no apparent phosphorylation of nNOS could be detected, despite the fact that PKC can phosphorylate partially purified nNOS *in vitro* and increase enzyme activity. Such data indicate that nNOS is not susceptible to PKC-dependent phosphorylation in cerebellar slices. They also indicate that the PKC-mediated alteration in nNOS activity is related not to direct phosphorylation of the enzyme but rather to the phosphorylation of unknown proteins that regulate the Ca^{2+} sensitivity of nNOS in the cerebellum. The apparent insensitivity of nNOS to PKC-mediated phosphorylation *in situ* may be explained by the finding that PKC preferentially phosphorylates the non-H_4B-stabilized nNOS dimer, and H_4B-dependent dimer stabilization prevents nNOS from PKC-dependent phosphorylation *in vitro.*

nNOS can also be phosphorylated on tyrosine residues. For example, in human astrocytoma T67 cells the enzyme is basally tyrosine phosphorylated, and the tyrosine phosphatase inhibitor vanadate increases nNOS phosphorylation and decreases NO production. Tyrosine kinases inhibitors, on the other hand, reduce the tyrosine phosphorylation of nNOS and enhance enzyme activity. However, no information on the site of nNOS tyrosine phosphorylation has been provided.

Not only the phosphorylation of nNOS, but also that of CaM determine enzyme activity. CaM is known to be constitutively phosphorylated, and the binding of phosphorylated CaM to nNOS increases its V_{max} by approximately 2.6-fold with respect to that of the nonphosphorylated CaM:nNOS. Phosphorylation of CaM has very little effect on binding of calcium to the enzyme despite the fact that the two main phosphorylated amino acids Ser-101 and Tyr-99 are located in the third calcium-binding loop of CaM.

Inducible NOS

Inflammatory mediators such as interleukin-1β and tumor necrosis factor-α induce the formation of NO in vascular cells as well as in many other types of mammalian cells such as hepatocytes, chrondrocytes and cardiomyocytes. The cytokine-inducible NOS isoform iNOS is generally thought to be unregulated by any cellular signaling processes other than those that determine its expression and/or degradation. Thus, from the moment of dimer formation to the moment of dimer disruption, this enzyme produces excessive amounts of NO.

Ca^{2+}-Independent NO Production by iNOS Activation

The iNOS isoform is to all intents and purposes a Ca^{2+}-independent enzyme, even though this statement is not strictly speaking true. Indeed, although iNOS is fully active at basal $[Ca^{2+}]_i$ and although increases in cellular Ca^{2+} cannot further enhance the production of NO, the activity of iNOS can be completely inhibited in the presence of EDTA and EGTA, just as for the other NOS isoforms.

What accounts for the differential sensitivity of iNOS to physiological concentrations of Ca^{2+}, and why, once expressed, is this enzyme fully active? The difference may well be attributable to the fact that this NOS isoform does not contain the autoinhibitory peptide insert found within the so-called Ca^{2+}-regulated isoforms (which is thought to restrict access to the CaM-binding domain). Indeed, whereas the binding of CaM to eNOS and nNOS is generally described as Ca^{2+}-dependent and reversible, the binding of calmodulin to iNOS is Ca^{2+}-independent and irreversible. The introduction of the eNOS CaM-binding domain into iNOS has been shown to render the inducible enzyme Ca^{2+}/CaM dependent, whereas the deletion of this sequence in eNOS results in a CaM-independent enzyme. However, although the deletion of the autoinhibitory peptide sequence from nNOS results in a less Ca^{2+}-sensitive isoform, the introduction of the autoinhibitory loop peptide into iNOS only slightly modifies its enzymatic properties, and a significant Ca^{2+}-independent enzyme activity is retained. Therefore, the presence of the autoinhibitory loop may only partly determine the Ca^{2+} sensitivity of the three NOS isoforms, and evidence exists to suggest that the CaM which is so tightly bound to iNOS interacts with two domains, the first being the CaM-binding domain and the second appearing to be contained within the flavoprotein domain.

Other Modulators of iNOS Function

THE INTERACTION OF iNOS WITH CAVEOLIN

Unlike the other NOS isoforms, the activity of iNOS is not thought to be dependent on its subcellular localization, and the enzyme is generally assumed to be distributed throughout the cell cytosol. Evidence exists for the localization of iNOS in the cytoplasm, perinuclear space, Golgi complex, mitochondria, plasma membrane, and along contractile fibers of cardiomyocytes, as well as for the appearance of iNOS staining of the cell nuclei in the course of cultivation. The residues in eNOS that bind to caveolin-1 and -3 are conserved in iNOS, and there is some structural evidence to suggest that caveolin binding to the reductase domain of iNOS inhibits the transfer of electrons to heme. Scaffolding domain peptides derived from caveolin-1 and caveolin-3 inhibit iNOS activity *in vitro.* However, it is not

entirely clear whether caveolin is actually involved in the physiological regulation of iNOS-mediated NO production, as iNOS and the caveolins do not necessarily come into close contact *in vivo.*

REGULATION OF iNOS BY PHOSPHORYLATION

Although numerous investigations into the role of various kinases in the induction of iNOS have been performed, almost nothing is known about the acute regulation of iNOS activity by phosphorylation. iNOS does appear to be phosphorylated on serine and tyrosine residues. In macrophages, iNOS protein can be phosphorylated via a phosphatidylinositol 3-kinase-independent but FKBP12-rapamycin-associated protein-dependent pathway, although no information regarding the specific amino acid residue phosphorylated is currently available. A little more is known about the tyrosine phosphorylation of iNOS. This is thought to be an early event coinciding with the appearance of newly synthesized iNOS. The tyrosine phosphorylation of iNOS is, as expected, enhanced by the protein tyrosine phosphatase inhibitor vanadate, which also increases iNOS activity.

Endothelial NOS

The functional eNOS, like the other isoforms, is a dimer composed of two identical subunits, both of which are myristoylated and palmitoylated. H_4B may well prove to be essential for the dimerization of eNOS, and only the dimer retains the ability to bind substrate and cofactors. Maintenance of the integrity of the H_4B-binding site on the eNOS oxygenase domain appears to involve a zinc tetrathiolate or $Zn[S–cysteine]_4$, positioned equidistant from each heme and H_4B. The identification of ZnS_4 in eNOS may well be of physiological relevance. It is feasible that increasing nitrosative stress *in vivo* may result in the release of the zinc from the oxygenase domain, may destabilize the H_4B, and/or may deplete eNOS of this essential cofactor while favoring the generation of superoxide anion (O_2^-).

Comparative analysis of NOS domain interactions shows that subunit association of NOS may involve head-to-tail interactions between the oxygenase and reductase domains. In order to properly align reductase and oxygenase domains for NO synthesis, electrons are transferred from the flavin to heme of adjacent subunits.

Ca^{2+}-Dependent eNOS Activation

Until recently, regulation of the constitutive NOS in endothelial cells was attributed solely to changes in endothelial $[Ca^{2+}]_i$. Indeed, the increase in endothelial NO production elicited by endothelial agonists, such as bradykinin and acetylcholine, mirrors endothelial $[Ca^{2+}]_i$ (Fig. 2) and has been demonstrated to be strictly dependent on the formation of a Ca^{2+}/CaM complex. Unstimulated endothelial cells continuously produce NO, suggesting that the intracellular Ca^{2+} level under resting conditions is just sufficient to allow the NOS to turn over and produce low amounts of NO.

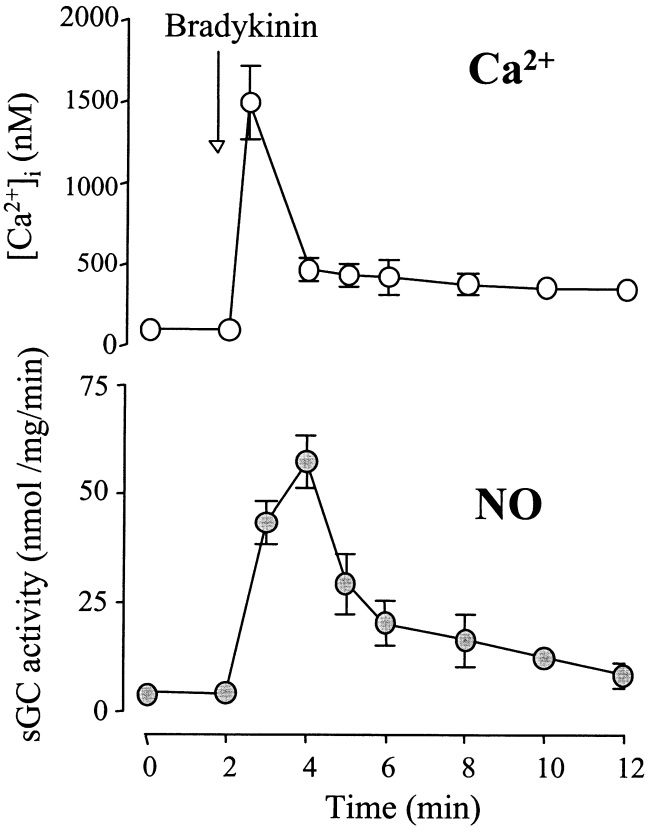


Figure 2 Temporal relationship between the agonist (bradykinin)-induced increase in endothelial concentration of free Ca^{2+} ($[Ca^{2+}]_i$) and the production of NO assessed as the activity of the intracellular NO receptor the soluble guanylyl cyclase (sGC).

THE INTERACTION OF eNOS WITH CALMODULIN

The identification of a CaM-binding domain in the primary structure of eNOS together with the finding that CaM-binding proteins inhibited enzyme activity suggested that the binding of a Ca^{2+}/CaM complex is essential to activate the constitutive enzyme.

Alterations in the CaM-binding domain determine the Ca^{2+} sensitivity of the various NOS isoforms, and substitution of eNOS and iNOS CaM-binding domains in eNOS–iNOS chimeric proteins produces major alterations in the Ca^{2+}/CaM dependence of the intact enzymes (see earlier). The CaM-binding domain of eNOS has also been reported to directly bind phospholipids, and thus it may determine the membrane association of the enzyme. Both a direct physical interaction and an association dependent on the phosphorylation of both the CaM-binding domain of eNOS and CaM have been proposed.

The most distinctive difference between the Ca^{2+}-regulated and Ca^{2+}-independent NOS isoforms is the existence of the polypeptide insert (40–50 amino acids) in the FMN-binding domains of the Ca^{2+}-dependent enzymes. Three-dimensional molecular modeling suggested that the insert originates from

a site immediately adjacent to the CaM-binding sequence, and synthetic peptides derived from the 45-amino acid insert were found to potently inhibit the binding of CaM to eNOS as well as enzyme activity. On the basis of these observations, it was suggested that the polypeptide insert is an autoinhibitory control element, which physically impedes CaM binding and thus enzyme activation. Such a control mechanism would imply that Ca^{2+}/CaM must displace the insert on binding to eNOS. This hypothesis is supported by the fact that the concentration of Ca^{2+} that is required to activate eNOS is markedly reduced following deletion of the autoinhibitory loop, and maximal enzyme activity is significantly (up to fourfold) enhanced. The autoinhibitory sequences in the two Ca^{2+}-dependent isoforms are not identical, and since the basal activity of nNOS is greater than that of eNOS, it is assumed that the eNOS inhibitory sequence is a much more effective inhibitor of enzyme activity. Indeed, synthetic peptides derived from the eNOS insertion sequence proved to be much more effective inhibitors of eNOS and nNOS activity than comparable peptides based on the nNOS insert. It is currently thought that the autoinhibitory loop regulates eNOS activity via at least two mechanisms, one related to the Ca^{2+}-dependent binding of CaM to the CaM-binding domain and the second to a constant partial inhibition of reductase activity.

The insert peptide is also a potential site for phosphorylation (12 of the 45 amino acids are either serine or threonine) and contains recognition motifs for PKC and casein kinase II. Therefore, phosphorylation and/or dephosphorylation may influence the affinity of insert peptides for binding and thus the sensitivity and/or affinity of eNOS/CaM binding.

The Interaction of eNOS with Caveolin-1

The subcellular localization of eNOS and alterations in its cellular compartmentalization following cell stimulation are controversial. Previous reports have assigned eNOS to the Golgi apparatus, whereas others have localized eNOS in plasma membranes or partially or exclusively in plasmalemmal caveolae. The truth lies somewhere in between, as immunostaining of porcine coronary arteries for eNOS reveals an association with the plasma membrane and with a perinuclear region, identified as the Golgi apparatus. Moreover, costaining of endothelial cells with antibodies raised against eNOS and caveolin-1 shows that not all eNOS is colocalized with caveolin-1, and a significant proportion of eNOS can be found within cell–cell contacts. In which fraction eNOS is active in unstimulated cells and can account for the basal production of NO is also controversial, since the eNOS in caveolae is thought to be mostly inactive (see below), and disruption of the Golgi apparatus in intact arterial segments reportedly fails to affect NO-mediated relaxation.

The binding of caveolin-1 to a consensus site (**F**^{350}XAAP**F**XXW) in the eNOS oxygenase domain is proposed to antagonize CaM binding and thereby inhibit enzyme activity. This motif in eNOS lies between the heme and the CaM-binding domain adjacent to a glutamate residue that is necessary for the binding of L-arginine, suggesting that caveolin-1 may interfere with the reduction of heme iron. Analysis of the crystal structure of the eNOS oxygenase domain revealed that the putative caveolin-1 recognition motif is primarily inaccessible to solvent. Therefore, it is relatively unlikely that caveolin-1 is able to interact with this sequence. Other groups have, however, detected interaction between both the N- and C-terminal domains of caveolin-1 and the oxygenase domain of eNOS. In experiments to examine how a caveolin-1 scaffolding domain peptide (amino acids 82–101) would affect NO synthesis, it was demonstrated that caveolin-1 must bind to the reductase domain of eNOS in order to inhibit enzyme activity. Therefore, caveolin-1 binding to the reductase domain of eNOS compromises its ability to bind CaM and to donate electrons to the heme subunit, thereby inhibiting NO synthesis. The interaction of caveolin-1 with the reductase domain was independent of the caveolin binding motif and was reversed by CaM. Coexpression of eNOS and caveolin-1 leads to a marked inhibition of enzyme activity. This inhibitory interaction is reported to be reversed by the addition of CaM, suggesting that eNOS activity may be determined by the relative proportion of eNOS–Ca^{2+}/CaM to eNOS–caveolin-1-binding.

One additional posttranslational mechanism thought to govern the interaction between eNOS and caveolin-1 is tyrosine phosphorylation, although it is not clear at the moment whether or not one or both of the proteins must be tyrosine phosphorylated.

Although there is overwhelming biochemical evidence for the existence of such a reversible protein–protein interaction, the extent of the eNOS–caveolin-1 interaction *in vivo* is still uncertain. For example, caveolae contain relatively large concentrations of CaM, and whether the stoichiometry of CaM to caveolin-1 in the vicinity of eNOS could ever reach the state at which eNOS is mostly inhibited is, at the moment, unclear. Moreover, the ongoing debate regarding the competitive antagonistic roles of CaM and caveolin-1 undermines the possible relevance of the autoinhibitory loop in the vicinity of the CaM-binding domain in regulating eNOS function.

What happens to eNOS after dissociation from the caveolin-1 complex is also largely unresolved. Although the dissociation of eNOS from a membrane fraction and its translocation to the cytosol following cell stimulation with bradykinin has been described, this finding is very controversial.

At this stage it should be stressed that although the points discussed earlier are applicable to the endothelium on the whole, the endothelium of one vessel or vascular bed is not always identical with that of another. The intracellular localization of eNOS differs in endothelial cells within one organ, as do the specific proteins expressed by the endothelium from one organ to the next.

Other Modulators of eNOS Function

There is a clear disparity in the temporal relationship between agonist-induced increases in endothelial $[Ca^{2+}]_i$ and the activation of eNOS, with the duration of the Ca^{2+} re-

sponse being significantly shorter than that of the subsequent NO production. Indeed, apart from changes in intracellular levels of Ca^{2+}, a number of posttranslational mechanisms have been proposed to regulate eNOS activity, including the interaction of eNOS with associated proteins, interaction with membrane phospholipids, and phosphorylation. In addition, relatively small pH changes in the physiological range (from pH 6.7 to pH 7.4) markedly alter the activity of the eNOS. High concentrations of NO may also affect eNOS activity by interacting with the heme prosthetic group, and the acute withdrawal of inhaled NO is associated with an increase in pulmonary vascular resistance.

Associated Proteins The concept that eNOS may complex proteins which determine cellular targeting or regulate its activity is somewhat analogous to the situation described for nNOS, which associates with α1-syntrophin, the postsynaptic density proteins (PSD-95 and PSD-93).

ENAP-1 ENAP-1 (endothelial NOS-associated protein-1) is a tyrosine-phosphorylated, approximately 90-kDa protein observed to interact with eNOS immunoprecipitated from cultured bovine aortic endothelial cells. The Ca^{2+}-elevating, receptor-dependent agonist bradykinin is reported to induce the association of ENAP-1 with eNOS, but the functional significance of this remains to be elucidated.

HSP90 Heat-shock proteins (Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsp/α-crystallins), so called because they are preferentially synthesized by organisms exposed to heat or other physiological stresses, are also expressed constitutively and function as molecular chaperones, able to mediate many cellular processes by influencing higher order protein structure and acting as a scaffolding protein. Several signal transduction systems, especially steroid receptors, utilize an interaction with Hsp90 as an essential component of the signaling pathway. A number of signaling molecules such as G-protein βγ subunits and protein kinases, including Src and Raf components of the mitogen-activated protein kinase (MAP) cascade, are also bound to Hsp90. Geldanamycin and herbimycin, commonly accepted as inhibitors of the Src family of tyrosine kinases, do not inhibit the kinase activity of Src but bind in a specific manner to Hsp90, inhibiting Src–Hsp90 heterocomplex formation and increasing Src turnover.

Hsp90 has been identified as an eNOS-associated protein, and its binding to the enzyme increases catalytic activity. A certain amount of Hsp90 appears to complex with eNOS in unstimulated endothelial cells, as immunoprecipitation of Hsp90 results in the recovery of eNOS, and vice versa. In response to cell stimulation with histamine or vascular endothelial growth factor (VEGF), Hsp90 is associated with eNOS, and exposure of endothelial cells to fluid shear stress stimulates the association of both proteins, albeit with a slower time course. In all cases the association of Hsp90 with eNOS increased NO production and was prevented by pretreatment with geldanamycin. The eNOS-associated Hsp90 may also serve as a scaffolding protein, facilitating the organization of additional associated regulatory proteins.

Given the similarity in the molecular weight of ENAP and Hsp90, it is possible that these are one and the same protein. Although Hsp90 has been described as a serine/threonine phosphorylated protein, coimmunoprecipitation experiments showed that the tyrosine kinase inhibitor genistein inhibited ligand-induced release of Hsp90 from the glucocorticoid receptor. Thus, the interaction of proteins with Hsp90 may also be regulated by a tyrosine kinase-dependent pathway. Preliminary reports also indicate that the Hsp90 which forms a complex with eNOS following the application of fluid shear stress is indeed tyrosine phosphorylated.

Phosphorylation The role of protein kinases in the regulation of endothelial NO production is a topic of intense current investigation, because several consensus sequence sites for phosphorylation by PKA, Akt/PKB (see later), PKC, and CaM kinase II (CaM-KII) are found in eNOS (Fig. 3). Although eNOS was initially reported to be basally phosphorylated solely on serine residues, it is now clear that eNOS is also phosphorylated on threonine and tyrosine residues. Whereas some progress has been made in elucidating the kinases that phosphorylate eNOS on serine and threonine residues, the amino acids they phosphorylate, and the consequences of phosphorylation on enzyme activity, elaborating the functional consequences of eNOS tyrosine phosphorylation is extremely complex, as this modification is only evident in endothelial cells *in situ* or in primary cultured cells.

PKC In cultured endothelial cells, the Ca^{2+}-elevating receptor-dependent agonist bradykinin was initially described to enhance the serine phosphorylation of eNOS, an effect which was maximal after 5 min and was maintained for at least 20 min. This bradykinin-induced phosphorylation of eNOS appears to be a Ca^{2+}-dependent phenomenon and is inhibited either by a calmodulin antagonist or by the removal of extracellular Ca^{2+}. A rough comparison of the time course of NO production and eNOS activation in response to bradykinin suggests that at least this serine phosphorylation of eNOS may be an inactivating mechanism and fits well with the observations that PKC (which phosphorylates eNOS *in vitro*) negatively regulates endothelial NO production. In addition, the exposure of pulmonary artery endothelial cells to sodium nitroprusside (SNP) enhances the serine phosphorylation of eNOS in a PKC-dependent manner and attenuates eNOS activity. PKC inhibitors significantly enhance agonist-induced NO release. However, the serine residue phosphorylated by PKC is unknown.

AMPK The AMP-activated protein kinase (AMPK) can be categorized equally well under the heading NOS-associated protein as under the heading eNOS kinase. This rather unusual kinase is part of a family generally described as ''metabolite sensing kinases'' that are activated following heat shock, vigorous exercise, hypoxia/ischemia, and starvation. Initially, AMPK was thought to be an indicator of intracellular energy demands, but it also appears to modulate intracellular energy levels, as it initiates changes to maintain intracellular ATP levels and prevent complete ATP deple-

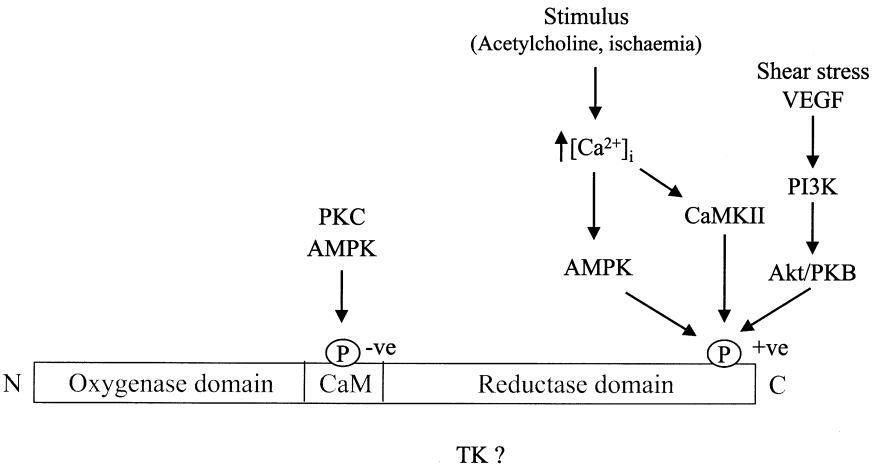


Figure 3 Regulation of eNOS phosphorylation and activity by different stimuli. Stimuli that elevate endothelial Ca^{2+} levels activate AMP-activated protein kinase (AMPK) and CaM-dependent kinase II (CaM-KII) to phosphorylate Ser-1177 and enhance NO production. Phosphorylation of the same residue is achieved in the absence of an increase in endothelial Ca^{2+} following stimulation with mechanical stimuli (such as shear stress) as a consequence of the sequential activation of phosphatidylinositol 3-kinase (PI3K) and Akt/PKB. eNOS appears to be basally phosphorylated by both AMPK and PKC on Thr-495. This residue is situated within the CaM-binding domain, and thus inhibits enzyme activity by interfering with the association of CaM with eNOS. (Modified from Michell *et al.,* 1999.)

tion; that is, once activated the enzyme switches off ATP-consuming anabolic pathways and switches on ATP-producing catabolic pathways. Functionally, the activity spectrum of AMPK has not been fully elucidated, but AMPK is clearly a multisubstrate enzyme.

What has a kinase that regulates the cellular ATP balance to do with the regulation of eNOS? This is actually quite a complicated process as, depending on the cellular conditions, AMPK can activate or inactivate eNOS. Fitting with the known role of AMPK as a metabolite sensor, the interaction between eNOS and AMPK was first demonstrated in rat hearts in response to ischemia. AMPK coprecipitates with eNOS from cardiac homogenates as well as the eNOS isolated from endothelial cells of different origins. Activation of AMPK in the presence of Ca^{2+}/CaM following the induction of ischemia results in the serine phosphorylation of eNOS and its activation. The serine residue phosphorylated by AMPK is Ser-1177, which is located in the carboxyl-terminal region of the reductase domain.

Although various forms of metabolic stress are expected to activate eNOS via its AMPK-dependent phosphorylation, the full spectrum of physiological stimuli resulting in the activation of AMPK and the AMPK-dependent phosphorylation of eNOS in endothelial cells remains to be fully elucidated.

Ca^{2+}-Independent eNOS Activation

Although classified as a Ca^{2+}/CaM-dependent enzyme, a basal eNOS activity was originally reported at Ca^{2+} concentrations as low as 10 n*M* in lysates prepared from native endothelial cells, indicating that a significant portion of the NO produced by unstimulated endothelial cells may be formed via a Ca^{2+}-independent pathway. Little physiological relevance was attributed to this phenomenon, especially after the identification of a CaM-binding domain in the primary structure of eNOS. More recent thorough biochemical studies have, however, reinforced the original observation that eNOS may produce NO in an apparently Ca^{2+}-independent manner. Although eNOS, like the neuronal isoform, requires the binding of Ca^{2+}/CaM to achieve maximal activity, a basal level of NO production can be demonstrated when Ca^{2+}/CaM is omitted and 0.5 m*M* EDTA is present in the assay solution. Moreover, eNOS binds CaM so tightly that the activity of the purified enzyme is about 80% of maximum, and immunoprecipitation of eNOS results in the coprecipitation of CaM, unless stringent washing procedures are used.

Experimental evidence has demonstrated that a number of stimuli can activate eNOS in an apparently Ca^{2+}-independent manner; that is, NO production can be elicited in the absence of an increase in intracellular Ca^{2+}, and/or in the presence of an intracellular Ca^{2+} chelator. These stimuli include mainly physical stimuli, such as shear stress and isometric stretch, as well as growth factors and hormones such as VEGF and estrogen. It is important to stress, however, that although there are obviously two completely separate pathways that can lead to the activation of eNOS, both are, strictly speaking, Ca^{2+} dependent, as eNOS activity (like that of iNOS) is abolished in the presence of high concentrations of EDTA and EGTA.

Fluid Shear Stress, Stretch, and Strain

Blood flow through a vascular segment generates a viscous drag at the luminal surface of endothelial cells that can

be expressed as shear stress (τ) and can be calculated according to Poiseuille's law (see Fig. 4). This relation highlights the fact that relatively small decreases in vessel diameter at constant flow can markedly increase shear stress at the endothelial surface. It should be noted, however, that shear stress is calculated under the assumption that the flow profile is parabolic, a situation which is never realized *in vivo* owing to the pulsatile nature of blood flow and the fact that blood vessels are distensible. Branching and bifurcations along the vascular tree together with a gradual decrease in diameter also disfavor the establishment of a stable flow profile.

Although it is sometimes assumed that pressure per se is a proper physiological stimulus for the vascular wall, it must be emphasized that the application of pressure in the physiological range (below 1 atm) to a tissue which is essentially incompressible due to its high water content has no direct effect on cell function. Rather, the pressure exerted on a compliant, anisotropic tissue elicits deformation, that is, distension (stretch). As a result of pulsatile pressure changes and the anisotropy of the tissue, cells within the vascular wall are subjected to three-dimensional cyclic strain in the radial, longitudinal, and circumferential directions.

There are also hemodynamically relevant cell–cell-generated forces that cannot be expressed by a simple physical relationship. One example is the isometric contraction, in which the development of contractile force within the smooth muscle cell layer counteracts the distending transmural pressure. Under such conditions there is a relative displacement of opposing cell layers within the vascular wall (e.g., smooth muscle cells versus elastic lamina and endothelial cells), despite the fact that no net movement occurs. Although the displacement induced may be subtle, the close physical arrangement of endothelial focal adhesion contacts and the smooth muscle suggests that the forces developed at the abluminal surface of endothelial cells may be greater than those generated by shear stress on the luminal surface.

In principle, the relative contribution of pulsatile stretch and wall shear stress to the adjustment of local vascular tone is likely to depend on a number of factors including vessel architecture, moment-to-moment changes in smooth muscle activity, and the ability of endothelial and smooth muscle cells to sense hemodynamic stimuli and to produce vasoactive factors. During each cardiac cycle, the ventricles eject a given volume of blood into the aorta and the pulmonary artery. At each site in the vasculature through which this pulse wave travels, three basic wave phenomena can be observed: a pressure pulse, a flow pulse, and a volume pulse (which is a change in cross-sectional area). In an artery *in situ,* two extreme situations that result in an increase in either shear stress or circumferential strain at a given pulse pressure are conceivable; in other words, in a vessel exhibiting high tone and thus low distensibility, the endothelium would be exposed to shear stress levels greatly elevated above those observed in a highly distensible vessel with low tone. The converse is, of course, the case for circumferential strain or stretch.

However, although it is tempting to break down hemodynamically generated forces into singular physical compo-

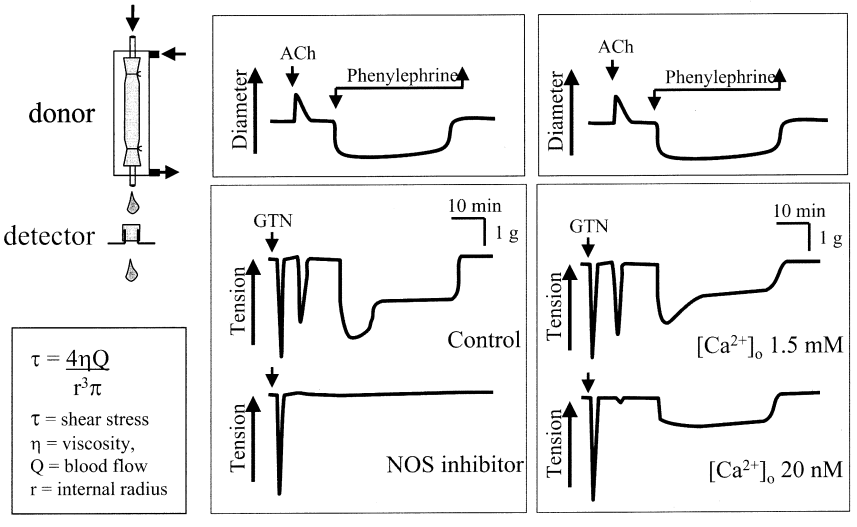


Figure 4 Bioassay system for the detection of NO released from endothelium-intact rabbit iliac artery segments. The upper tracing represents the change in external diameter of the endothelium-intact donor segment following the bolus application of acetylcholine (ACh) and superfusion with phenylephrine to contract the artery and thus increase shear stress at constant flow according to Poiseuille's law (insert). The lower traces represent the relaxation of an endothelium-denuded detector ring in response to topical application of glyceryl trinitrate (GTN) or superfusion with the effluate from the donor. The tracings shown demonstrate that the relaxations of the detector ring are completely inhibited by a NOS inhibitor, and that the removal of extracellular Ca^{2+} abolishes the ACh-induced production of NO by the donor segment but not that elicited by an increase in fluid shear stress. (Modified from Ayajiki *et al.,* 1996.)

nents such as circumferential stretch or strain and wall shear stress, it must be stressed that such a procedure is purely theoretical, as changes in pulsatile stretch and shear stress are inextricably linked.

Fluid Shear Stress and Ca^{2+}-Independent NO Production

Endothelial cells are continuously exposed to shear stress, and not to agonists such as acetylcholine or bradykinin, and it is shear stress (together with other mechanical stimuli such as cyclic stretch) that is the physiologically most relevant determinant of endothelial activation, NO production, and, as a consequence, the local regulation of vascular tone and organ perfusion. The first demonstration of an apparently Ca^{2+}-independent eNOS activation in native endothelial cells (i.e., endothelial cells in an isolated intact arterial segment) was in response to fluid shear stress in a classic bioassay system where the NO produced in a perfused, endothelium-intact donor segment was detected by monitoring alterations in the tone of an isometrically contracted endothelium-denuded detector ring (Fig. 4). In this bioassay system, constriction of the donor segment at a constant perfusion rate increases fluid shear stress at the endothelial surface and evokes a pronounced vasodilation in the detector ring. This response consisted of an initial transient peak followed by a stable plateau phase that was maintained as long as the donor remained constricted. The removal of the endothelium from the donor artery, or pretreatment with a NOS inhibitor, abolished both the acetylcholine and the shear stress-induced relaxation of the detector segment.

Replacing the solution perfusing the donor segment with a nominally Ca^{2+}-free solution (i.e., lowering the extracellular Ca^{2+} concentration from 1.6 to $\sim$10 n*M*) completely abolished the acetylcholine-induced release of NO and the peak component of NO release in response to vasoconstriction. The plateau phase of NO release, however, was unaffected by the absence of extracellular Ca^{2+}.

Phosphorylation

As the transmembranous influx of Ca^{2+} does not appear to regulate eNOS activity following the application of fluid shear stress, other intracellular signaling pathways must be involved in the regulation of NO production by eNOS. Because fluid shear stress is known to alter the phosphorylation of numerous endothelial cell proteins, and a similar and apparently Ca^{2+}-independent activation of eNOS can be achieved by incubating endothelial cells with inhibitors of either serine or tyrosine phosphatases, it seemed logical to assume that Ca^{2+}-independent eNOS activity may be modulated by eNOS phosphorylation or by the phosphorylation of an eNOS-regulatory protein.

AKT/PKB

Akt (otherwise known as protein kinase B or PKB) is a downstream target of at least two kinases [the phosphatidylinositol 3-kinase (PI3K) and the p38 MAP kinase], and it phosphorylates serine residues within a defined recognition sequence, RXRXXS. This motif is present in the sequence surrounding RIRTQS(1177)F in eNOS. PI3K and Akt/PKB can be activated by some, but not all, of the stimuli that result in the activation of eNOS. For example, the classic Ca^{2+}-elevating agonists bradykinin, histamine, and Ca^{2+} ionophores do not activate Akt/PKB in arterial endothelial cells, whereas mechanical stimuli, such as fluid shear stress and isometric contraction, and the growth factors VEGF, insulin, and angiopoietin do. In other words, stimuli that elicit the apparently Ca^{2+}-independent activation of eNOS generally also activate Akt/PKB.

In order for an Akt/PKB-mediated phosphorylation of eNOS to be accountable for the alteration in eNOS Ca^{2+} sensitivity, a number of criteria must be fulfilled:

1. Akt/PKB must be rapidly activated following the onset of the stimulus.
2. Activation of Akt/PKB must be maintained as long as the stimulus duration (at least in the case of shear stress).
3. Activation of Akt/PKB should occur independently of the presence of extracellular Ca^{2+}.
4. Akt/PKB should associate with eNOS and phosphorylate it.
5. The Akt/PKB-mediated phosphorylation of eNOS should alter the sensitivity of the enzyme to Ca^{2+}.

All of these criteria are met by Akt/PKB. Therefore, to all intents and purposes, the Ca^{2+}-independent activation of eNOS can be attributed to the activation of PI3K and its downstream target Akt/PKB. Indeed, inhibiting the activation of PI3K prevents the activation of Akt/PKB, the phosphorylation of eNOS Ser-1177, and the shear stress- or VEGF-induced production of NO. Specific mutation of Ser-1177 to aspartate in order to mimic the continuous phosphorylation of the protein significantly augments enzyme activity (Fig. 5). On the other hand, overexpression of a construct in which Ser-1177 phosphorylation was inhibited by replacement with alanine resulted in an enzyme activity that was consistently lower than that of the wild-type enzyme.

There are, however, a number of points that remain to be clarified, the first being how the phosphorylation of Ser-1177 alters the affinity of eNOS for Ca^{2+}. One possibility under investigation is whether phosphorylation increases the affinity of the eNOS reductase domain for CaM. If this is the case, how can the phosphorylation of a serine residue located at the carboxy-terminal reductase domain influence CaM binding? Perhaps the best current hypothesis is that Ser-1177 is part of an additional inhibitory motif that stabilizes the autoinhibitory loop in its ''closed position'' and thus prevents CaM from obtaining access to its binding domain (Fig. 6). Phosphorylation or Ser-1177 may alleviate this intrinsic inhibition, so that the autoinhibitory loop can be displaced and the CaM present in the vicinity of eNOS is able to bind and enhance eNOS activity. Such a sequence of events, should it be proved correct, is likely to be a general eNOS activation mechanism, as this one residue (Ser-1177) can be phosphorylated by more than one protein kinase. Although

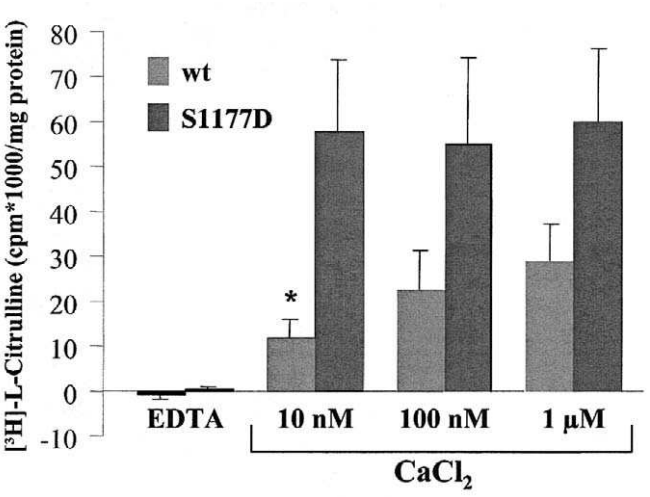


Figure 5 The wild-type (wt) eNOS is less active and markedly more sensitive to alterations in Ca^{2+} concentration than the eNOS phosphomimetic (replacement of Ser-1177 with aspartate). NO activity was assessed as the production of L-[^{3}H]citrulline from L-[^{3}H]arginine. (Modified from Dimmeler *et al.,* 1999.)

Akt/PKB currently appears to be the only kinase that can phosphorylate this residue without an increase in intracellular Ca^{2+}, AMPK and CaM kinase are also able to phosphorylate it and enhance NO production in response to other stimuli.

Despite several convincing demonstrations that Akt/PKB directly phosphorylates eNOS *in vitro,* it is possible that other signal transduction pathways must also be activated in parallel in order to facilitate NO production. For example, insulin and angiopoietin which, as mentioned earlier, are potent activators of Akt/PKB in native and cultured endothelial cells, have little or no effect on NO production. Although few data are available at the moment, it is more than likely that stimulus-induced alterations in the association of eNOS with other signaling or adapter molecules will play a decisive role in the regulation of endothelial NO production.

AMPK

It is no mistake that the AMP-activated kinase appears under two different subheadings, as AMPK does differentially regulate eNOS activity depending on the availability of Ca^{2+}/CaM. AMPK actually phosphorylates eNOS on two distinct sites. The first, as mentioned earlier, is Ser-1177. However, in the absence of Ca^{2+}/CaM, AMPK phosphorylates eNOS on Thr-495. Thr-495 is an amino acid situated within the CaM-binding domain and is also thought to be phosphorylated by PKC. In both cases, phosphorylation, which attenuates enzyme activity, most probably by interfering with the binding of Ca^{2+}/CaM. The stimuli that result in the activation of AMPK and eNOS Thr-495 phosphorylation are completely unknown, as the induction of ischemia, which is a classic activator of AMPK, does not affect the threonine phosphorylation of eNOS.

TYROSINE KINASES

Nonreceptor tyrosine kinases play a central role in endothelial cell signaling and may functionally link rearrangement of the cytoskeleton or focal adhesion points with more

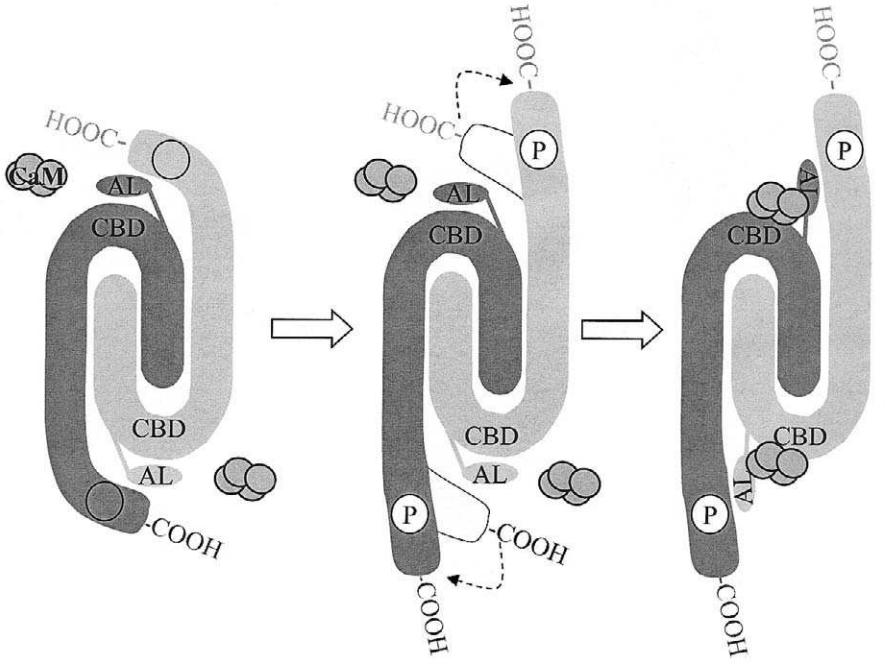


Figure 6 Proposed model for the activation of eNOS and the conferral of apparent Ca^{2+} independency on eNOS by the phosphorylation of Ser-1177. CaM, calmodulin; CBD, CaM-binding domain; AL, autoinhibitory loop; P, phosphorylation. The open circle represents the Akt/PKB recognition motif.

classic signal transduction pathways (see later). Results obtained in the shear stress bioassay suggest a role for tyrosine kinases in mediating shear stress-induced NO production, as tyrosine kinase inhibitors almost completely abolish shear stress-dependent NO production. Indeed, the application of fluid shear stress to cultured endothelial cells results in both transient and maintained alterations in the protein tyrosine phosphorylation of detergent-soluble and -insoluble proteins and has been shown to enhance eNOS tyrosine phosphorylation. The tyrosine residues phosphorylated following the application of shear stress remain to be elucidated, as does the kinase which phosphorylates it.

How Does an Endothelial Cell Sense Physical Stimuli?

The endothelial cell can be viewed as a membrane stretched over a frame composed of microtubules, intermediate filaments, and actin fibers that transverse the cells and appear to end in characteristic adhesion complexes. Even under nonstimulated conditions the entire endothelial cytoskeleton is maintained under tension, and in response to an externally applied stimulus intracellular tension is redistributed over the cytoskeletal network. This tensegrity architecture within cells permits forces to be directly transmitted from the cell surface, through the cytoskeleton, and across physically interconnecting filaments. Thus, extracellular forces are superimposed on preexisting forces within cells attached to the extracellular matrix at focal adhesion points, and to each other at cell–cell contacts. Generally, signaling molecules are clustered around and inherent to these contact sites, so that it is conceivable that the application of a stress, which is transmitted through the entire cell by the actin cytoskeleton, activates signal transduction cascades without the need of a specific shear stress or stretch receptor (see Fig. 7). Molecular connections between integrins, cytoskeletal filaments, and nuclear scaffolds have been proposed to provide a pathway for signal transfer, thus raising the possibility that mechanical stimuli may be passed on to the nucleus in the absence of or simultaneously with mechanochemical signaling processes.

Focal adhesion sites, cell–cell contacts, and caveolae have been suggested to play a critical role in the differential activation of signaling cascades in response to fluid shear stress. However, rather than there being one major site of shear sensing, it is more than likely that the signaling pathways activated by the mechanical perturbation of endothelial cells are determined by the sites where there is the greatest alteration in force. Thus, the apparent selective regulation of cellular signaling pathways is in fact predetermined by the localization of signaling molecules within caveolae, cell–cell contacts, and focal adhesion plaques.

Mechanosensitive ion channels are present in endothelial cells and respond primarily to membrane tension, rendering them appropriate transducers for forces derived from osmotic or hydraulic gradients and shear stress. These channels are usually cation selective, passing Ca^{2+} as well as monovalent ions, but some are K^+ selective. The open probability

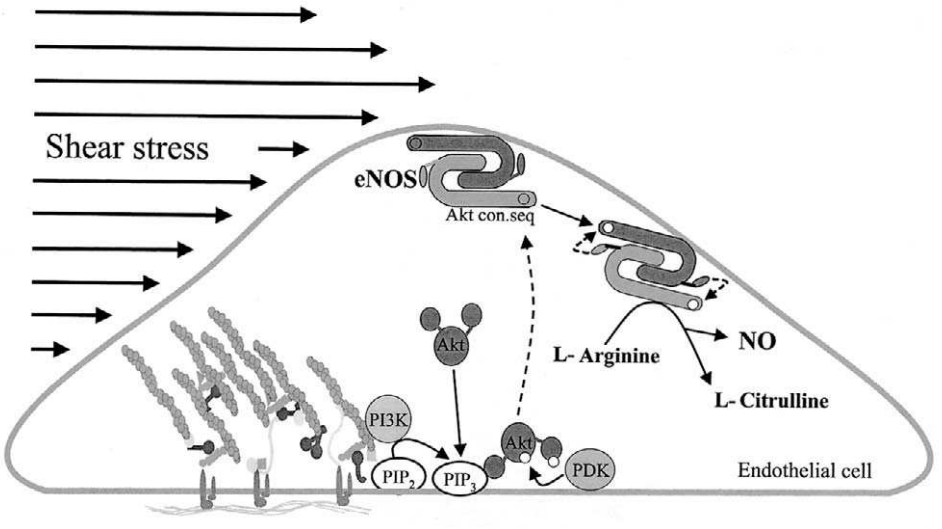


Figure 7 Proposed model for the activation of Akt/PKB and the phosphorylation of eNOS in response to the mechanical stimulation of an endothelial cell by fluid shear stress. The symbols on the left-hand side represent the cytoskeletal structure of the endothelial cell in the vicinity of a focal adhesion plaque, where many adaptor molecules and inactive protein kinases are localized (see text for explanation). Deformation of the endothelial cell by shear stress leads to a redistribution of stress across the endothelial structure, resulting in changes in the association of actin filaments and cytoskeleton-associated proteins. This leads to the activation of phosphatidylinositol 3-kinase (PI3K), which converts phosphatidylinositol bisphosphate (PIP_2) to phosphatidylinositol trisphosphate (PIP_3). The newly formed PIP_3 promotes the translocation of the inactive kinase Akt/PKB to the plasma membrane, where it is phosphorylated by phosphatidylinositol-dependent kinases (PDKs). The now activated Akt/PKB is able to dissociate from the PI3K and phosphorylate eNOS within the Akt/PKB recognition motif on Ser-1177 and stimulates NO production.

of these channels may be modulated by membrane potential and various ligands, although stress-activated ion channels may also be activated by virtue of cytoskeletal interconnections rather than directly through the plasma membrane. Other possible mechanisms by which endothelial cells may sense shear is through the glycocalyx, a layer of glycoproteins extending into the extracellular space, which may be displaced by shear stress to elicit an intracellular response.

Additional Reading

Andries, L. J., Brutsaert, D. L., and Sys, S. U. (1998). Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. *Circ. Res.* **82,** 195–203.

Ayajiki, K., Kindermann, M., Hecker, M., Fleming, I., and Busse, R. (1996). Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circ. Res.* **78,** 750–758.

Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* **82,** 743–752.

Dawson, V. L., and Dawson, T. M. (1998). Nitric oxide in neurodegeneration. *Prog. Brain Res.* **118,** 215–229.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399,** 601–605.

Fleming, I., and Busse, R. (1999). Signal transduction of eNOS activation. *Cardiovasc. Res.* **43,** 532–541.

García-Cardeña, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S. W., Lisanti, M. P., and Sessa, W. C. (1997). Dissecting the interaction between nitric oxide synthase (NOS) and caveolin—Functional significance of the NOS caveolin binding domain in vivo. *J. Biol. Chem.* **272,** 25437–25440.

García-Cardeña, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **292,** 821–824.

Griscavage, J. M., Fukuto, J. M., Komori, Y., and Ignarro, L. J. (1994). Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. *J. Biol. Chem.* **269,** 21644–21649.

Ingber, D. E. (1997). Tensegrity: The architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.* **59,** 575–599.

Kandel, E. S., and Hay, N. (1999). The regulation and activities of the mutifunctional serine/threonine kinase Akt/PKB. *Exp. Cell Res* **253,** 210–229.

Michell, B. J., Griffiths, J. E., Mitchelhill, K. I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., Ortiz de Montellano, P. R., Kemp, B. E., and Pearson, R. B. (1999). The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr. Biol.* **9,** 845–848.

Nishida, C. R., and deMontellano, P. R. (1998). Electron transfer and catalytic activity of nitric oxide synthases—Chimeric constructs of the neuronal, inducible, and endothelial isoforms. *J. Biol. Chem.* **273,** 5566–5571.

Pratt, W. B. (1997). The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptor signaling via MAP kinase. *Annu. Rev. Pharmacol. Toxicol.* **37,** 297–326.

Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997). An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J. Biol. Chem.* **272,** 29769–29777.

Xie, Q.-W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256,** 225–228.

CHAPTER 39

Role of Nitric Oxide in Ischemia–Reperfusion Injury

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THE IMMUNE SYSTEM CONSISTS OF A MULTIFACETED DEFENSE INVOLVING CIRCULATING LEUKOCYTES, BLOOD-BORNE FACTORS, AND CELL-BOUND MEDIATORS. ALTHOUGH THE IMMUNE SYSTEM PRIMARILY FUNCTIONS TO KILL PATHOGENS, INVASION OF MICROORGANISMS IS NOT A PREREQUISITE FOR IMMUNE ACTIVATION. ISCHEMIA–REPERFUSION, OR THE INTERRUPTION AND RESTORATION OF TISSUE BLOOD FLOW, IS A COMMON CLINICAL SITUATION (EXAMPLES INCLUDE ORGAN TRANSPLANTATION, BYPASS PROCEDURES, AND CEREBROVASCULAR DISEASES) THAT APPEARS TO RESULT IN IMMUNE SYSTEM ACTIVATION. THIS REGIONAL (E.G., STROKE) OR SYSTEMIC (E.G., HEMORRHAGIC SHOCK) VASCULAR DISORDER IS ASSOCIATED WITH AN OVERPRODUCTION OF OXYGEN FREE RADICALS AND PRODUCTION OF INFLAMMATORY MEDIATORS BY ACTIVATED ENDOTHELIAL CELLS, AS WELL AS THE ACTIVATION AND ENDOTHELIAL CELL ADHESION OF CIRCULATING BLOOD CELLS, SUCH AS LEUKOCYTES AND PLATELETS. THE INFILTRATION OF PLATELETS AND LEUKOCYTES INTO POSTISCHEMIC TISSUE IS ALSO FACILITATED BY AN INCREASED EXPRESSION OF ADHESION GLYCOPROTEINS AND MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) MOLECULES ON THE SURFACE OF ENDOTHELIAL CELLS.

NITRIC OXIDE (NO), WHICH IS PRODUCED BY A VARIETY OF CELLS THAT PARTICIPATE IN THE IMMUNE RESPONSE, APPEARS TO CONTRIBUTE TO THE PATHOPHYSIOLOGICAL CHANGES ASSOCIATED WITH ISCHEMIA–REPERFUSION. NITRIC OXIDE EXHIBITS BOTH ANTI- AND PROINFLAMMATORY PROPERTIES THAT CAN INFLUENCE THE SEVERITY AND DURATION OF THE IMMUNE ACTIVATION ELICITED BY ISCHEMIA–REPERFUSION. THIS CHAPTER WILL ADDRESS THE IMMUNE SYSTEM ACTIVATION THAT OCCURS AFTER REPERFUSION OF ISCHEMIC TISSUES AND DISCUSS THE CONTROVERSIAL ROLE OF NO IN MODULATING THIS RESPONSE.

Ischemia–Reperfusion Enhances the Production of Cytotoxic Oxygen Radicals

Endothelium-Derived Oxygen Radicals

Tissue ischemia results in the depletion of intracellular adenosine triphosphate (ATP) stores, which subsequently compromises the function of membrane-associated, ATP-dependent ionic pumps in endothelial cells. This membrane dysfunction allows entry of calcium, sodium, and water into the cells. The resultant accumulation of calcium and other ions in the cell can result in cell swelling and the inappropriate activation of cellular enzymes. One enzyme that is activated by the rise in intracellular calcium during ischemia is xanthine dehydrogenase (XDH). Under normal conditions, hypoxanthine (a breakdown product of ATP metabolism) is oxidized by XDH, in an NADH-dependent manner, to produce xanthine and uric acid. However, during the hypoxic

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condition of ischemia, hypoxanthine levels rise within the cell due to ATP hydrolysis, and there is a calcium-dependent activation of proteases that convert the NADH-reducing XDH to an oxygen-reducing form of the enzyme, namely, xanthine oxidase (XO) (Granger, 1988). On restoration of blood flow (reperfusion) to the tissue and with the reintroduction of molecular oxygen, XO will convert hypoxanthine to xanthine and uric acid, and it will catalyze the reduction of molecular oxygen to form both superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) (Fig. 1).

This XO-dependent mechanism of oxygen radical production has been invoked to explain the involvement of O_2^- and H_2O_2 in reperfusion injury to a variety of organs, including intestine, brain, heart, and skeletal muscle (Granger and Korthuis, 1995). The contention that XO contributes to the enhanced generation of oxygen radicals is largely based on studies demonstrating diminished oxygen radical-dependent tissue injury in animal models of reperfusion injury treated with XO inhibitors such as allopurinol or oxypurinol.

The tissue injury that is elicited by ischemia–reperfusion does not appear to be mediated by O_2^- or H_2O_2 per se, but by a product of the reaction of these two species which is more unstable and hence more reactive. The hydroxyl radical (·OH) is formed by a spontaneous reaction between O_2^- and H_2O_2 that is catalyzed by metals such as iron (the iron-catalyzed Haber–Weiss or Fenton reaction) (Fig. 1). There is ample evidence that iron is mobilized from ferritin and other iron-sequestering proteins during ischemia and reperfusion. Hence, the metal is available for facilitation of ·OH formation in postischemic tissues. A number of investigators have proposed that the highly cytotoxic ·OH may directly account for the microvascular and parenchymal cell dysfunction that is manifested as reperfusion injury, whereas others attribute a more important role for XO-derived oxygen radicals (O_2^- and H_2O_2) as signaling molecules that subsequently recruit and activate neutrophils (Granger and Korthuis, 1995).

Neutrophil-Derived Oxygen Radicals

Neutrophils possess the membrane-associated enzyme NADPH oxidase, which allows leukocytes to generate large quantities of oxygen radicals (Granger and Korthuis, 1995). It is activated when neutrophils are exposed to inflammatory mediators, such as the leukotriene B_4 (LTB_4) and platelet activating factor (PAF) produced by endothelial cells. Neutrophilic NADPH oxidase reduces molecular oxygen to yield O_2^-, which in turn undergoes spontaneous dismutation to form H_2O_2. Unlike H_2O_2 in endothelial cells, neutrophil-derived H_2O_2 does not participate in iron-catalyzed reactions to form ·OH, but rather participates in a reaction that yields hypochlorous acid (HOCl), which possesses 100 times the oxidizing and chlorinating potential of H_2O_2. In the presence of myeloperoxidase (MPO), which is released from neutrophil granules on cellular activation, H_2O_2 reacts with chloride ions to form HOCl. Antioxidant enzymes are present in cells to decompose O_2^- and H_2O_2, but similar detoxification mechanisms do not exist for HOCl.

Although both XO and neutrophilic NADPH oxidase contribute to the enhanced oxygen radical production observed in postischemic tissues, the two sources generate radicals at different times after the onset of reperfusion. Endothelial cell-associated XO appears to account for the enhanced oxidant flux that is observed within the first few

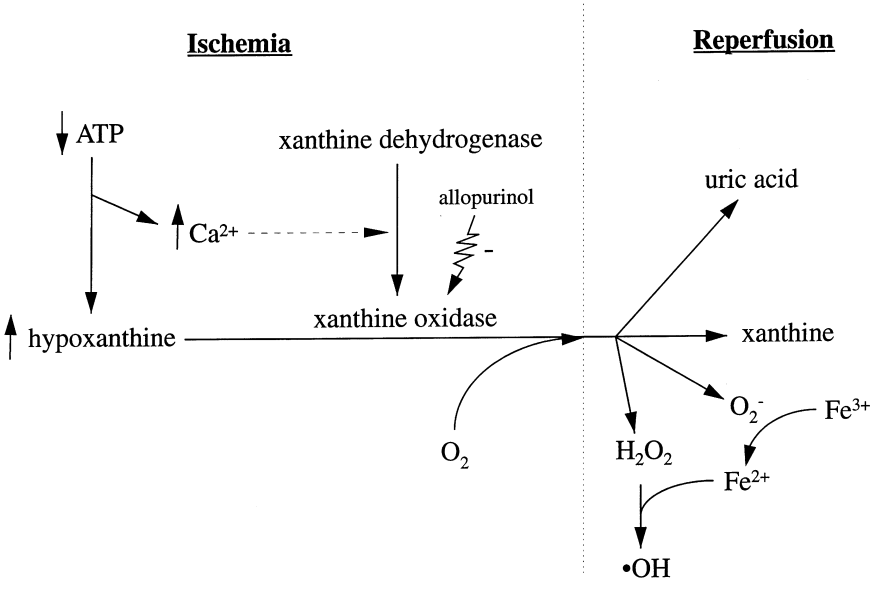


Figure 1 The xanthine oxidase pathway of oxygen radical production following ischemia–reperfusion. During ischemia, hypoxanthine levels build up as ATP is metabolized and xanthine dehydrogenase is converted to xanthine oxidase in a calcium-dependent manner. On reintroduction of oxygen to the tissue when it is reperfused, the xanthine oxidase converts the hypoxanthine to oxygen radicals (O_2^- and H_2O_2), xanthine, and uric acid.

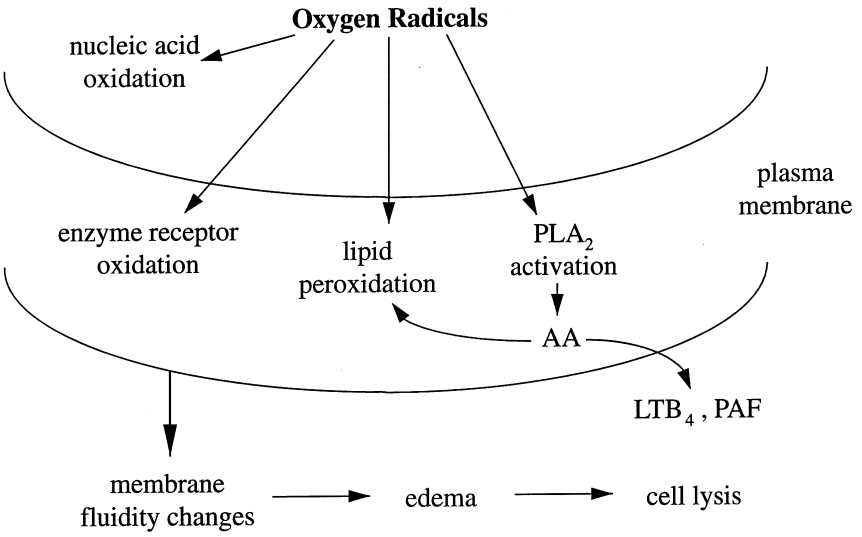


Figure 2 The mechanisms of oxidant-induced injury. The oxygen radicals attack the cell membrane, causing lipid peroxidation and release of inflammatory mediators such as LTB_4 and PAF. The net result of this is decreased cell membrane integrity, increased membrane permeability, and ultimately cell death.

minutes after reperfusion, while neutrophils account for the oxidant generation detected at 1–5 hours after reperfusion.

Mechanisms of Oxygen Radical-Mediated Tissue Injury

The plasma membrane is not only a site for the generation of oxygen radicals, but it is also a prime target for radical attack and damage (Fig. 2). Oxygen radicals can initiate injury by promoting lipid peroxidation of cell membranes and through oxidative modification of nucleic acids and enzyme receptors. Oxygen radicals can activate phospholipase A_2 (PLA_2) by increasing intracellular calcium. This activated enzyme releases lipid peroxyl free radicals, which promote further lipid peroxidation. Activation of PLA_2 also causes arachidonic acid (AA) to split off from membrane phospholipids. The AA is then metabolized by several enzymes to yield inflammatory mediators such as PAF and LTB_4. The lipid peroxidation initiated by oxygen radicals results in cell dysfunction, which is characterized by a loss of cell membrane integrity resulting from alterations in membrane fluidity and cell compartmentalization. Vascular endothelial cells may swell or lyse as a consequence of the aforementioned events. Consequences of these changes include a diminished endothelial barrier function, an increased vascular permeability, and interstitial edema.

Oxidants can also initiate leukocyte–endothelial cell adhesion. This action may result from oxidant-mediated generation of inflammatory mediators, which increase the expression of adhesion molecules on leukocytes, endothelial cells, or both. Alternatively, oxygen radicals may activate nuclear transcription factors [e.g., nuclear factor κB (NF-κB), activator protein-1 (AP-1)] that promote the biosynthesis of specific endothelial cell adhesion molecules (CAMs) such as E-selectin and vascular cell adhesion molecule (VCAM-1). Both mechanisms (inflammatory mediator production and nuclear transcription factor activation) can result in the recruitment of inflammatory cells, which in turn can mediate the parenchymal cell and microvascular dysfunction observed in postischemic tissues. Neutrophil-derived HOCl and H_2O_2 can also disturb the balance of extracellular proteases and antiproteases, toward more proteolytic power. These oxidants can inactivate antiproteases (e.g., antitrypsin), thereby allowing neutrophilic proteases (e.g., elastase) to hydrolyze components of the vascular wall and interstitial matrix. This protease-promoting action is manifested at the interface between adherent leukocytes and vascular endothelial cells. A microenvironment exists between adherent leukocytes and the blood surface of vascular endothelial cells that is not subject to the ''washout'' effect of the flowing blood. As a result, the damage inflicted on endothelial cells by neutrophilic proteases and oxidants can be quite severe, as extracellular antiproteases and antioxidants are rapidly consumed in the protected microenvironment.

Antioxidants

The generation of oxygen radicals in postischemic tissues appears to overcome the capacity of endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione, and NO to protect endothelial and parenchymal cells (Fig. 3). Exogenous antioxidants such as SOD and catalase have been shown to attenuate the leukocyte infiltration and tissue injury elicited by ischemia and reperfusion. Indeed, antioxidants have been successfully employed in preservation solutions for prevention of reperfusion injury to transplanted organs. Superoxide dismutase, which is normally found in the cytosol and mitochondria, catalytically dismu-

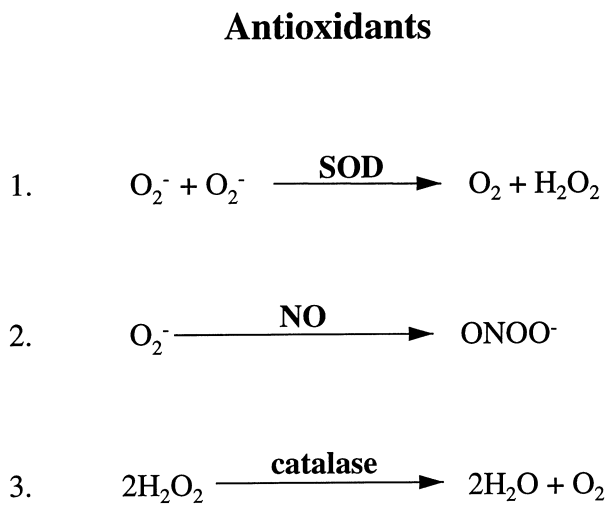


Figure 3 Endogenous antioxidants, SOD, NO, and catalase are normally able to protect the endothelium from endogenous release of oxidants by participating in their conversion to less harmful metabolites. During ischemia–reperfusion, however, oxygen radical release overcomes the capacity of the antioxidants to prevent events such as leukocyte infiltration and tissue injury. Administration of exogenous sources of these antioxidants protects against ischemia–reperfusion-induced injury.

tates two molecules of O_2^- to form oxygen and H_2O_2. Nitric oxide can also react with O_2^-, but this reaction occurs at a rate that is three times faster than that catalyzed by SOD. The inactivation of O_2^- by NO results in the formation of peroxynitrite, $ONOO^-$, a highly toxic oxidant. Catalase catalyzes the breakdown of H_2O_2 to water and oxygen rather than allowing it to be converted to the highly damaging ·OH.

Nitric Oxide Production Is Altered by Ischemia–Reperfusion

Biological Properties of Nitric Oxide

Nitric oxide is a highly reactive gas generated by endothelial cells, leukocytes, and platelets, among other cells. The NO produced by activated macrophages is used by these cells to kill phagocytosed bacteria. The huge intracellular production of NO results in oxidative damage to the invading microorganisms. Under normal physiological conditions, NO is continuously produced from oxidation of L-arginine by the enzyme nitric oxide synthase (NOS). The end products of this reaction are citrulline, nitrites, and nitrates (Fig. 4). Nitric oxide normally regulates its own release from endothelial cells through negative feedback on constitutive endothelial NOS (eNOS). Owing to its short half-life (3–5 s in a physiological salt solution), NO acts locally. It is both water and lipid soluble, and hence it readily permeates cells. Nitric oxide is important in maintaining vascular tone as a vasodilator. It has antiplatelet aggregation, antiplatelet activation, and antileukocyte adherence properties (Moncada and Higgs, 1993). Nitric oxide also appears to attenuate the accumulation of O_2^-, and it has been shown to prevent increases in cellular membrane permeability (Fig. 4). However, there are also some cytotoxic actions of NO that are associated with the interaction of NO with O_2^-, resulting from the formation of $ONOO^-$. Any imbalance in the production of NO and O_2^- can either lead to the formation of cytotoxic $ONOO^-$ or result in an accumulation of protective NO, thereby explaining the controversy concerning the detrimental versus beneficial role of NO in experimental models of ischemia–reperfusion injury.

Nitric Oxide in Ischemia–Reperfusion

L-ARGININE

Plasma and tissue levels of NO have been reported to decline in some tissues exposed to ischemia–reperfusion (Fig. 5). This diminution of NO may result from endothelial injury or dysfunction. Although L-arginine supplementation has been shown to afford protection in some models of ischemia–reperfusion injury, the available evidence suggests that L-arginine availability should not be a rate-limiting factor in the generation of NO by endothelial cells *in vivo.* This contention has been challenged on the basis of observations in hypercholesterolemic animals, which appear to have an elevated plasma level of dimethyl-L-arginine, a naturally occurring inhibitor of NO synthase.

NITRATES AND NITRITES

Some investigators have reported an increase in plasma nitrate and nitrite levels during reperfusion of an ischemic tissue, suggesting that NO metabolism is increased in the postischemic period. However, others have observed a reduction in the plasma levels of these same metabolites. Nitrates and nitrites are also produced when NO interacts with O_2^- to generate $ONOO^-$; hence, the decline in nitrates/nitrites cannot be attributed to the enhanced O_2^- production.

CONSTITUTIVE NITRIC OXIDE SYNTHASE ACTIVITY

The decrease in NO levels observed in some models of ischemia–reperfusion may reflect alterations at the level of NOS activity. It is only a matter of seconds from the engagement of cell membrane receptors by an appropriate stimulant before constitutive NOS (cNOS) activity is increased. Total cNOS activity has been shown to decrease following reperfusion, although this change is only detectable after 2 hours of reperfusion (Fig. 5). This explains why the NO synthesis inhibitor L-N^G-nitroarginine methyl ester only exacerbates ischemia–reperfusion injury if it is given early in the reperfusion period, but not at 4 hours of reperfusion when cNOS is already inhibited or depressed (Kanwar *et al.,* 1994). This may also explain why administration of L-arginine attenuates ischemia–reperfusion injury if given early, but not late, in the reperfusion period, as there is no cNOS to enzymatically convert it to NO.

INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY

Inducible NOS (iNOS) requires *de novo* synthesis, and at least 4 hours of stimulation by cytokines or bacterial prod-

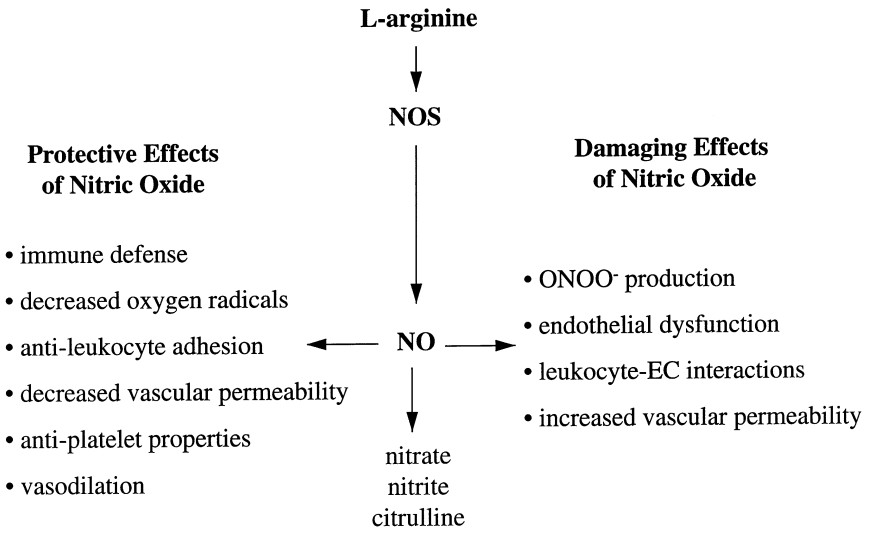


Figure 4 Nitric oxide can act as a protective or damaging agent following ischemia–reperfusion. It is generally accepted that too much NO release due to activation of iNOS following ischemia–reperfusion is the key to its deleterious effects, which include endothelial dysfunction and leukocyte–endothelial interactions. However, if a NO donor agent is administered early on in reperfusion it attenuates ischemia–reperfusion injury.

ucts such as endotoxin is normally required to detect iNOS activity in tissue. Once activated, iNOS can release NO from L-arginine at a constant rate for up to 36 hours. There is considerable controversy and uncertainty concerning the role of iNOS in ischemia–reperfusion injury. Some studies demonstrate that iNOS is undetectable after a few hours of reperfusion, despite the fact that several mediators of ischemia–reperfusion injury are known to induce iNOS activity (Kanwar *et al.,* 1994). Other studies show that it takes as little as 1 hour of reperfusion to demonstrate tissue activity of iNOS (Chandrasekar *et al.,* 1998), although studies measuring both cNOS and iNOS demonstrate a decrease in cNOS activity before iNOS activity is seen.

It has been suggested that iNOS activation may contribute to the chronic responses to ischemia–reperfusion, such as those changes noted after organ transplantation. Indeed, excess NO production is one of the earliest signs of transplant rejection. Immunosuppressive drugs such as cyclosporine exert an inhibitory effect on iNOS, possibly by blocking the activation of the transcription factor NF-κB. However, such an action would enable cyclosporine to interfere with the biosynthesis of a variety of inflammatory molecules (e.g.,

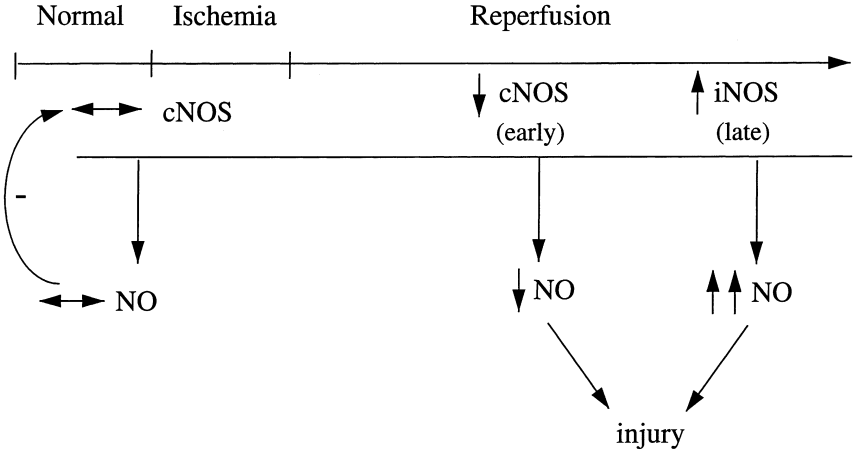


Figure 5 Nitric oxide synthase activity following ischemia–reperfusion. Constitutive NOS (cNOS) is continuously activated at basal levels. The resulting NO regulates its own production by negative feedback on cNOS. On the other hand, inducible NOS (iNOS) requires *de novo* synthesis and so takes longer to increase in activity. Early in reperfusion (a matter of hours) the levels of cNOS and NO fall. It is likely that iNOS levels increase after the drop in cNOS in the postischemic tissue, releasing large amounts of NO. Both of these changes in NOS activity may contribute to the ischemia–reperfusion injury.

cytokines, adhesion molecules) in addition to iNOS. Hence, the protection afforded by these compounds is not specific for iNOS.

Oxygen Radicals and Nitric Oxide Interact during Ischemia–Reperfusion

Damaging Effects

The interaction between NO and oxidants can result in either protective or damaging responses in postischemic tissue, depending on the source and production of NO and O_2^-. As discussed earlier, there is a burst of oxidant production and a decrease in NO levels immediately at the onset of reperfusion. Although endogenous SOD can inhibit O_2^--induced inactivation of NO, reperfusion may also result in an inhibition of endogenous SOD and catalase activity. Consequently, the relative fluxes of O_2^- and NO generated by endothelial cells is likely to favor O_2^- accumulation during the early phase of reperfusion. This would limit the accumulation of NO and prevent any of its beneficial actions. Hence, NO-dependent vasodilation would be attenuated, and there would be a tendency for leukocytes to adhere to vascular endothelium and for platelets to aggregate. In the absence of NO, the O_2^- produced immediately after reperfusion will undergo spontaneous dismutation to form H_2O_2. The H_2O_2 formed in this manner can promote the activation of PLA_2 and result in the generation of inflammatory mediators (e.g., PAF and LTB_4). In addition, peroxide can activate NF-κB to promote the biosynthesis of endothelial CAMs. The net result of these changes is recruitment and activation of neutrophils into the postischemic tissues (Fig. 4).

Protective Effects

Nitric oxide can act in a tissue protective manner through vasodilation, attenuation of platelet aggregation, and leukocyte adherence (Fig. 4). It can also attenuate the accumulation of H_2O_2 in postischemic tissues by reacting with O_2^-, thereby interfering with the spontaneous or SOD-catalyzed dismutation of O_2^-. This O_2^- scavenging property of NO is supported by observations that inhibition of eNOS results in an oxidant stress in endothelial cells, and that NO-donating compounds can attenuate or abolish O_2^--induced inflammatory responses such as leukocyte–endothelial cell adhesion.

Nitration of tyrosine residues produces nitrotyrosine, which is a sensitive marker of $ONOO^-$ activity. Nitrotyrosine levels are increased in postischemic tissues. Some studies have shown that blocking reperfusion-induced $ONOO^-$ production by inhibiting NOS activity worsens the injury response, presumably because the deleterious actions of decreased NO levels outweigh the diminished $ONOO^-$ levels. Ischemia per se does not increase the amount of nitrotyrosine in the tissue, as O_2^- is only released on reperfusion. The production of NO is what limits $ONOO^-$ generation in postischemic tissues. Although a 10-fold increase in both O_2^- and NO should result in a 100-fold increase in $ONOO^-$ levels, such large increments in $ONOO^-$ generation are not likely to occur at the onset of reperfusion because of the early reduction in NO production that accompanies reperfusion. Although the protective effects of NO in reperfusion injury have often been attributed to its O_2^--scavenging actions, evidence suggests that the product of this reaction ($ONOO^-$) may be protective in its own right, since it has been shown, in small quantities, to inhibit P-selectin-dependent leukocyte–endothelial adhesion and platelet aggregate formation (Lefer *et al.,* 1997).

Different Inflammatory Mediators Are Released during Ischemia–Reperfusion

Arachidonic Acid Metabolites

Reperfusion, probably as a consequence of enhanced oxidant production and increased intracellular calcium levels, causes the activation of PLA_2. This in turn causes AA to be split off from membrane phospholipids. The AA is metabolized to produce eicosanoids via two pathways with different end products (Fig. 6). The first enzyme, lipoxygenase, produces leukotrienes from AA. Cyclooxygenase catalyzes the second pathway, producing prostaglandins (PGs) and thromboxane (Tx). Oxygen radicals are also involved in the subsequent conversion of certain eicosanoids such as TxA_2 and prostacyclin (PGI_2) to stable breakdown products called prostanoids, TxB_2 and 6-keto-PGF1α, respectively. Most of these factors can be released from endothelial cells and neutrophils.

LEUKOTRIENES

Leukotrienes act as chemoattractants and chemoactivators. Leukotriene B_4 is a widely documented chemoattractant for neutrophils, attracting them to the vascular endothelium where they can come in contact with many AA metabolites and other stimulants. It may also directly activate the neutrophils to produce other chemoactivators such as TxA_2, PGE_2, PAF, and oxygen radicals, as well as cytotoxic proteases such as elastase and collagenases (Karasawa *et al.,* 1991). An outcome of the accumulation of these inflammatory substances is adhesion of neutrophils, damage to the endothelium, increased permeability, and subsequent transmigration of the neutrophils into the tissue where they can cause further damage. Nitric oxide does not prevent LTB_4-induced neutrophil adhesion to the endothelium of postcapillary venules, and so its protective role in reperfusion injury is not likely to involve direct interference with LTB_4 responses. The inability of NO to interfere with LTB_4-mediated inflammatory responses likely reflects the fact that LTB_4 is a weak stimulant of neutrophilic O_2^- formation.

THROMBOXANE

Thromboxane A_2 is a vasoconstrictor that opposes any vasodilatory actions of NO. It is released from neutrophils,

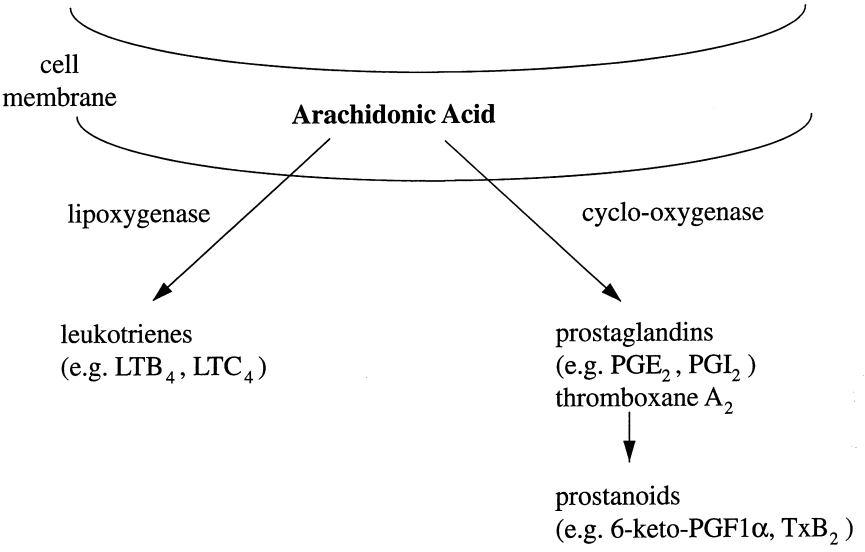


Figure 6 The arachidonic acid cascade. On exposure to oxygen radicals, PLA_2 is activated and splits AA from membrane phospholipids. The AA is metabolized by two different enzymes. Lipoxygenase produces leukotrienes from AA. Cyclooxygenase produces prostaglandins (PGs) and thromboxane (Tx) from the AA. Many of these products lead to leukocyte–endothelial interactions and microvascular permeability. Prostacyclin (PGI_2) has been shown to protect against these injuries.

platelets, and endothelial cells. It can cause injury by increasing vascular permeability both via a direct action on endothelial barrier function and by promoting the transmigration of neutrophils through the vascular wall.

Prostaglandins

Most prostaglandins are proinflammatory and cause vasoconstriction. However, PGI_2, or prostacyclin, is anti-inflammatory and vasodilatory. Hence, this cyclooxygenase product shares many of the properties of NO. Following reperfusion, more vasoconstricting than vasodilating substances may be released, which may lead to further ischemia. Thus, the relative levels of PGI_2, NO, and TxA_2 in postischemic tissues are important determinants of the extent and duration of reperfusion after an ischemic insult.

Other Mediators

Platelet Activating Factor

Platelet activating factor is also formed from a membrane phospholipid by the action of PLA_2 (Hourani and Cusack, 1991). Newly synthesized PAF is placed on the blood front of vascular endothelial cells, where it is accessible to neutrophils that are rolling along the cell surface (Fig. 7). Platelet activating factor is a potent chemoattractant and activator of neutrophils, which respond with a large flux of O_2^-. Unlike for LTB_4, NO-donating compounds attenuate the oxidant-dependent PAF-induced adhesion of neutrophils to endothelium. Superoxide dismutase exerts the same action on PAF-induced inflammation; therefore, the beneficial effects of NO may relate to its ability to scavenge O_2^-.

Mast Cell Products

Ischemia–reperfusion elicits an increased microvascular permeability that exhibits both leukocyte adhesion-dependent and -independent components. Nitric oxide has been shown to blunt both the adhesion-dependent and -independent components of this vascular permeability response. The early, leukocyte-independent microvascular leak observed in postischemic tissues may result from the activation of mast cells, which release oxygen radicals and inflammatory mediators such as histamine (Fig. 7). Nitric oxide appears to regulate the stability of mast cell membranes. Hence, the decrease in NO levels that occurs early after reperfusion may contribute to the concomitant mast cell degranulation that is observed along the length of postcapillary venules. The fact that mast cell-stabilizing agents afford some protection against the early microvascular leak in much the same manner as NO-donating agents lends support to the concept that mast cell products contribute to reperfusion injury.

Proteolytic Enzymes

Activated neutrophils produce proteolytic enzymes, including elastase, collagenase, and cathepsin G (Fig. 7). With neutrophils, these proteases can be released into the microenvironment between adherent neutrophils and the endothelium. This allows for an accumulation of proteases on the endothelial cell surface, where different glycoproteins (e.g., glycocalyx, adhesion molecules) are hydrolyzed. The proteases also attack the intercellular endothelial and interstitial matrix proteins. Elastase, for example, digests elastin, fibronectin, fibrinogen, and collagen. This causes destruction of the basement membrane to which endothelial cells are attached. Oxygen radicals promote the release of neutrophilic

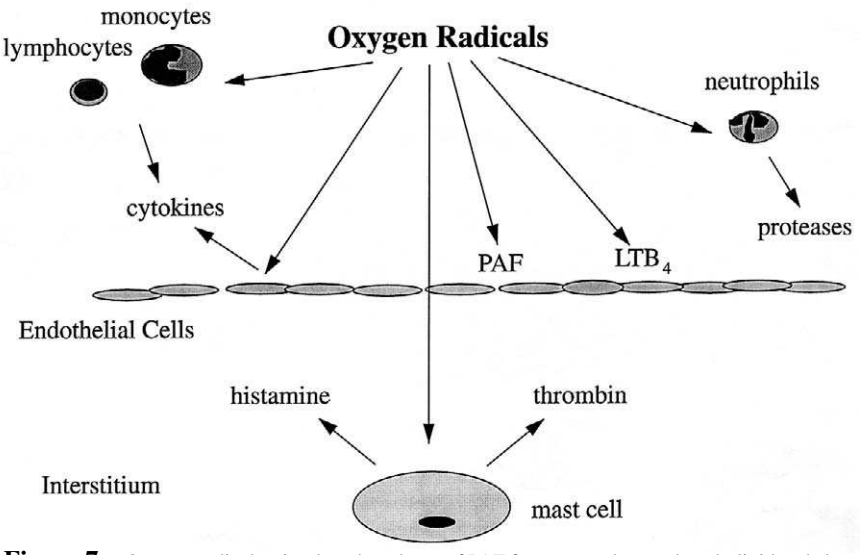


Figure 7 Oxygen radicals stimulate the release of PAF from a membrane phospholipid and also stimulate AA activation. In addition, neutrophils are stimulated to produce proteases that attack proteins, including those of the basement membrane matrix. Monocytes, lymphocytes, and endothelial cells release cytokines in response to oxidants. The oxygen radicals released following ischemia–reperfusion also cause mast cell degranulation, resulting in the release of yet more inflammatory mediators.

proteases and inactivate protease inhibitors such as α_1-protease, which inhibits elastase (Korthuis *et al.,* 1994). Nitric oxide can both inhibit and stimulate the release of proteases. Its protective effects may be mediated through an intracellular cGMP-dependent signal transduction pathway.

CYTOKINES

Lymphocytes, macrophages, endothelial cells, and other cells produce and secrete cytokines such as tumor necrosis factor alpha (TNF-α), γ-interferon (IFN-γ), and interleukin 1 (IL-1). These cytokines can contribute to the pathobiology of ischemia–reperfusion injury by engaging with their specific receptors on endothelial cells and macrophages to promote the increased biosynthesis of proteins such as endothelial CAMs and iNOS. Both the production and actions of cytokines are linked to activation of NF-κB. Oxygen radicals, which are potent stimuli for the production and release of cytokines, promote cytokine generation in postischemic tissues by activating NF-κB. This transcription factor normally resides in the cytosol bound to an inhibitory factor, IκB. The degradation of IκB allows NF-κB to translocate into the nucleus, where it binds to response elements on cytokine genes to promote their synthesis.

The iNOS gene has two binding sites for NF-κB. Although cytokines could potentially participate in a positive feedback loop involving oxygen radicals, NF-κB, and iNOS, there is evidence to suggest otherwise, at least within the first hours of reperfusion. However, days after an ischemic insult, cytokines and iNOS have been noted to increase in parallel with the increased expression of endothelial CAMs. These observations suggest that cytokines enhance, in an NF-κB-dependent fashion, the biosynthesis of iNOS, which in turn produces large quantities of NO in the late postischemic period (Chandrasekar *et al.,* 1998). Conversely, there is some evidence that NO can inhibit IL-1 and TNF-α release from macrophages. In addition, NO attenuates reoxygenation-induced upregulation of the CAM intercellular adhesion molecule-1 (ICAM-1), probably by decreasing the oxidative stress-induced NF-κB activation that can lead to increased ICAM-1 expression (Kupatt *et al.,* 1997). These studies support the contention that NO may exert both pro- and anti-inflammatory effects in tissues exposed to ischemia and reperfusion.

Nitric Oxide Modulates Leukocyte–Endothelial Interactions Induced by Ischemia–Reperfusion

Leukocyte Infiltration in Postischemic Tissue

One of the key events in the pathogenesis of reperfusion injury is the interaction between activated leukocytes and microvascular endothelium. Because of their abundance and chemotactic attraction to complement 5a, which is mobilized quickly to sites of damage, neutrophils are usually the dominant cell type observed in the initial phases of an inflammatory response. Neutrophils may mediate both morphological and functional lesions during reperfusion, as neutrophil depletion blocks the increased capillary leak caused by ischemia–reperfusion. Leukocytes must adhere to vascular endothelium in order to accumulate in tissues and cause damage. To facilitate this accumulation of leukocytes, the expression of adhesion glycoproteins is increased on the surface of activated leukocytes and endothelial cells. The endothelial CAMs act as receptors for ligands expressed on

leukocytes or platelets, and they allow leukocytes not only to adhere but also to emigrate through the endothelial barrier into the interstitium, where they can injure parenchymal cells (Panes and Granger, 1998).

Not all adherent leukocytes transmigrate into the tissue. Some detach and reenter the circulation. Since activated leukocytes are stiffer than their unactivated counterparts, trapping within the pulmonary vasculature or in other distant capillary beds is often a result of ischemia–reperfusion. Platelets also aggregate to each other and to leukocytes in the microvasculature of postischemic tissues, where they release various mediators of inflammation and increase vascular permeability. A second wave of leukocyte infiltration follows, with monocytes/macrophages and lymphocytes playing a role in the maintenance of the ischemia–reperfusion-induced injury. This pattern of cellular infiltration is associated not only with the expression of adhesion molecules but also with MHC upregulation (Takada *et al.,* 1997).

Forces Acting on the Leukocytes

Shear force, provided by the flowing blood parallel to the endothelial cell surface, normally limits the contact area between endothelial cells and leukocytes. After reperfusion of ischemic tissues, shear rate is reduced in both arterioles and venules. Since endothelial NO production is directly related to shear rate, the low blood flow associated with reperfusion may explain the accompanying decline in NO production.

Studies of leukocyte adhesion over a wide range of venular shear rates have demonstrated an inverse relationship between the number of adherent leukocytes and shear rate; that is, leukocyte adhesion rises as shear rate is reduced, whereas it decreases when shear rate is elevated. This observation suggests that the lower shear rate that occurs in the microcirculation after reperfusion serves to facilitate the recruitment of adherent leukocytes. Although the tendency for leukocytes to adhere to endothelium at low shear rates is often attributed to the ability of leukocytes to establish stronger adhesive contacts with the endothelial cell surface, it is also possible that a diminished production of NO at low shear rates could promote the adhesion of leukocytes.

Mechanical Disturbances

Apart from the release of harmful substances, neutrophils are thought to contribute to reperfusion injury by mechanical means, that is, by plugging capillaries. The increased stiffness of activated neutrophils, coupled with endothelial swelling, may account for the propensity for neutrophil trapping in capillaries after reperfusion (''capillary no-reflow''). The tendency for platelets to form aggregates with other platelets and to form platelet–leukocyte aggregates after reperfusion of ischemic tissues may also explain the capillary obstruction and malperfusion that is observed on reperfusion. Reperfusion-induced capillary no-reflow can be attenuated by administration of SOD or NO donor agents, suggesting that alterations in the balance between NO and O_2^- production may be a critical determinant of leukocyte capillary plugging.

Leukocyte–Endothelial Interactions

As noted in Fig. 8, the interaction between leukocytes and vascular endothelium can be divided into three steps, each governed by different families of CAMs. Initially, the leukocytes become ''sticky'' and roll along the endothelial cell surface at a velocity that is lower than the movement of red blood cells. This tentative initial interaction is mediated by members of the selectin adhesion molecule family, which are found on the surface of both the leukocytes and endothelial cells. Their corresponding ligands on the other cell include sialylated Lewis X and A blood group antigens. Adherence of leukocytes to the vessel wall follows, where the firm attachment is primarily mediated by integrins on the leukocytes and members of the immunoglobulin superfamily on the endothelial cell. These CAMs are also involved in the subsequent transmigration of leukocytes through the vascular endothelial barrier, although the nature of the bonds is likely to be different from those that mediate adherence. The levels of expression of CAMs on both the leukocyte and endothelial cell are increased under inflammatory conditions such as ischemia–reperfusion (Panes and Granger, 1998).

Adhesion Molecules

ROLLING LEUKOCYTES

Adhesion of neutrophils to cultured endothelial cells is rapidly initiated by agents that increase cytosolic calcium. Thrombin, H_2O_2, and LTB_4 are examples of agents that cause a rapid (1–2 min) fusion of Weibel–Palade bodies (storage granules for P-selectin and von Willebrand factor) with the endothelial cell membrane, leading the subsequent release of von Willebrand factor and the translocation of P-selectin [or granule membrane protein-140 (GMP-140)] to the surface of the endothelial cells. This adhesion molecule is a member of the selectin family that mediates leukocyte rolling, which is a prerequisite for (and precedes) firm attachment of leukocytes to the vessel wall. Once mobilized, P-selectin probably acts as a ligand to glycoproteins such as P-selectin glycoprotein ligand (PSGL) or sialylated Lewis X on the surface of neutrophils and monocytes. In the absence of these inflammatory cells, P-selectin rapidly disappears (within 5 to 10 min) poststimulation. P-selectin can also be upregulated after cytokine stimulation, via a transcription-dependent pathway, requiring about 4 hours for peak expression.

Another member of the selectin family, L-selectin, is constitutively expressed on neutrophils, monocytes, lymphocytes, and other myeloid cells. It is involved in initial tethering of leukocytes. It binds to P-selectin and E-selectin among others and is rapidly shed from the leukocyte surface on activation.

The neutrophil-specific ligand endothelial–leukocyte adhesion molecule-1 (ELAM-1 or E-selectin) is not present in normal tissue, but its expression is increased on cytokine-

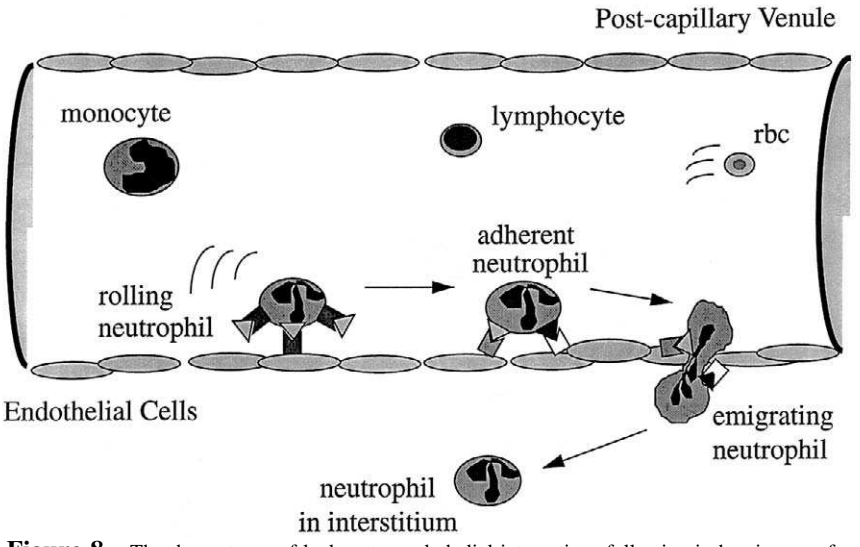


Figure 8 The three stages of leukocyte–endothelial interactions following ischemia–reperfusion. (1) Rolling is supported by the selectin group of CAMS and sialylated Lewis A and X blood group antigens. (2) In adherence, the leukocytes adhere firmly to endothelial cells. This interaction is mediated by integrins on the leukocyte surface and immunoglobulins on the endothelial cells. (3) In transmigration, the adhesion bonds change as the leukocytes emigrate between or through endothelial cells into the interstitium beyond. Neutrophils are the first cells to interact with the vessel wall following ischemia–reperfusion. Hours or days later monocytes and lymphocytes also adhere and emigrate as the injury progresses.

activated venular and capillary endothelial cells. It is induced in a transcription (NF-κB)-dependent manner. Following reperfusion, peak expression of E-selectin coincides with peak neutrophil infiltration into the tissue, and it precedes the transmigration of monocytes and lymphocytes.

Adhesion and Emigration of Neutrophils

Complement 5a, PAF, LTB_4, and a variety of other inflammatory mediators are known to increase the expression and/or avidity of adhesion glycoproteins (β_2-integrins) on leukocytes that facilitate firm adhesion to endothelial cells. The β_2-integrins share a common β (CD18) subunit that is noncovalently linked to an α subunit (CD11a, CD11b, CD11c). These heterodimers can bind to ICAM-1 and ICAM-2 on endothelial cells. The ubiquitously distributed immunoglobulin ICAM-1 is basally expressed at low levels on endothelial cells, whereas ICAM-2 is constitutively expressed in moderate quantities. The expression of ICAM-1 on endothelial cells is transcription-dependent, with cytokines and oxygen radicals serving as potent stimuli for upregulation. Expression is significantly increased after ischemia–reperfusion, reaching a stable level by 24 hours postischemia. In different models of ischemia–reperfusion, ICAM-1 knockout mice exhibit a blunted leukocyte recruitment response and protection against microvascular and parenchymal cell injury. Similar protection is observed in CD11/CD18-deficient mice and in mice receiving monoclonal antibodies directed against either ICAM-1 or CD11/CD18. Hence, the interaction between CD11/CD18 on leukocytes and ICAM-1 on endothelial cells is considered to be the major determinant of reperfusion-induced leukocyte recruitment and the consequent tissue injury.

Another endothelial CAM, platelet–endothelial cell adhesion molecule (PECAM-1), may also be involved in the adhesion and transmigration of neutrophils in postcapillary venules. It is expressed on neutrophils, platelets, and endothelial cells, and it is homophilic in its interaction, that is, binds only to other PECAM-1 molecules. Unlike other CAMs, the expression of PECAM-1 is unaltered by cytokines, oxygen radicals, or ischemia–reperfusion.

Initial rapid recruitment of neutrophils from the circulation is mediated by P-selectin and L-selectin, PAF, and the β_2-integrins. The more sustained wave of leukocyte recruitment is mediated by E-selectin, IL-8, and β_2-integrin–ICAM-1 interactions. It has been proposed that neutrophils binding through E-selectin to cytokine-activated endothelial cells encounter IL-8, activate the β2-integrins, and undergo transmigration. At later times, E-selectin declines, but ICAM-1 and VCAM-1 continue to rise. This sequence of events creates an endothelial cell surface that favors neutrophil adhesion initially then becomes more adhesive for monocytes and lymphocytes.

Role of Other Leukocyte Populations in Ischemia–Reperfusion Injury

Lymphocytes, including T cells and lymphokine-activated killer cells, can injure the endothelial cells via oxygen-independent mechanisms. The adhesion molecule CD11a/CD18 (also known as lymphocyte function-associated anti-

gen, LFA-1) can undergo molecular activation in T lymphocytes, markedly increasing the avidity for its ligands, ICAM-1 and ICAM-2. T-cell-dependent cytotoxicity may be influenced by ICAM-1 and can be completely inhibited by an anti-ICAM-1 monoclonal antibody. Another immunoglobulin adhesion molecule, VCAM-1, is normally found on epithelial cells but not on vascular endothelium. However, VCAM-1 is synthesized and expressed on endothelial cells following transcription in response to IL-1 or TNF-α treatment. Both ICAM-1 and VCAM-1 appear to show some complementary distribution: ICAM-1 is constitutively expressed on capillaries and can be induced on epithelium, whereas the opposite holds for VCAM-1 expression. The VCAM-1 ligand on the leukocyte is the β_1-integrin very late antigen (VLA-4), which is expressed by lymphocytes and monocytes, and probably neutrophils. The T lymphocytes that migrate into inflammatory sites may be memory T cells, that is, those that have been previously stimulated by antigen. These memory T cells are more adherent than naive T cells to cytokine-activated endothelial cells *in vitro,* and they uniquely appear to induce increased endothelial permeability by a contact-dependent pathway. Compared to naive cells, memory T cells have diminished expression of L-selectin but increased expression of CD44. Both VLA-4 and CD44 can mediate the binding of T cells to inflamed venules.

Transmigration of lymphocytes appears to involve the interaction of LFA-1 and ICAM-1 *in vitro.* Nevertheless, the endothelial ICAM-1–lymphocyte LFA-1 pathway is probably not of critical importance, as humans lacking the LFA-1 receptor still have lymphocytes that infiltrate inflammatory sites, whereas their neutrophils cannot. The β_1-integrins such as VLA-4 and extracellular matrix components, for example, laminin, collagen, and fibronectin, are probably involved in lymphocyte transmigration. P- and E-selectin may also be involved directly or indirectly in lymphocyte emigration, as blocking these receptors inhibits tissue infiltration by lymphocytes.

Role of Nitric Oxide in Leukocyte–Endothelial Interactions

NEUTROPHILS

Inhibition of NOS activity with L-arginine analogs elicits microvascular and inflammatory responses that are very similar to those observed after ischemia–reperfusion, including neutrophil adherence to venular endothelium and emigration into the interstitium. Nitric oxide synthase inhibition also has been shown to elicit an oxidant stress in venular endothelium, presumably due to the absence of the highly efficient O_2^- scavenger, NO. Mast cell degranulation and platelet–leukocyte aggregation are additional features that are shared by ischemia–reperfusion and NOS inhibition. In some models of reperfusion injury, NOS inhibition exacerbates certain aspects of the injury response, including neutrophil adhesion and vascular permeability. Additional support for a role for NO in reperfusion injury comes from reports that NO-donating compounds attenuate the leukocyte–endothelial cell adhesion, platelet–leukocyte aggregation, mast cell degranulation, and increased vascular permeability that are normally elicited by ischemia–reperfusion (Kurose *et al.,* 1994). These actions may also reflect the O_2^--scavenging properties of NO, as SOD administration is also effective in blunting the same responses to ischemia–reperfusion.

The observation that NO is protective only in those reperfusion injury models that require the presence of neutrophils suggests that NO exerts its beneficial actions on the leukocyte, endothelial cell, or both. Since it has been shown that NO can inhibit the expression of P-selectin (Gauthier *et al.,* 1994), ICAM-1, and VCAM-1 on endothelial cells and E-selectin, ICAM-1, and VCAM-1 in plasma (Engelman *et al.,* 1995) *in vivo,* it appears likely that endothelial cells are a target of NO action in models of ischemia–reperfusion. It has been difficult to demonstrate such an inhibitory effect of NO donors on CAM expression in cultured endothelial cells, suggesting that the inhibitory actions observed *in vivo* may reflect an effect of NO on cytokine and/or oxygen radical production/accumulation. Spieker *et al.* (1998) showed that NO can block the TNF-α-induced upregulation of E-selectin, ICAM-1, and VCAM-1, probably by promoting the activity of the IκB-α promoter and thereby preventing NF-κB translocation into the nucleus and subsequent CAM gene transcription.

OTHER LEUKOCYTE POPULATIONS

There is growing evidence that lymphocytes may contribute to the pathogenesis of ischemia–reperfusion injury. Studies performed in severe combined immunodeficient (SCID) mice, which have no lymphocytes, have revealed an attenuated response to ischemia–reperfusion. Reconstitution of the T cell, but not B cell, population in SCID mice restores the normal pathological alterations to ischemia–reperfusion. Furthermore, it has been shown that depletion of $CD4^+$, but not $CD8^+$, T cells blunts the tissue injury associated with ischemia–reperfusion. These observations suggest that $CD4^+$ T cells contribute to the microvascular and parenchymal cell dysfunction associated with ischemia and reperfusion. The mechanisms involved in $CD4^+$ T-cell-mediated, reperfusion-induced tissue injury are poorly defined. However, some evidence suggests that the T cells may act to modulate the recruitment of neutrophils by modulating the expression of certain endothelial CAMs. Although NO plays an important role in regulating neutrophil recruitment and function, it appears to exert little influence on lymphocytes (Cartwright *et al.,* 1997), possibly because lymphocytes do not produce oxygen radicals.

Ischemia–Reperfusion Promotes Leukocyte–Platelet Interactions

Platelet Interactions

Ischemia–reperfusion is known to activate and promote the aggregation of platelets. In addition, reperfusion is often

associated with the formation of platelet–leukocyte aggregates. These aggregates form on the surface of endothelial cells lining postcapillary venules of the postischemic tissue, where they are dislodged by flowing blood to enter the circulation. The dislodged aggregates are then trapped within capillaries of the liver, lung, or other organs. The plugging of capillaries by these aggregates can lead to a reduction in blood flow and cellular necrosis. In addition, the aggregates can produce and release a variety of mediators that can elicit an increased vascular permeability. P-selectin, which is synthesized, stored in α-granules, and expressed on the surface of activated platelets, mediates the heterotypic aggregation of leukocytes and platelets. P-selectin-deficient mice and mice receiving monoclonal antibodies directed against P-selectin produce fewer leukocyte–platelet aggregates within postcapillary venules exposed to ischemia–reperfusion.

The Influence of Nitric Oxide on Platelet Function

Activated platelets release adenosine diphosphate and serotonin. These substances can bind to endothelial cell receptors to stimulate the formation and release of NO, which modulates platelet function (Cheung *et al.,* 1997). Nitric oxide can act synergistically with PGI_2, metabolites of ectonucleotidase, and lipoxygenase to influence platelet function. Nitric oxide prevents platelet aggregation and promotes disaggregation of preformed aggregates. In addition, NO attenuates platelet adhesion to leukocytes and to endothelium. These actions of NO may be partially mediated by the activation of soluble guanylate cyclase. Nitric oxide is known to inhibit P-selectin expression on platelets and endothelial cells. This may account for the inhibitory action of NO on the binding of platelets to endothelial cells and leukocytes, but not the homotypic aggregation of platelets.

Major Histocompatibility Complexes in Ischemia–Reperfusion Injury: Does Nitric Oxide Play a Role?

What Are Major Histocompatibility Complexes?

Nearly all vertebrate species studied have been shown to possess highly polymorphic cell surface molecules. These antigenic molecules are coded for by the human leukocyte antigen (HLA) genes as alleles of HLA-A, -B, and -C (class I) and HLA-DR, -DQ, and -DP (class II) loci. The HLA genes are also known as the MHCs, and the plasma membrane proteins they encode are often referred to as MHC I and MHC II. Class I antigens are found on virtually every cell in the body except for erythrocytes. Class II antigens are found only on the surfaces of macrophages and a few other cell types, including B cells. Identical MHC genes are not shared except between identical twins. The main role of these molecules is to govern immune recognition by presenting self and nonself antigenic peptides to the immune system. Only when an antigen is bound to an MHC protein on an antigen presenting cell (e.g., a macrophage to form an MHC–peptide complex) can a T cell recognize and bind to it. This binding is necessary to trigger a transduction cascade leading to T-cell activation, differentiation, and initiation of the antigen-specific immune response. The different subsets of T cells require the peptide to be complexed to different MHC molecules. Cytotoxic and suppressor T cells bind to MHC I-associated antigens and are CD8-positive, and helper T cells bind to MHC II-associated antigens and are $CD4^+$. The CD4 and CD8 molecules exist in a complex with the T-cell receptor (TCR) and CD3 (T cell phenotype) (Fig. 9). Also on the T cells are other accessory molecules, LFA-1 and CD2. The VLA-4 molecule mediates lymphocyte recirculation patterns and increases binding of the T cell to MHC antigens, contributing to antigen-dependent immunity.

The MHC I molecules have a high affinity to endogenous, synthesized antigens such as viral antigens, whereas MHC II types primarily present peptides derived via endocytosis. Cross-linking of the TCR–CD3 complex by binding the MHC molecule transiently converts the β_2-integrin (LFA-1) from low avidity to high avidity. This provides a mechanism for regulating cellular adhesion and deadhesion in an antigen-specific manner. It also allows lymphocytes to adhere more strongly to vascular endothelium and transmigrate. Anti-LFA-1 monoclonal antibodies decrease the T cell response by inhibiting T-cell-directed cytolysis and adhesion of target cells (Heemann *et al.,* 1994). The presence of either CD4 or CD8 molecules in the TCR complex produces a considerably stronger impulse for T-cell activation on binding the MHC proteins than binding of the TCR–CD3 complex alone. The signal transduction that occurs after the TCR binds to the antigen is regulated by the CD3 part of the TCR–CD3 complex, not by the TCR molecule. The CD2 molecule binds to LFA-3 on the antigen-presenting cells.

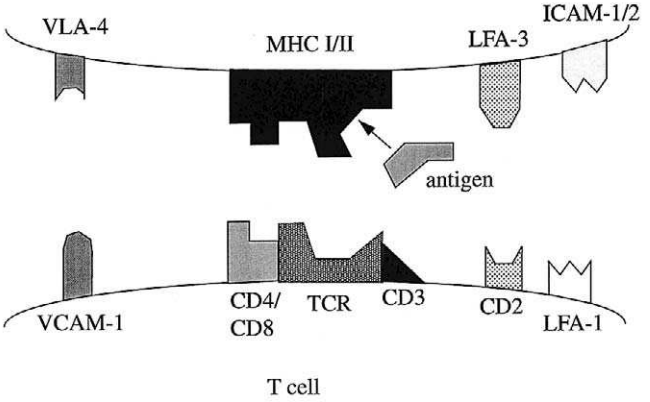


Figure 9 Major histocompatibility complexes are upregulated following ischemia–reperfusion. Normally they present antigens to T cells. The MHC–antigen complex binds a complex of CD4/CD8, TCR, and CD3 on the lymphocytes. Enhancing this interaction, the accessory molecules VCAM-1, LFA-3, and ICAM-1/-2 bind their respective ligands on the lymphocytes. The LFA-1 molecule is converted from low to high avidity when the MHC receptor is cross-linked with the TCR complex, allowing the lymphocytes to adhere to vascular endothelium and transmigrate.

Major Histocompatibility Complexes in Ischemia–Reperfusion

The upregulation of ICAM-1 and recruitment of neutrophils that is elicited by ischemia–reperfusion precedes the increased expression of MHC molecules (Takada *et al.,* 1997). This MHC upregulation is associated with lymphocyte and macrophage infiltration. Since it follows the expression of lymphocyte products such as IL-2, IFN-γ, and TNF-α, some of these products may be responsible for the increase in MHC expression. Blocking both P- and E-selectin blocks the infiltration of all leukocytes into the postischemic tissue and prevents both MHC II induction and the expression of the inflammatory products of the leukocytes. Although these findings demonstrate profound changes in MHC II induction after ischemia–reperfusion, the significance of these changes to the pathobiology of reperfusion injury remain poorly defined.

Regulation of Major Histocompatibility Complex Expression: Role of Nitric Oxide

Nitric oxide has a controversial role in the regulation of MHC expression. It has been shown to downregulate MHC expression in several models of immune activation such as IFN-γ-induced MHC expression. However, IFN-γ can also cause NO production, and TNF-α can further increase IFN-γ-induced MHC II and NO upregulation, although these are through different signaling pathways (Hellendall and Ting, 1997). The NO produced in response to IFN-γ seems to be protective against other pathways in inflammation. Conversely, NO may cause MHC II upregulation in certain cells, although this is important for the development of immunocompetent activity of the cells. Other studies show no influence of NO on MHC expression induced by IFN-γ, TNF-α, or IL-1. What about the other way around? In macrophages, cross-linkage of MHC I or II actually triggers NO release. Macrophages can produce NO by two different pathways that involve T-cell-derived cytokines. The MHC II molecule may act as a transmembrane signal transducer that ultimately causes NO production when the T cell engages the macrophage MHC II receptor. Hence, these findings indicate that the role of NO in reperfusion-induced MHC upregulation remains poorly understood.

Nitric Oxide Modulates Reperfusion-Induced Tissue Injury

Vascular Permeability

A well-characterized response of the microvasculature to ischemia–reperfusion is endothelial barrier dysfunction leading to increased vascular permeability. Leukocytes appear to be key mediators of the increased permeability associated with ischemia–reperfusion. Several lines of evidence support this view: (1) the magnitude of the increased albumin leakage across venules exposed to ischemia–reperfusion is significantly and positively correlated with the number of adherent and emigrated leukocytes in these vessels, (2) monoclonal antibodies directed against leukocyte or endothelial CAMs attenuate reperfusion-induced increases in vascular permeability, and (3) a variety of agents (e.g., SOD, adenosine, allopurinol) that blunt the leukocyte recruitment response to ischemia–reperfusion also diminish the vascular permeability response. Nitric oxide-donating compounds have also proved to be very effective in reducing reperfusion-induced increases in leukocyte adhesion and the concomitant increase in vascular permeability in certain vascular beds. Since NO donors are so effective in reducing leukocyte adhesion, it is generally assumed that the attenuated permeability is directly related to the blunted leukocyte traffic across the vascular wall. However, the molecular basis for the protection afforded by NO in this leukocyte-dependent increase in vascular permeability remains unclear. This is made evident by reports demonstrating that in leukocyte-independent models of acute inflammation, nitric oxide per se appears to promote endothelial barrier dysfunction. A potential explanation for this discrepancy is that the elevated O_2^- fluxes in leukocyte-dependent models may favor a protective role effect of NO, whereas the low O_2^- fluxes generally associated with non-leukocyte-dependent models may minimize the contribution of NO.

Cell Death

There are two types of cell death following ischemia–reperfusion (Fig. 10). Necrosis is characterized by cell swelling and membrane rupture, allowing the contents of the cells to leak into the surrounding environment. The contents include damaging oxygen radicals, proteases, and other inflammatory mediators that can cause damage to the surrounding tissue, possibly leading to the death of the tissue or organ. The other process leading to cell death is apoptosis (Duke *et al.,* 1996). This is known as non-necrotic cell suicide and is a form of programmed cell death. This process is associated with cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. The cells split into plasma membrane-bound vesicles known as apoptotic bodies. These are phagocytosed by neighboring cells and tissue macrophages, and so nearby cells are not exposed to harmful cellular contents.

APOPTOSIS

Following brief ischemic episodes, apoptosis usually prevails as the dominant cause of cell death in injured cells. However as the length or severity of ischemia increases, the scales tip toward necrotic death. Apoptosis can be seen within half a day following just a few minutes of ischemia. However, necrosis requires longer than that to be detected in most tissues, and it can be seen within a day of the injury. The oxidative stress that is elicited by ischemia–reperfusion

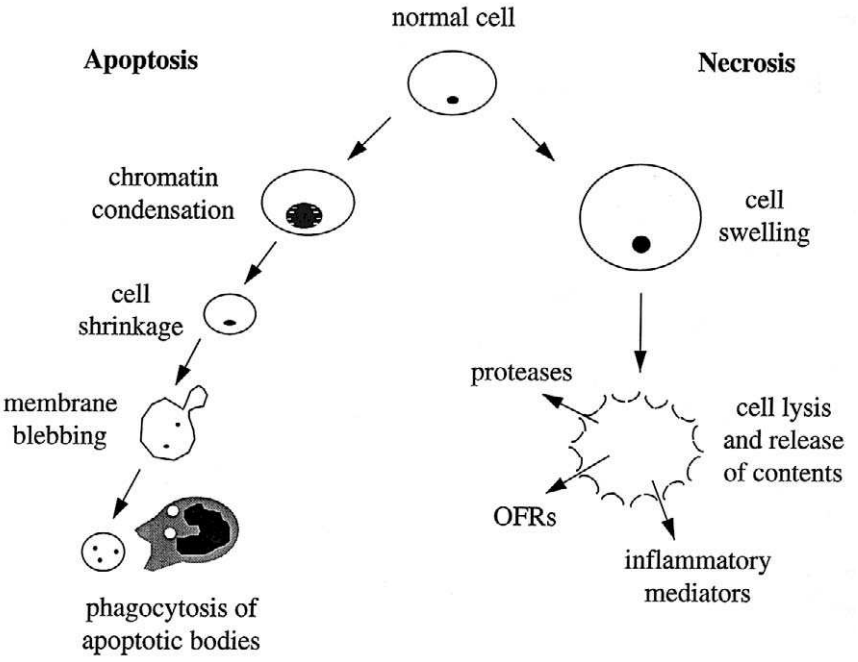


Figure 10 Cell death following ischemia–reperfusion. Cells that die by apoptosis break up into membrane-bound vesicles that are phagocytosed by neighboring cells and tissue macrophages. When cells die by necrosis, however, they swell and lyse, releasing their harmful contents into the surrounding milieu, which can damage other cells or cause them to die.

is known to induce apoptosis. Nitric oxide appears to be both beneficial and detrimental to the process of apoptosis; that is, it is protective if released from cNOS and harmful if released from iNOS. The increase in iNOS activity that is observed after reperfusion may be caused by TNF-α, which also induces apoptosis (Ferrari *et al.,* 1998). Many treatments that block iNOS activity also block apoptosis. On the other hand, L-arginine has been shown to prevent the apoptosis elicited by reperfusion; however, it remains unclear whether this action is directly related to NO production (Calabrese *et al.,* 1997).

NECROSIS

After a prolonged ischemic period, less than an hour in many cases, cells die due to necrosis. Nitric oxide can prevent this cellular necrosis if administered early in the reperfusion period. However, if administered at a time when iNOS is significantly upregulated, then the exogenous NO could prove toxic and exacerbate the tissue injury.

Organ Dysfunction

Ischemia–reperfusion-induced oxygen radical generation may result in endothelial cell injury and leukocyte activation. These cells release other harmful mediators that attract other leukocytes and that promote the release of other inflammatory substances. The leukocytes infiltrate the tissue, where they can inflict injury to parenchymal cells. In the heart, for example, reperfusion is associated with myocyte injury and cardiac dysfunction (Lefer *et al.,* 1993), whereas enterocytes have a reduced capacity to transport electrolytes and nutrients in the postischemic intestine. The parenchymal cell alterations and organ dysfunction that are associated with ischemia–reperfusion can be attenuated by administration of NO-donating compounds or by L-arginine. There are other reports, however, that describe an improved organ function after treatment with NOS inhibitors, suggesting that NO accumulation contributes to the organ dysfunction associated with ischemia–reperfusion. Whether these different responses to NO manipulation reflect different contributions of cNOS versus iNOS to the organ dysfunction remains unclear.

Conclusion

Ischemia–reperfusion leads to an increased production of oxygen radicals, endothelial cell injury, leukocyte infiltration, and organ dysfunction. Nitric oxide bioavailability appears to be reduced in the early period after reperfusion, which is likely due to a decline in endothelial NO production and an increased inactivation of NO by endothelial cell-derived O_2^-. The limited bioavailability of NO may contribute to the abnormal cell–cell interactions and vascular dysfunction in the initial moments of reperfusion. However, as iNOS expression is increased later during reperfusion, the fluxes of NO generated by tissues is greatly increased. In some models of reperfusion injury, this increased NO flux is detrimental,

perhaps related to $ONOO^-$ generation. Nitric oxide-donating compounds have shown promise as protective agents in experimental models of regional ischemia–reperfusion and organ transplantation. The contradictory results obtained with NOS inhibitors in reperfusion injury models are difficult to explain. However, resolution of this issue must await a better understanding of the relative rates of production of NO and O_2^- as well as the level of dependence of injury on leukocytes, platelets, mast cells, and macrophages in different tissues and experimental models of reperfusion injury.

References

Calabrese, F., Valente, M., Pettenazzo, E., Ferraresso, M., Burra, P., Cadrobbi, R., Cardin, R., Bacelle, L., Parnigotto, A., and Rigotti, P. (1997). The protective effects of L-arginine after liver ischaemia/reperfusion injury in a pig model. *J. Pathol.* **183,** 477–485.

Cartwright, J. E., Whitley, G. S., and Johnstone, A. P. (1997). Endothelial cell adhesion molecule expression and lymphocyte adhesion to endothelial cells: Effect of nitric oxide. *Exp. Cell Res.* **235,** 431–434.

Chandrasekar, B., Streitman, J. E., Colstan, J. T., and Freeman, G. L. (1998). Inhibition of nuclear factor κB attenuates proinflammatory cytokine and inducible nitric-oxide synthase expression in postischemic myocardium. *Biochim. Biophys. Acta* **1406,** 91–106.

Cheung, P. Y., Salas, E., Schulz, R., and Radomski, M. W. (1997). Nitric oxide and platelet function: Implications for neonatology. *Semin. Perinatol.* **21,** 409–417.

Duke, R. C., Ojcius, D. M., and Young, J. D. (1996). Cell suicide in health and disease. *Sci. Am.* **275,** 80–87.

Engelman, D. T., Watanabe, M., Maulik, N., Cordis, G. A., Engelman, R. M., Rousou, J. A., Flack III, J. E., Deaton, D. W., and Das, D. K. (1995). L-Arginine reduces endothelial inflammation and myocardial stunning during ischemia–reperfusion. *Ann. Thorac. Surg.* **60,** 1275–1281.

Ferrari, R., Agnoletti, L., Comini, L., Gaia, G., Bachetti, T., Cargnoni, A., Ceconi, C., Curello, S., and Visioli, O. (1998). Oxidative stress during myocardial ischaemia and heart failure. *Eur. Heart J.* **19,** B2–B11.

Gauthier, T. W., Davenpeck, K. L., and Lefer, A. M. (1994). Nitric oxide attenuates leukocyte–endothelial interaction via P-selectin in splanchnic ischemia–reperfusion. *Am. J. Physiol.* **267,** G562–G568.

Granger, D. N. (1988). Role of xanthine oxidase and granulocytes in ischemia–reperfusion injury. *Am. J. Physiol.* **255,** H1269–H1275.

Granger, D. N., and Korthuis, R. J. (1995). Physiologic mechanisms of postischemic tissue injury. *Annu. Rev. Physiol.* **57,** 311–332.

Heemann, U. W., Tullius, S. G., Azuma, H., Kupeic-Weglinsky, J., and Tilney, N. L. (1994). Adhesion molecules and transplantation. *Ann. Surg.* **219,** 4–12.

Hellendall, R. P., and Ting, J. P. (1997). Differential regulation of cytokine-induced major histocompatibility complex class II expression and nitric oxide release in rat microglia and astrocytes by effectors of tyrosine kinase, protein kinase C, and cAMP. *J. Neuroimmunol.* **74,** 19–29.

Hourani, S. M., and Cusack, N. J. (1991). Pharmacological receptors on blood platelets. *Pharmacol. Rev.* **43,** 243–298.

Kanwar, S., Tepperman, B. L., Payne, D., Sutherland, L. R., and Kubes, P. (1994). Time course of nitric oxide production and epithelial dysfunction during ischemia/reperfusion of the feline small intestine. *Circ. Shock* **42,** 135–140.

Karasawa, A., Guo, J. P., Ma, X. L., Tsao, P. S., and Lefer, A. M. (1991). Protective actions of a leukotriene B4 antagonist in splanchnic ischemia and reperfusion in rats. *Am. J. Physiol.* **261,** G191–G198.

Korthuis, R. J., Anderson, D. C., and Granger, D. N. (1994). Role of neutrophil–endothelial cell adhesion in inflammatory disorders. *J. Crit. Care* **9,** 47–71.

Kurose, I., Wolf, R., Grisham, M. B., and Granger, D. N. (1994). Modulation of ischemia–reperfusion-induced microvascular dysfunction by nitric oxide. *Circ. Res.* **74,** 376–382.

Kupatt, C., Weber, C., Wolf, D. A., Becker, B. F., Smith, T. W., and Kelly, R. A. (1997). Nitric oxide attenuates reoxygenation-induced ICAM-1 expression in coronary microvascular endothelium: Role of NFkappaB. *J. Mol. Cell. Cardiol.* **29,** 2599–2609.

Lefer, A. M., Siegfried, M. R., and Ma, X. L. (1993). Protection of ischemia–reperfusion injury by sydnonimine NO donors via inhibition of neutrophil–endothelium interaction. *J. Cardiovasc. Pharmacol.* **22,** S27–S33.

Lefer, D. J., Scalia, R., Campbell, B., Nossuli, T., Hayward, R., Salamon, M., Grayson, J., and Lefer, A. M. (1997). Peroxynitrite inhibits leukocyte–endothelial cell interactions and protects against ischemia–reperfusion injury in rats. *J. Clin. Invest.* **99,** 684–691.

Moncada, S., and Higgs, A. (1993). The L-arginine–nitric oxide pathway. *N. Engl. J. Med.* **329,** 2002–2012.

Panes, J., and Granger, D. N. (1998). Leukocyte–endothelial cell interactions: Molecular mechanisms and implications in gastrointestinal disease. *Gastroenterology* **114,** 1066–1090.

Spieker, M., Darius, H., Kaboth, K., Hubner, F., and Liao, J. K. (1998). Differential regulation of endothelial cell adhesion molecule expression by nitric oxide donors and antioxidants. *J. Leukocyte Biol.* **63,** 732–739.

Takada, M., Nadeau, K. C., Shaw, G. D., and Tilney, N. L. (1997). Early cellular and molecular changes in ischemia/reperfusion injury: Inhibition by a selectin antagonist, P-selectin glycoprotein ligand-1. *Transplant. Proc.* **29,** 1324–1325.

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CHAPTER 40

Nitric Oxide: A Critical Determinant in Ischemia–Reperfusion

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BASAL PHYSIOLOGICAL LEVELS OF NITRIC OXIDE (NO) ARE ESSENTIAL TO THE MAINTENANCE OF CARDIOVASCULAR HOMEOSTASIS, ON THE MICRO- AND MACROCIRCULATORY LEVELS. NORMALLY, THESE PHYSIOLOGICAL CONCENTRATIONS OF NO, IN THE LOW NANOMOLAR RANGE, ORIGINATE FROM THE ENDOTHELIUM, AND THUS IT IS CALLED ENDOTHELIUM-DERIVED NITRIC OXIDE (EDNO). EDNO EXERTS A VARIETY OF IMPORTANT CIRCULATORY FUNCTIONS, INCLUDING THE MAINTENANCE OF VASODILATOR TONE, INHIBITION OF PLATELET AGGREGATION, ATTENUATION OF LEUKOCYTE ROLLING AND ADHERENCE TO THE ENDOTHELIUM, AND STABILIZATION OF ENDOTHELIAL LAYER PERMEABILITY. THE ONSET OF REPERFUSION OF AN ISCHEMIC VASCULAR BED, AS OCCURS IN MYOCARDIAL OR MESENTERIC ISCHEMIA-REPERFUSION, IN TRANSPLANTATION OF AN ORGAN (I.E., KIDNEY), OR IN REINFUSION OF SHED BLOOD FOLLOWING TOTAL BODY HEMORRHAGE, RESULTS IN A RAPID ENDOTHELIAL DYSFUNCTION CHARACTERIZED BY A LOSS OF FUNCTIONAL EDNO. THIS REDUCED EDNO LEADS TO A PROFOUND INCREASE IN EXPRESSION OF CELL ADHESION MOLECULES (E.G., P SELECTIN, ICAM-1) AND TO ENHANCED LEUKOCYTE–ENDOTHELIAL INTERACTION. THE CONSEQUENCE OF THESE EFFECTS IS MARKED STIMULATION OF THE INFLAMMATORY RESPONSE, WITH TRANSENDOTHELIAL MIGRATION OF NEUTROPHILS THROUGH THE DYSFUNCTIONAL ENDOTHELIUM TO THE AFFECTED REGION, WHERE THESE PMNS INDUCE TISSUE INJURY.

Introduction

When one markedly reduces or produces cessation of blood flow (i.e., induces ischemia) to a vascular bed and then rapidly reperfuses that vascular bed (i.e., reestablishes blood flow), a severe perturbation occurs to the organ perfused by that vascular bed. This phenomenon is known as reperfusion injury. Reperfusion injury has been documented in several vascular beds, including those of the coronary (Van Benthuysen *et al.,* 1987), mesenteric (Lefer and Ma, 1991), renal (Lieberthal *et al.,* 1989), cerebral (Rosenblum *et al.,* 1992), and skeletal muscle (Korthuis *et al.,* 1988) circulations. One of the earliest and most critical events occurring after reperfusion of an ischemic bed is a marked endothelial dysfunction

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characterized by the loss of endothelium-derived relaxing factor (EDRF) (Tsao *et al.,* 1990), now known to be nitric oxide (NO). This reduction in biologically active NO release has been observed in response to challenge with endothelium-dependent vasodilators [e.g., acetylcholine (ACh), A23187], the so-called agonist-mediated NO release, as well as in response to application of NO synthase (NOS) inhibitors [e.g., N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA)], which unmask the basal release of NO (Moncada *et al.,* 1990). Basal NO release is the more physiologically relevant form of NO release, but agonist-mediated NO is more widely used. Nevertheless, both indices of NO release are valid (Carey *et al.,* 1992) and are useful in quantifying the degree of endothelial dysfunction.

A dramatic and sustained endothelial dysfunction occurs following reperfusion of ischemic myocardial (Tsao *et al.,* 1990) or splanchnic vasculatures (Carey *et al.,* 1992). This endothelial dysfunction contributes significantly to the reperfusion injury of the underlying tissue, because NO promotes vasorelaxation, inhibits platelet aggregation, quenches superoxide radicals (Rubanyi and Vanhoutte, 1986; Gryglewski *et al.,* 1986), and attenuates the adherence of polymorphonuclear (PMN) leukocytes to the endothelium (McCall *et al.,* 1988; Kubes *et al.,* 1991). The endothelial dysfunction occurring in ischemia–reperfusion is characterized by a rapid decrease in biological activity of NO released from the affected endothelium. This profound decrease in NO activity is an important event that signals early pathophysiological changes and serves as the hallmark of the first phase of reperfusion injury, termed the endothelial trigger. This endothelial trigger initiates a cascade of major pathophysiological events in reperfusion injury, and it directly leads to a second major event resulting in tissue injury (Lefer and Lefer, 1993). This second major event, which is directly dependent on the endothelial dysfunction, is known as the neutrophil amplification step. This relationship was observed initially in splanchnic ischemia–reperfusion (Bulkley, 1989), but it clearly pertains to myocardial ischemia–reperfusion and to that ischemia–reperfusion occurring in other regional vascular beds (e.g., renal, cerebral) (Lieberthal *et al.,* 1989; Rosenblum *et al.,* 1992). This two-stage sequence also occurs in total body ischemia–reperfusion such as that occurring in hemorrhage and reinfusion (Wang *et al.,* 1993) and to some extent in endotoxemia and sepsis (Siegfried *et al.,* 1992a; Wang *et al.,* 1995). Figure 1 is a time course of ischemia–reperfusion showing the temporal relationships among endothelial dysfunction, leukocyte adherence, neutrophil infiltration, and tissue injury. These relationships, which are discussed in more detail in the next section, occur not only in myocardial ischemia–reperfusion (Tsao *et al.,* 1990) but also in mesenteric ischemia–reperfusion (Hayward and Lefer, 1998a), in hemorrhage–reperfusion (Scalia *et al.,* 1999), and in trauma (Scalia *et al.,* 1996).

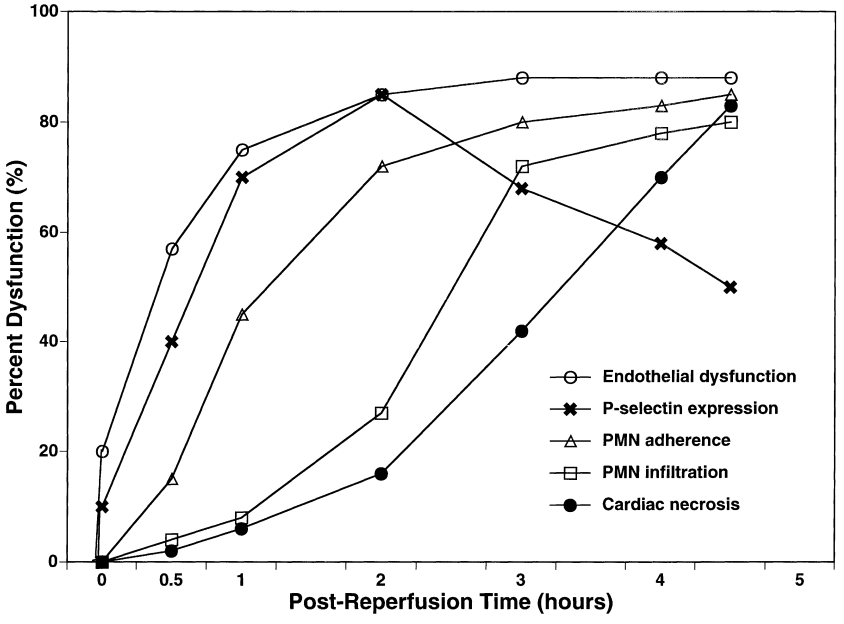


Figure 1 Time course of key mileposts in feline myocardial ischemia–reperfusion: onset of effects in percentage of maximal effect versus time following reperfusion of the 90-min occluded LAD coronary artery. Endothelial dysfunction was tested in isolated coronary artery rings (vasorelaxation response to acetylcholine). P-selectin expression was analyzed immunohistochemically. PMN adherence [i.e., autologous polymorphonuclear (PMN) leukocytes] to the coronary endothelium was measured in isolated coronary artery segments. PMN infiltration was studied histologically, and cardiac necrosis was assessed by nitroblue tetrazolium staining.

NO and the Endothelium in Ischemia–Reperfusion

Time Course of NO Changes in Ischemia–Reperfusion

The time course of loss of endothelial-derived nitric oxide (EDNO) in ischemia–reperfusion is well established (Tsao *et al.,* 1990). The first complete study of this type was reported by Tsao *et al.,* (1990) in myocardial ischemia–reperfusion in cats. Cats were subjected to 90 min of complete occlusion of the left anterior descending (LAD) coronary artery followed by either no reperfusion (i.e., 0 min) or reperfusion of 2.5, 5, 10, 20, 60, 180, and 270 min. LAD coronary artery rings were challenged with two endothelium-dependent vasodilators: ACh, a receptor mediated endothelium-dependent dilator, and A23187, a non-receptor-mediated endothelium-dependent dilator. Responses to these vasodilators were compared to an endothelium-independent dilator, sodium nitrite ($NaNO_2$) at pH 2.0. These vasorelaxant responses were also compared to those obtained with these same three vasodilators in the nonischemic control left circumflex coronary (LCX) artery. In nonischemic LCX arteries, there was a complete vasorelaxation response (i.e., 90–100%) to all vasodilators. Surprisingly, total occlusion of the LAD for 90 min in the absence of reperfusion did not diminish the vasorelaxant response to either ACh or $NaNO_2$. However, 90 min of ischemia followed by only 2.5 min of reperfusion markedly attenuated the vasorelaxation response to ACh and A23187, but not to $NaNO_2$. This pattern of response (i.e., attenuated relaxation to an endothelium-dependent dilator but normal relaxation to an endothelium independent dilator) is characteristic of ''endothelial dysfunction'' and is a consequence of the reduced NO release by the affected endothelium. Over the remaining postreperfusion time (i.e., 5 to 270 min) the vasorelaxation response to ACh and A23187 diminished further, but at no time was there a reduction in the vasorelaxant response to $NaNO_2$. Therefore, endothelial dysfunction commenced in the first 2.5 min of reperfusion, and it was exacerbated and sustained over the entire 4.5-hour postreperfusion observation period. Intravenous administration of recombinant human superoxide dismutase (hSOD) just prior to reperfusion prevented much of the endothelial dysfunction (Tsao *et al.,* 1990). Thus, endothelial dysfunction was observed in response to agonist-mediated NO release by the endothelium (i.e., pulsed release).

The endothelium, however, normally releases NO continuously at low concentrations (i.e., basal release). Basal release is more difficult to quantify, but it can be done by measuring the degree of vasocontraction of vascular rings in response to a NOS inhibitor (e.g., L-NMMA, L-NAME). Using this technique, vasocontraction of cat LAD coronary arteries to L-NAME was found to be markedly diminished 5 min postreperfusion following 90 min of ischemia (Ma *et al.,* 1993). The response was significant at all postreperfusion times from 5 to 270 min, just as in the case of reduced ACh and A23187 responses. Thus, the endothelial dysfunction occurring following reperfusion of an ischemic coronary vasculature can be observed both in agonist-mediated (i.e., pulsed release of NO) and in basal release of NO (i.e., low level continuous release of NO). This concordance provides strong evidence that decreased NO release is an important early consequence of postischemic reperfusion.

Additional important information on the mechanism of the endothelial dysfunction was obtained in isolated rat hearts perfused with Krebs–Henseleit buffer rather than with blood (Tsao and Lefer, 1990). Hearts were subjected to total cessation of coronary flow for 20 min followed by reperfusion (for periods of 2.5 to 45 minutes) with Krebs–Henseleit solution gassed with a mixture of either 95% O_2 plus 5% CO_2 or 95% N_2 plus 5% CO_2. Additionally, superoxide radical release in these hearts was determined by chemiluminescence. As expected, there was a profound endothelial dysfunction 2.5 min following reperfusion with oxygenated Krebs–Henseleit buffer characterized by markedly attenuated vasorelaxation of the coronary microcirculation to ACh but not to the direct vasodilator nitroglycerin (NTG). This endothelial dysfunction gradually worsened over the ensuing 45-min postreperfusion period. This could be attributed to the large pulse of superoxide radical observed in the ischemic–reperfused vasculature within the first minute of reperfusion and which lasted for about 1 to 2 min. This burst of superoxide radical generation preceded the endothelial dysfunction and, in the absence of blood cells in the perfusate, appeared to be produced by the endothelium itself. Moreover, infusion of recombinant hSOD, but not the hydroxyl radical scavenger *N*-(2-mercaptopropionyl)-2 glycine (MPG), given at the time of reperfusion, abolished both the pulse of superoxide release, and the endothelial dysfunction.

An important follow-up finding in this study was that reperfusion with 95% N_2 plus 5% CO_2 abolished both the superoxide release and the subsequent endothelial dysfunction, clearly pointing to reoxygenation (i.e., reintroduction of oxygen) as a major factor responsible for the endothelial dysfunction. In additional studies, it was determined that myocardial ischemia–reperfusion in intact rats results in a comparable degree of coronary microvascular endothelial dysfunction as observed in isolated perfused rat hearts (Tsao and Lefer, 1991). Moreover, the time course of onset of endothelial dysfunction observed in myocardial ischemia–reperfusion in cats and rats is not unique to the coronary circulation, but is very similar to comparable events occurring in the mesenteric vasculature. Thus, splanchnic artery occlusion–reperfusion also results in a profound endothelial dysfunction in the superior mesenteric artery (SMA) of the rat (Lefer and Ma, 1991, 1993), occurring as early as 2.5 min postreperfusion and progressing to its maximal state by 60 min postreperfusion (Lefer and Ma, 1993). A comparable profile of endothelial dysfunction has been observed in cats subjected to splanchnic artery occlusion–reperfusion (Carey *et al.,* 1992; Karasawa *et al.,* 1991), and thus neither the splanchnic nor the myocardial endothelial dysfunction is species specific.

In summary, a severe form of endothelial dysfunction occurs within the first 5 min after reperfusion of the ischemic coronary or mesenteric circulation. This endothelial dysfunction is characterized by a reduced release of NO, assessed either by agonist stimulation or by basal release. The trigger event most likely responsible for the endothelial dysfunction is rapid reoxygenation of the previously ischemic vasculature, resulting in a burst of superoxide radicals that inactivate NO, and that perhaps may damage the NO biosynthetic process. Evidence suggests that a deficit in or sequestration of either tetrahydrobiopterin (TB_4), a cofactor for NO biosynthesis, or L-arginine (Hayward and Lefer, 1998b; Weyrich *et al.,* 1992; Tiefenbacher *et al.,* 1996) may be a key factor in bringing about the endothelial dysfunction (Tiefenbacher *et al.,* 1996).

One of the key aspects contributing to ischemia–reperfusion injury is the contribution of neutrophils (i.e., PMN leukocytes). Their contribution is multifaceted and clearly amplifies the reperfusion injury. Although not widely appreciated, PMNs also contribute to endothelial dysfunction under conditions of reperfusion injury. Figure 2 illustrates these principles in isolated cat coronary artery rings bathed with PMNs under a variety of conditions. When PMNs are added to the solution bathing a coronary artery ring (Fig. 2A), no vasoactive effect is observed until the PMNs are activated by a chemotactic agent (e.g., leukotriene B_4, LTB_4). On activation, the PMNs induce a significant vasocontraction of the vascular smooth muscle in the coronary artery ring. This vasocontraction is well sustained, but it can be further augmented by addition of other vasoconstrictors including the thromboxane-mimetic (U-46619). Addition of ACh to the fully contracted ring produces only a very small (i.e., $<10\%$) vasorelaxation, in contrast to a 100% vasorelaxation in response to acidified $NaNO_2$. This verifies the severe endothelial dysfunction.

The remainder of the recordings (Fig. 2B–E) are used to elucidate the mechanism of this PMN induced vasocontraction and related endothelial dysfunction. In Fig. 2B, employing a coronary artery ring that is de-endothelialized (−Endo), neither the vasocontraction nor the endothelial dysfunction occurs in response to activated PMNs. This suggests that the endothelium is essential for these phenomena. Figure 2C illustrates the importance of PMN interaction with the endothelium for triggering these phenomena. Thus, if one blocks PMN adherence to the endothelium with a monoclonal antibody (MAb) to CD18, the β-chain of the β_2-integrins (i.e., the major adhesion molecule of the neutrophil), neither the PMN-induced vasocontraction nor the endothelial dysfunction occur. This experiment shows the essential nature of PMN adherence to the endothelium to provoke these phenomena. In Fig. 2D, the importance of superoxide radicals to the vasocontraction and endothelial dysfunction is unmasked, since introduction of recombinant hSOD, an enzyme which inactivates superoxide radicals, blocks both of these phenomena. Finally, in Fig. 2E, we see the critical importance of nitric oxide to these events. Inhibition of nitric oxide synthase with L-NAME produces a marked vasocontraction comparable to that induced by activated PMNs.

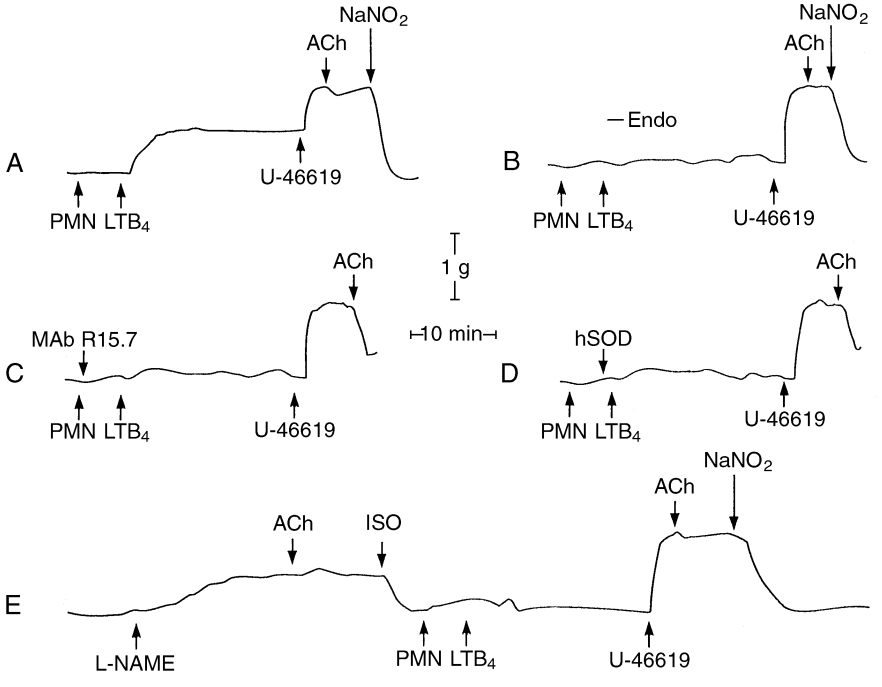


Figure 2 Representative recordings of isolated cat LAD coronary artery rings in isolated muscle baths at 37°C in Krebs–Henseleit buffer. Calibrations for developed force (1 g) and time (10 min) are shown. ACh, acetylcholine; LTB_4, leukotriene B_4; U46619, 9,11-methanoepoxy-PGH_2; $NaNO_2$, acidified sodium nitrite; MAb 15.7, anti-CD18 monoclonal antibody; ISO, isoproterenol; PMN, polymorphonuclear leukocytes; −Endo, de-endothelialized; L-NAME, N^G-nitro-L-arginine methyl ester.

This is verified by the lack of response to ACh, but not to isoprotenol (ISO), a nonendothelium dependent vasodilator. However, in this milieu lacking NO, activated PMNs fail to induce either the vasocontraction or augment the endothelial dysfunction usually observed in response to activated PMNs.

Integrating all these findings into a sequence of events, one can see that activated PMNs adhere to the endothelium, where they release superoxide radicals which then quench basal NO continuously produced by the endothelium. This shift in balance toward superoxide and away from NO contracts the subjacent vascular smooth muscle, reflecting the loss of NO that acts as a vasodilator counteracting the basal tone of the vascular smooth muscle. Superoxide radicals are released from the activated adhered PMNs, where they can quench EDNO. If the endothelium is removed, injured, or is inhibited from producing NO, activated neutrophils cannot exacerbate the existing endothelial dysfunction. This model is a microcosm of ischemia–reperfusion injury, and it reflects many of the key elements in the pathophysiology of reperfusion injury (Lefer and Lefer, 1996). Moreover, it shows how physiological amounts of NO are important in preserving vascular homeostasis and maintenance of control of vascular tone.

Role of NO in Modulating Cell Adhesion Molecules

Considerable evidence has accumulated showing that NO attenuates leukocyte–endothelium interaction (i.e., inhibits adherence of leukocytes to the endothelium). Among the earliest evidence obtained in this area is that NO inhibits formylmethionylleucylphenylalanine (fMLP)-induced PMN aggregation (McCall *et al.,* 1988). Kubes *et al.* (1991) later showed that superfusion with L-NMMA, a NOS inhibitor, over the cat mesenteric microvasculature resulted in a dramatic increase in leukocyte adherence to the microvascular endothelium. This enhanced adhesiveness was overcome by a monoclonal antibody directed against the leukocyte β_2-integrin CD18, the major neutrophil adhesion molecule responsible for firm cellular adhesion. This was further investigated by Davenpeck and colleagues (Davenpeck *et al.,* 1994a,b; Gauthier *et al.,* 1994), who clearly showed that suppression of endogenous NO synthesis either by pharmacological inhibition of NOS or by splanchnic ischemia–reperfusion results in increased leukocyte rolling along and adherence to the endothelium. In an elegant series of studies of intravital microscopy coupled with immunohistochemistry, this was largely attributable to upregulation of P-selectin on the affected endothelium in the absence of EDNO.

Additional evidence has since been obtained in the coronary vasculature. In a series of studies employing nitric oxide donor agents, Siegfried and co-workers (Siegfried *et al.,* 1992b,c; Johnson *et al.,* 1990) showed that several different types of NO donor agents (e.g., sydnonimine, cysteine, nitrate) inhibited the release of superoxide radicals by cat neutrophils, resulting in a NO-sparing effect on the vascular endothelium (Carey *et al.,* 1992). This maintenance of endothelium-derived NO led to a marked attenuation of myocardial necrosis (i.e., less severe reperfusion injury). This suppression of free radical release has also been found in human neutrophils in the case of superoxide (Clancy *et al.,* 1992; Moilanen *et al.,* 1993) and hydrogen peroxide (H_2O_2) (Forslund and Sundqvist, 1995). These data strongly point toward an important antineutrophil effect of NO, although they do not identify the specific cell adhesion molecules involved in this phenomenon.

One interesting question being answered now is which cell adhesion molecules are downregulated by nitric oxide. In this regard, either NO donor agents or the NO precursor L-arginine decreased basal intracellular adhesion molecule-1 (ICAM-1) surface expression (Lefer *et al.,* 1993a) in cultured human aortic endothelial cells (HAECs). DeCaterina *et al.* (1995) showed that NO donor agents also downregulate mRNA and protein expression for ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells. Downregulation of these important endothelial cell adhesion molecules was linked to inhibition of the transcription factor NF-κB and can be triggered by oxidized low density lipoproteins (oxo-LDL) (Liao *et al.,* 1995). Along these lines, Armstead *et al.* (1996) reported that NO donor agents or L-arginine downregulated the mRNA and protein expression of P-selectin in human cultured iliac artery and vein endothelial cells. Moreover, L-NAME upregulated P-selectin expression in these same cell types. These studies conducted in cultured human endothelial cells are important in understanding the cell signaling mechanisms responsible for the regulatory effects of NO on cell adhesion molecules. However, they do not yield critical information on whether these interactions pertain to the intact animal under physiologically relevant conditions. To answer these questions, studies have been conducted *in vivo* employing techniques such as intravital microscopy and flow cytometry. These have been mentioned earlier (Davenpeck *et al.,* 1994a,b; Gauthier *et al.,* 1994), and coupled with immunolocalization of cell adhesion molecule expression on the microvascular endothelium, they provide powerful reinforcement of the concept that NO functionally suppresses endothelial cell adhesion molecule expression, ablating much of the leukocyte–endothelium interaction occurring soon after the onset of reperfusion.

Role of NO in Endothelial Dysfunction and Neutrophil-Mediated Tissue Necrosis

Since endothelial dysfunction is the hallmark of the early events following reperfusion of a previously ischemic vasculature, it clearly is a crucial event in reperfusion injury. This applies to the coronary, the mesenteric, and other circulations. As mentioned earlier, the key event in this endothelial dysfunction is a loss of endothelial derived NO. One of the major triggers of endothelial dysfunction is a large early burst of superoxide radicals. This early superoxide burst is thought to originate from the hypoxic–reoxygenated

endothelium itself (Zweier *et al.,* 1987). This ''endothelial trigger'' sensitizes the microvasculature for the consequences of endothelial dysfunction, since superoxide is known to quench NO (Rubanyi and Vanhoutte, 1986; Gryglewski *et al.,* 1986) and thus inactivate EDNO. One of the likely generators of the endothelial superoxide is the xanthine oxidase system (Bulkley, 1989). Endothelial production of superoxide anions is an important event in early endothelial dysfunction, usually occurring within the first minute of reperfusion (Tsao *et al.,* 1990; Tsao and Lefer, 1990; Lefer *et al.,* 1990). This early loss of NO is necessary but not sufficient by itself to induce profound tissue injury. In the isolated perfused cat heart, made globally ischemic and then reperfused with and without neutrophils, about 25% of the endothelial dysfunction occurs in the absence of neutrophils, and 75% of the dysfunction occurs on perfusion with activated PMNs (Tsao *et al.,* 1992). These data are consistent with the findings of Tsao and Lefer (1990) showing that PMNs play a key role in propagating the endothelial dysfunction and in contributing to the cell injury of the underlying parenchymal cells (i.e., cardiac myocytes in the case of myocardial ischemia–reperfusion, and liver and intestinal cells in the case of splanchnic ischemia–reperfusion).

A further aspect of the interaction of PMNs with endothelium-derived NO in the setting of ischemia–reperfusion is the interesting effect the oxidative burst of neutrophils has on residual NO from the dysfunctional endothelium. This large secondary burst of superoxide from activated PMNs blocks any further release of NO from the endothelium, and it results in a vasospasm-like contraction of coronary arteries (Ma *et al.,* 1991; Ohlstein and Nichols, 1989). As mentioned earlier, results obtained in both cat (Ma *et al.,* 1991) and rabbit (Ohlstein and Nichols, 1989) coronary arteries clearly show that activated PMNs induce a marked and sustained vasoconstriction in isolated arterial segments. The basis for this constriction, somewhat analogous to a coronary vasospasm, is that the PMNs activated by fMLP or by leukotriene B_4 (LTB_4) release an oxidative burst of superoxide radicals, which antagonize the basal release of NO by the endothelium (see Fig. 2). This vasospasm does not occur in an artery denuded of endothelium or when the PMNs are not activated, indicating that the vasocontraction is clearly due to the superoxide released by activated PMNs acting on endothelium-derived NO (EDNO). This vasoconstriction can be washed out, and the arteries can be tested for their ability to relax to endothelium-dependent dilators (e.g., acetylcholine).

Similar results were obtained on activation of the endothelium, namely, with thrombin or hydrogen peroxide, without activating the PMNs (Murohara *et al.,* 1994). In this model of endothelial dysfunction, either the thrombin or the H_2O_2 upregulated P-selectin and promoted the adherence of unstimulated autologous PMNs to this activated cat coronary endothelium, despite the fact that the PMNs were normal and unstimulated. The result is a vasocontraction followed by a marked endothelial dysfunction (i.e., attenuated vasorelaxation to ACh, but not to acidified $NaNO_2$). Monoclonal antibodies directed against either P-selectin (PB1.3) or L-selectin (DREG-200), but not non-neutralizing control isotype antibodies, blocked both the vasocontraction and the endothelial dysfunction. Additionally, a sialyl $Lewis^x$ oligosaccharide (i.e., a selectin antagonist), but not its nonsialylated analog, also attenuated both phenomena. Coupled with flow cytometry of cultured cat endothelial cells, these results were found to be largely due to the activation of endothelial P-selectin subsequent to loss of EDNO. The PMN–endothelium interaction mediated largely by P-selectin leads to the release of superoxide radicals (Guo *et al.,* 1994). Recombinant hSOD abolished these phenomena, as did denudation of the endothelium, as in the case of the vasospasm and endothelial dysfunction induced by activated PMNs. Interestingly, a platelet activating factor (PAF) receptor antagonist (WEB-2170) also blocked these thrombin- and H_2O_2-induced vasospastic phenomena. This is not surprising because PAF is coexpressed with P-selectin on the vascular endothelium and contributes to PMN capture (Lorant *et al.,* 1991). Thus, either activating PMNs or activating the endothelium can bring about neutrophil–endothelium interaction that promotes severe endothelial dysfunction and vasospasm. Moreover, blockade of adherence of PMNs to the coronary endothelium with a neutralizing monoclonal antibody directed against either ICAM-1 (Ma *et al.,* 1992) or P-selectin (Weyrich *et al.,* 1993) or with an antiselectin oligosaccharide (Buerke *et al.,* 1994) preserves vascular endothelial function as well as attenuates myocardial injury on reperfusion. Moreover, critical reductions in basal NO release to the coronary vascular endothelium were shown to promote PMN adhesion to the ischemic–reperfused endothelium (Ma *et al.,* 1993). Thus, preservation of endogenous NO in the coronary vascular endothelium is a salient feature of the protection against reperfusion injury in myocardial ischemia–reperfusion.

One of the key mechanisms of propagation of reperfusion injury is the marked effect reduced EDNO exerts on the expression of endothelial cell adhesion molecules (CAMs). Thus, when biologically effective NO is reduced in the first 2.5 to 5 min after reperfusion of an ischemic vascular bed, expression of important endothelial cell adhesion molecules occurs rapidly. Thus, P-selectin is markedly upregulated on the endothelial cell surface within 10 to 20 min. ICAM-1, which is constitutively present on the endothelium, is upregulated more slowly (DeCaterina *et al.,* 1995; Weyrich *et al.,* 1995). Figure 3 illustrates these principles for P-selectin. In this regard, most P-selectin is usually contained within Weibel–Palade (WP) bodies located within endothelial cells (i.e., about 90% of the P-selectin is normally internalized). However, about 50% of the P-selectin can be translocated to the endothelial cell surface in 10–20 min by ischemia–reperfusion (Weyrich *et al.,* 1993, 1995). Stimuli that promote translocation of P-selectin to the endothelial cell surface are superoxide radicals, which act via conversion to H_2O_2, histamine, and thrombin. Once P-selectin is expressed on the surface of the endothelial cell, it can interact with its high affinity ligand P-selectin glycoprotein ligand-1 (PSGL-1), which is located constitutively on the microvilli of PMNs, to effect the slowing down and rolling (i.e., primary capture) of

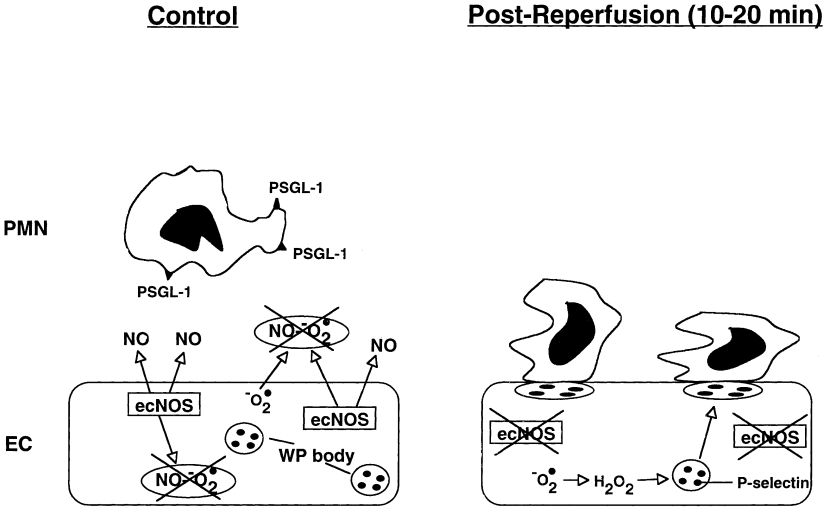


Figure 3 Scheme for explaining the postreperfusion upregulation of P-selectin on the endothelial cell surface. PMN, polymorphonuclear leukocytes; EC, endothelial cells; WP body, Weibel–Palade body; $^{-}O_2^{\cdot}$, superoxide radicals; ecNOS, endothelial constitutive nitric oxide synthase (black dots inside WP bodies and at cell surface are molecules of P-selectin); PSGL-1, P-selectin glycoprotein ligand-1; H_2O_2, hydrogen peroxide. When NO is quenched by superoxide radicals and H_2O_2 is formed, WP bodies translocate to the EC surface, where they bring P-selectin in contact with its PMN high-affinity ligand PSGL-1, facilitating PMN adherence to the endothelium.

leukocytes by the endothelium (Moore *et al.,* 1995). This starts the cascade of PMNs adhering to the endothelium and eventually migrating across the endothelium to the subjacent parenchymal cells (i.e., cardiomyocytes or intestinal cells), whereupon they can degranulate and release toxic mediators which injure or kill these parenchymal cells. A soluble PSGL-1 protein was found to markedly attenuate leukocyte–endothelium interaction in the rat mesenteric microvasculature following traumatic shock (Scalia *et al.,* 1999). These results point toward a key role of NO in this early response system.

Administration of NO or NO Precursors in Ischemia–Reperfusion

Nitric oxide is one of the major endogenous humoral agents that is important in preserving microvascular homeostasis and preventing reperfusion injury. Nitric oxide is normally produced in low nanomolar concentrations by the vascular endothelium (Kelm and Schrader, 1990). NO can diffuse to the basilar surface of the endothelial cells and then on to vascular smooth muscle cells, where it promotes vasorelaxation of the vascular smooth muscle cells (i.e., vasodilation). NO can also diffuse to the luminal surface of the endothelium, where it interfaces with the blood and can nitrosate circulating substances such as glutathione and serum albumin (Stamler *et al.,* 1992). NO can be later released by these carriers, whence it is able to exert several important physiological actions including inhibition of platelet aggregation (Radomski *et al.,* 1991), quenching of superoxide radicals (Rubanyi and Vanhoutte, 1986; Gryglewski *et al.,* 1986; Stewart *et al.,* 1988), and attenuation of leukocyte adherence to the endothelium (McCall *et al.,* 1988; Kubes *et al.,* 1991; Davenpeck *et al.,* 1994a,b; Gauthier *et al.,* 1994; Gaboury *et al.,* 1993). These effects occur at low nanomolar concentrations (i.e., 1 to 10 n*M*), which are even below concentrations of NO necessary to produce vasodilation (Davenpeck *et al.,* 1994a,b; Gauthier *et al.,* 1994; Gaboury *et al.,* 1993).

Table I summarizes the type of humoral agents that can directly release NO or promote synthesis of NO by the endothelium. In ischemia–reperfusion, authentic NO gas dis-

Table I Modes of Augmenting Nitric Oxide in Biological Systems

1. Authentic nitric oxide
   a. NO gas in physiological solution
   b. Inhaled NO
2. NO donating substances
   a. Inorganic NO releasing agents (e.g., acidified $NaNO_2$)
   b. Classic NO donor agents (e.g., nitroglycerin, sodium nitroprusside)
   c. Newer NO donor agents 9e.g., sydnonimines, furoxans, NO NOates)
   d. Peroxynitrite as a NO donor agent (concept of nitrosothiols as NO carriers)
3. Stimulators of NO biosynthesis
   a. L-Arginine
   b. Tetrahydrobiopterin
4. Gene therapy
   Local ecNOS or iNOS administration
5. Agents that stabilize ecNOS mRNA
   HMGCoA reductase inhibitors (e.g., simvastatin)

solved in physiological solutions (e.g., 0.9% NaCl), yielding local NO concentrations of 2 to 10 n*M,* has been shown to exert protective effects both in myocardial ischemia–reperfusion (Johnson *et al.,* 1991) and in splanchnic ischemia–reperfusion (Aoki *et al.,* 1990). Inhaled NO gas at 20–80 ppm has been found to exert antiadhesive effects and protect distant microvascular beds following ischemia–reperfusion (Fox-Robichaud *et al.,* 1998). The major effect was an attenuation of tissue injury and improved biochemical sequelae. In the case of myocardial ischemia–reperfusion, this was related to reduced neutrophil infiltration into the reperfused myocardium (Johnson *et al.,* 1991). These results were first observed following the administration of the inorganic NO-releasing agent $NaNO_2$ at pH 2.0 (Johnson *et al.,* 1990). These were the earliest reports that addition of physiological amounts of NO could exert beneficial effects in severe life-threatening forms of ischemia–reperfusion. However, these agents require significant improvement and a more readily controlled delivery system in order to be clinically useful in situations such as ischemia–reperfusion and shock.

The next major advance occurred with the synthesis of second and third generation organic NO donor agents. These are organic nitrates that release NO in solution, and they are much more potent than classic NO donor agents (e.g., nitroglycerin, nitroprusside), in addition to inducing much less ''tolerance'' than these agents (i.e., less tachyphylaxis). Also, these newer types of NO donor agents are more stable compounds, and they do not release superoxide radicals or cyanide, as do some of the earlier NO donors [e.g., 3-morpholinosydnonimine (SIN-1)]. Several of these newer NO donor agents have been carefully studied in myocardial ischemia–reperfusion. In this regard, C87-3754, a sydnonimine NO donor, but not its non-NO-releasing control compound C88-3934, markedly protected the ischemic cat myocardium from necrosis and preserved the integrity of the coronary vascular endothelium (Siegfried *et al.,* 1992b). Another different class of NO donor agent, a cysteine analog SPM-5185, was very effective in the same cat model of myocardial ischemia–reperfusion (Siegfried *et al.,* 1992c). In this latter study, the major mechanism of the cardioprotection was identified as inhibition of neutrophil–endothelium interaction and subsequent release of superoxide by activated PMNs (Siegfried *et al.,* 1992c). This finding was subsequently confirmed using the same NO donor agent in dogs (Lefer *et al.,* 1993b) and using a furoxan NO donor agent in isolated rat hearts perfused with PMNs (Pabla *et al.,* 1996). In both cases, nondonating control compounds having the same organic chemical backbone but lacking the NO moiety failed to exert a cardioprotective effect.

The same principles apply to splanchnic ischemia–reperfusion because the sydnonimine C87-3754 also protected cats subjected to splanchnic artery occlusion–reperfusion (Carey *et al.,* 1992). This NO donor agent increased survival and attenuated the formation of a myocardial depressant factor (MDF) (Lefer, 1987) in this lethal form of shock. Interestingly, the NO donor agent preserved the integrity and function of the superior mesenteric arterial endothelium (Carey *et al.,* 1992). In all cases, infusion of the NO donor agent occurred at a rate that did not exert any detectable effect on arterial blood pressure (i.e., was subvasodilatory). Thus, low concentrations of NO donor agents can be very effective in ischemia–reperfusion states.

The amino acid L-arginine, the precursor in the biosynthesis of NO, has been studied in myocardial ischemia–reperfusion (Weyrich *et al.,* 1992; Nakanishi *et al.,* 1992). In the same cat model of myocardial ischemia–reperfusion as that employed in the NO donor agent studies, intravenous infusion of L-arginine was shown to be effective in reducing infarct size as well as in attenuating neutrophil infiltration into the ischemic reperfused myocardium and in preserving coronary vascular endothelial function (Weyrich *et al.,* 1992). These effects were confirmed concurrently in a dog model of myocardial ischemia–reperfusion injury (Nakanishi *et al.,* 1992) and later in a rat model of traumatic shock (Hayward and Lefer, 1998b). Thus, availability of arginine may be important in maintaining endothelial NO in ischemia–reperfusion. Tiefenbacher *et al.,* (1996) showed that there is also a deficit in TB_4, a critical cofactor in the biosynthesis of NO. When TB_4 is replaced, much of the postreperfusion endothelial dysfunction is prevented.

Although there are now many published reports showing that either low doses of NO gas or NO donor agents or infusions of L-arginine or TB_4 are protective in ischemia–reperfusion (Tsao *et al.,* 1990; Gauthier *et al.,* 1994; Siegfried *et al.,* 1992b,c; Johnson *et al.,* 1990; Lefer *et al.,* 1993b; Pabla *et al.,* 1996), there are some dissenting studies in which NO synthase inhibitors (e.g., L-NAME) also have been reported to be protective in myocardial hypoxia–reoxygenation models (Mathies *et al.,* 1992; Schulz and Wambolt, 1995) and in either hypoxic–reoxygenated pigs or isolated perfused rat hearts, suggesting that NO may not be good under all conditions. It is difficult to distinguish among several possible explanations explaining these findings, including the possibility that (a) L-NAME may be cardiotoxic at high doses due to coronary constrictor or other effects, (b) NO may exert different effects in ischemia–reperfusion from hypoxia–reoxygenation, or (c) there is cardiotoxicity in these models due to formation of peroxynitrite ($ONOO^-$). However, the last potential explanation is unlikely, owing to findings showing physiologically relevant concentrations of peroxynitrite to be cardioprotective both *in vitro* (Lefer *et al.,* 1997a) and *in vivo* (Lefer *et al.,* 1997b).

Authentic $ONOO^-$ was added to a series of isolated perfused rat hearts to yield a local concentration of 800 n*M,* a concentration calculated to be comparable to biologically achievable concentrations (Lefer *et al.,* 1997a). In rat hearts subjected to ischemia for 30 min and reperfusion for 45 min, $ONOO^-$ had no detectable effect on left ventricular developed pressure (LVDP), *dP/dt* maximum, or coronary flow. When rat hearts were subjected to ischemia–reperfusion and perfused with rat neutrophils (i.e., PMNs), a severe reduction in LVDP, *dP/dt* maximum and coronary flow occurred. However, perfusion of ischemic–reperfused rat hearts with 800 n*M* $ONOO^-$ in the presence of PMNs resulted in a marked

amelioration of the cardiodepression observed with the pH matched vehicle for peroxynitrite ($ONOO^-$) (Lefer *et al.,* 1997a). Physiological concentrations of $ONOO^-$ clearly protected the heart against post- ischemia–reperfusion-induced cardiac stunning. These results also correlated closely with reduced myeloperoxidase (MPO) activity, indicative of fewer infiltrated PMNs in the ischemic rat hearts reperfused with PMNs (Lefer *et al.,* 1997a). Thus, $ONOO^-$ markedly attenuated accumulation of PMNs in the ischemic–reperfused rat heart, and it preserved left ventricular contractility in the face of ischemia–reperfusion with neutrophils.

These studies in isolated perfused hearts have been confirmed *in vivo* in cats subjected to myocardial ischemia–reperfusion. In these experiments, $ONOO^-$ was infused directly into the heart to achieve a local concentration of 400–1000 n*M*. Under these conditions, $ONOO^-$ significantly preserved the reperfused ischemic myocardium by 55% (Lefer *et al.,* 1997b). Figure 4 illustrates the potential cardiac cytoprotective mechanism of peroxynitrite in these studies. Basically, $ONOO^-$ is nitrosated to nitrosoglutathione, which then releases NO that is then available to the heart. These $ONOO^-$-treated cats also exhibited a significant preservation of coronary endothelial function (i.e., maintained NO release) as well as attenuated PMN infiltration into the ischemic–reperfused myocardium (Lefer *et al.,* 1997b). Thus, physiologically relevant concentrations of $ONOO^-$ (i.e., 400–800 n*M*) act as a cardioprotective agent in ischemia–reperfusion. Earlier studies demonstrating cytotoxic effects of $ONOO^-$ employed peroxynitrite concentrations of 500 μ*M* to 1.5 m*M* $ONOO^-$ to produce significant cell death (Szabo *et al.,* 1996). These extremely high concentrations are unlikely to ever occur *in vivo,* since $ONOO^-$ is formed from the equimolar combination of NO and superoxide radical (Pryor and Squadritto, 1995; Beckman *et al.,* 1990) and NO is present in blood at 1–10 n*M* (Kelm and Schrader, 1990). Even if NO levels increase by a factor of 100 to 1000, this could only yield maximal $ONOO^-$ concentrations of 1 to 10 μ*M*. With a half-life of <1 s, $ONOO^-$ would probably never achieve these cytotoxic levels *in vivo.*

Another way to generate NO at the site of injury *in vivo* is to transfect animals with the NOS enzyme, so that diseased blood vessels can produce their own NO. This has been proved to be effective in rat carotid arteries in a restenosis study (von der Leyen *et al.,* 1995) in which the injured vessel was able to respond to the transfected NOS by producing significant quantities of NO, which prevented myointimal proliferation, leading to a more patent artery.

Other types of agents have been identified [i.e., 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibition, ''statins''], such as simvastatin, which maintain endogenous NO levels by preserving or enhancing the stability of the mRNA for endothelial constitutive NOS (ecNOS). Figure 5 summarizes these relationships. Simvastatin pretreatment 18–24 hours before ischemia–reperfusion results in protection against myocardial–reperfusion injury in rats (Girod *et al.,* 1998). This has also been reported in experimental strokes or cerebral ischemia–reperfusion in mice (Endres *et al.,* 1998). The mechanism appears to be via inhibition of the biosynthesis of mevalonic acid and geranyl pyrophosphate, metabolites of the cholesterol biosynthesis pathway that cause rapid turnover of the mRNA for ecNOS. By inhibition of mevalonic acid and geranyl pyrophosphate, there is greater stability of the mRNA for ecNOS, and NO synthesis is maintained or enhanced. The enhanced NO is cytoprotective against subsequent reperfusion injury. Laufs

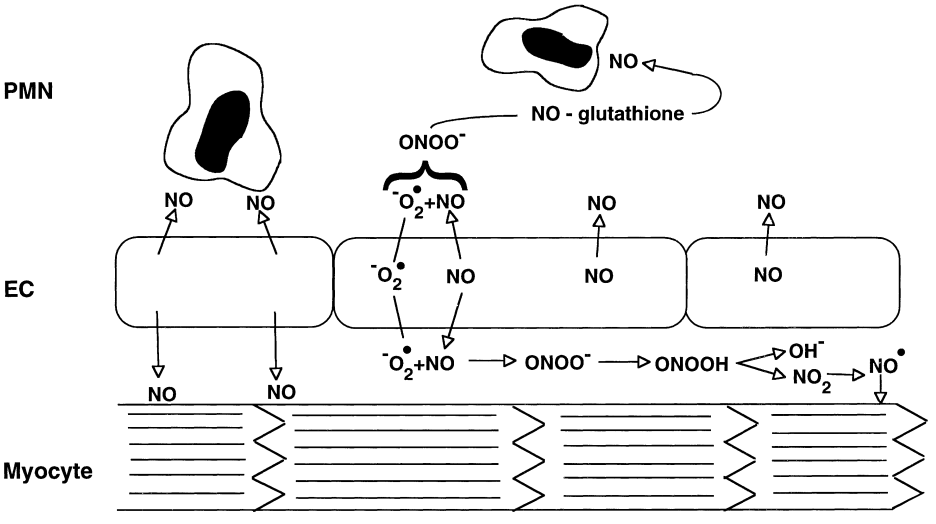


Figure 4 Schematic diagram of the mechanism of the cardiac cytoprotective effects of low concentrations (i.e., 400–1000 n*M*) of peroxynitrite. PMN, polymorphonuclear leukocytes; EC, endothelial cells; $ONOO^-$, peroxynitrite. Exogenously administered $ONOO^-$ nitrosates moieties such as glutathione to transport the $ONOO^-$ close to its site of action, where it dissociates to free NO. The free NO can diffuse to the endothelial cell surface, where it prevents PMN adherence, and to the cardiac myocytes, where it exerts additional cytoprotective effects.

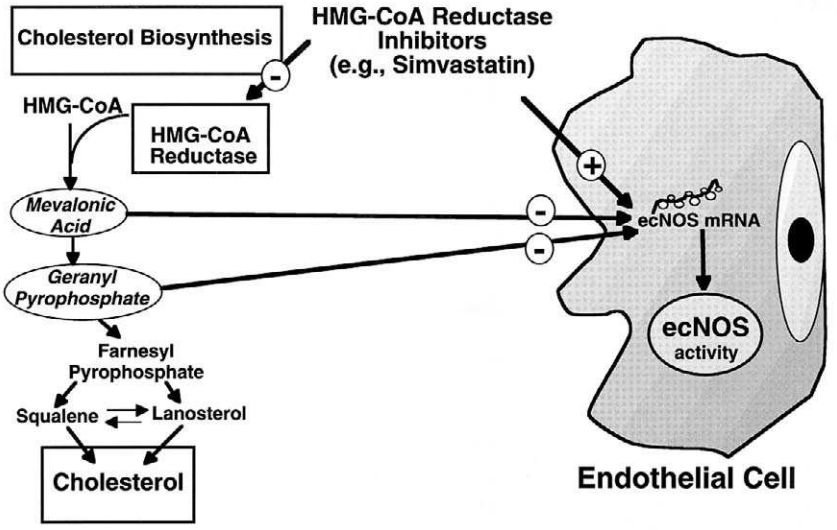


Figure 5 Schematic diagram for the mechanism of preserving NO biosynthesis by simvastatin, a HMG CoA reductase inhibitor. HMG CoA catalyzes the biosynthesis of the cholesterol precursors mevalonic acid and geranyl pyrophosphate. Both of these intermediates act on endothelial cells to destabilize the mRNA for ecNOS. By inhibiting mevalonic acid and geranyl pyrophosphate synthesis in normocholesterolemic states, NO biosynthesis is preserved, and more NO is bioavailable at the vascular endothelium. This enhanced NO protects the heart against ischemia–reperfusion injury.

et al. (1997) showed that this cytoprotective effect disappears in ecNOS gene-deleted mice, whereas Lefer *et al.* (1998) actually measured the enhanced NO release from aortic segments isolated from simvastatin-treated rats. Lefer *et al.* (1998) has shown that ecNOS gene-deleted mice develop greater myocardial reperfusion injury than wild-type mice. Thus, the evidence is accumulating that endogenous physiological NO is important in protecting the vasculature from reperfusion injury.

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References

Aoki, N., Johnson III, G., and Lefer, A. M. (1990). Beneficial effects of two forms of NO administration in feline splanchnic artery occlusion shock. *Am. J. Physiol.* **258,** G275–G281.

Armstead, V. E., Minchenko, A. G., and Lefer A. M. (1996). Nitric oxide physiologically attenuates P-selectin expression on human endothelial cells. *Circulation* **94**(Suppl.), I-634–I-635.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Buerke, M., Weyrich, A. S., Zheng, Z., Gaeta, F. C. A., Forrest, M. J., and Lefer, A. M. (1994). Sialyl Lewis^x-containing oligosaccharide attenuates myocardial reperfusion injury in cats. *J. Clin. Invest.* **93,** 1140–1148.

Bulkley, G. B. (1989). Mediators of splanchnic organ injury: Overview and perspective. *In* ''Splanchnic Ischemia and Multiple Organ Failure'' (A. Marston, G. B. Bulkley, R. G. Fiddian-Green, and U. H. Haglund, eds.), pp. 191–193. Arnold, London.

Carey, C., Siegfried, M. R., Ma, X.-L., Weyrich, A. S., and Lefer, A. M. (1992). Antishock and endothelial protective actions of a NO donor in mesenteric ischemia and reperfusion. *Circ. Shock* **38,** 209–216.

Clancy, R. M., Leszczynska-Piziak, J., and Abramson, S. B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action in the NADPH oxidase. *J. Clin. Invest.* **90,** 1116–1121.

Davenpeck, K. L., Gauthier, T. W., and Lefer, A. M. (1994a). Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation. *Gastroenterology* **107,** 1050–1058.

Davenpeck, K. L., Gauthier, T. W., Albertine, K. H., and Lefer, A. M. (1994b). Role of P-selectin in microvascular leukocyte-endothelial interaction in splanchnic ischemia–reperfusion. *Am. J. Physiol.* **267,** H622–H630.

DeCaterina, R., Libby, P., Peng, H.-B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, Jr., M. A., Shin, W. S., and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. No selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* **96,** 60–68.

Endres, M., Laufs, U., Huang, Z., Nakamura, T., Huang, P., Moskowitz, M. A., and Liao, J. K. (1998). Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8880–8885.

Forslund, T., and Sundqvist, T. (1995). Nitric oxide reduces hydrogen peroxide production from human polymorphonuclear neutrophils. *Eur. J. Clin. Invest.* **25,** 9–14.

Fox-Robichaud, A., Payne, D., Hasan, S. U., Ostrovsky, L., Fairhead, T., Reinhardt, P., and Kubes, P. (1998). Inhaled NO as a viable antiadhesive therapy for ischemia/reperfusion injury of distal microvascular beds. *J. Clin. Invest.* **101,** 2497–2505.

Gaboury, J., Woodman, R. C., Granger, D. N., Reinhardt, P., and Kubes, P. (1993). Nitric oxide prevents leukocyte adherence: Role of superoxide. *Am. J. Physiol.* **265,** H862–H867.

Gaboury, J., Niu, X. F., and Kubes, P. (1996). Nitric oxide inhibits numerous features of most cell-induced inflammation. *Circulation* **93,** 318–326.

Gauthier, T. W., Davenpeck, K. L., and Lefer, A. M. (1994). Nitric oxide attenuates leukocyte–endothelial interaction via P-selectin in splanchnic ischemia–reperfusion. *Am. J. Physiol.* **267,** G562–G568.

Girod, W. G., Jones, S. P., Huang, P. L., Fishman, M. C., and Lefer, D. J. (1998). The effects of nitric oxide synthase deficiency on myocardial reperfusion injury in mice. *Circulation* **98,** I-730.

Gryglewski, R. J., Palmer, R. M. J., and Moncada, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* **320,** 454–456.

Guo, J.-P., Siegfried, M. R., and Lefer, A. M. (1994). Endothelial preserving actions of a nitric oxide donor in carotid arterial intimal injury. *Methods Find. Exp. Clin. Pharmacol.* **16,** 347–354.

Hayward, R., and Lefer, A. M. (1998a). Time course of endothelial–neutrophil interaction in splanchnic artery ischemia–reperfusion. *Am. J. Physiol.* **275,** H2080–H2086.

Hayward, R., and Lefer, A. M. (1998b). L-Arginine attenuates endothelial dysfunction and prolongs survival in rats subjected to traumatic shock. *Endothelium* **6,** 71–79.

Johnson III, G., Tsao, P. S., Mulloy, D., and Lefer, A. M. (1990). Cardioprotective effects of acidified sodium nitrite in myocardial ischemia with reperfusion. *J. Pharmacol. Exp. Ther.* **252,** 35–41.

Johnson III, G., Tsao, P. S., and Lefer, A. M. (1991). Cardioprotective effects of authentic nitric oxide in myocardial ischemia with reperfusion. *Crit. Care Med.* **19,** 244–252.

Karasawa, A., Guo, J.-P., Ma, X.-L., and Lefer, A. M. (1991). Beneficial effects of transforming growth factor-β and tissue plasminogen activator in splanchnic artery occlusion and reperfusion in cats. *J. Cardiovasc. Pharmacol.* **18,** 95–105.

Kelm, M., and Schrader, J. (1990). Control of coronary vascular tone by nitric oxide. *Circ. Res.* **66,** 1561–1575.

Korthuis, R. J., Grisham, M. B., Zimmerman, B. J., Granger, D. N., and Taylor, A. E. (1988). Vascular injury in dogs during ischemia–reperfusion improvement with ATP-MgCl2 pretreatment. *Am. J. Physiol.* **254,** H702–H708.

Kubes, P., Suzuki, M., and Granger, D. N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4651–4655.

Laufs, U., La Fata, V., and Liao, J. K. (1997). Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. *J. Biol. Chem.* **272,** 31725–31729.

Lefer, A. M. (1987). Interaction between myocardial depressant factor and vasoactive mediators with ischemia and shock. *Am. J. Physiol.* **252,** R193–R205.

Lefer, A. M., and Lefer, D. J. (1993). Pharmacology of the endothelium in ischemia–reperfusion and circulatory shock. *Annu. Rev. Pharmacol.* **33,** 71–90.

Lefer, A. M., and Lefer, D. J. (1996). The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. *Cardiovasc. Res.* **32,** 743–751 (invited review).

Lefer, A. M., and Ma, X.-L. (1991). Endothelial dysfunction in the splanchnic circulation following ischemia and reperfusion. *J. Cardiovasc. Pharmacol.* **17**(Suppl. 3), S186–S190.

Lefer, A. M., and Ma, X.-L. (1993). Cytokines and growth factors in endothelial dysfunction. *Crit. Care Med.* **21**(Suppl.), S9–S14.

Lefer, A. M., Tsao, P., Aoki, N., and Palladino, Jr., M. A. (1990). Mediation of cardioprotection by transforming growth factor-β. *Science* **249,** 61–64.

Lefer, D. J., Klunk, D. A., Lutty, G. A., Merges, C., Schleimer, R. P., Bochner, B. S., and Zweier, J. L. (1993a). Nitric oxide (NO) donors reduce basal ICAM-1 expression on human aortic endothelial cells (HAEC's). *Circulation* **88**(Part 2), I–565.

Lefer, D. J., Nakanishi, K., Johnston, W. E., and Vinten-Johansen, J. (1993b). Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. *Circulation* **88,** 2337–2350.

Lefer, D. J., Scalia R., Campbell, B., Nossuli, T. O., Hayward, R., Salamon, M., Grayson, J., and Lefer, A. M. (1997a). Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia–reperfusion injury in rats. *J. Clin. Invest.* **99,** 684–691.

Lefer, A. M., Nossuli, T. O., and Hayward, R. (1997b). Peroxynitrite reduces myocardial infarct size and preserves coronary endothelium following ischemia and reperfusion. *FASEB J.* **11,** A432.

Lefer, A. M., Campbell, B., Shin, Y. K., Scalia, R., and Lefer, D. J. (1998). Simvastatin protects the ischemic reperfused myocardium from leukocyte–endothelium interaction and neutrophil induced cardiac dysfunction. *Circulation* **98**(Suppl.), I–114.

Liao, J. K., Shin, W. S., Lee, W. Y., and Clark, S. L. (1995). Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J. Biol. Chem.* **270,** 319–324.

Lieberthal, W., Wold, E. F., Rennke, H. G., Valeri, C. R., and Levinsky, N. G. (1989). Renal ischemia and reperfusion impair endothelium-dependent vascular relaxation. *Am. J. Physiol.* **256,** F894–F900.

Lorant, D. E., Patel, K. D., McIntyre, T. M., McEver, R. P., Prescott, S. M., and Zimmerman, G. A. (1991). Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: A juxtacrine system for adhesion and activation of neutrophils. *J. Cell Biol.* **115,** 223–234.

Ma, X.-L., Tsao, P. S., Viehman, G. E., and Lefer, A. M. (1991). Neutrophil-mediated vasoconstriction and endothelial dysfunction in low-flow perfusion-reperfused cat coronary artery. *Circ. Res.* **69,** 95–106.

Ma, X.-L., Lefer, D. J., Lefer, A. M., and Rothlein, R. (1992). Coronary endothelial and cardiac protective effects of a monoclonal antibody to intercellular adhesion molecule-1 in myocardial ischemia and reperfusion. *Circulation* **86,** 937–946.

Ma, X.-L., Weyrich, A. S., Lefer, D. J., and Lefer, A. M. (1993). Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ. Res.* **72,** 403–412.

McCall, T., Whittle, B. J. R., Boughton-Smith, N. K., and Moncada, S. (1988). Inhibition of fMLP induced aggregation of rabbit neutrophils by nitric oxide. *Br. J. Pharmacol.* **95,** 517P.

Mathies, G., Sherman, M. P., Buckberg, G. D., Haybron, D. M., Young, H. H., and Ignarro, L. J. (1992). Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am. J. Physiol.* **262,** H616–H620.

Moilanen, E., Vuorinen, P., Kankaanranta, H., MetsaKetela, T., and Vapatalo, H. (1993). Inhibition by nitric oxide-donors of human polymorphonuclear leukocyte function. *Br. J. Pharmacol.* **109,** 852–858.

Moncada, S., Palmer, R. M. J., and Higgs, E. H. (1990). Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Moore, K. L., Patel, K. D., Bruehl, R. E., Li, F., Johnson, D. A., Lichenstein, H. S., Cummings, R. D., Bainton, D. F., and McEver, R. P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J. Cell Biol.* **128,** 661–671.

Murohara, T., Buerke, M., and Lefer, A. M. (1994). Polymorphonuclear leukocyte-induced vasocontraction and endothelial dysfunction: Role of selectins. *Arterioscler. Thromb.* **14,** 1509–1519.

Nakanishi, K., Vinten-Johansen J., Lefer D. J., Zhao Z., Fowler III, W. C., McGee, D. S., and Johnston, W. E. (1992). Intracoronary L-arginine during reperfusion improves endothelial function and reduces infarct size. *Am. J. Physiol.* **263,** H1650–H1658.

Ohlstein, E. H., and Nichols, A. J. (1989). Rabbit polymorphonuclear neutrophils elicit endothelium-dependent contraction in vascular smooth muscle. *Circ. Res.* **65,** 917–924.

Pabla, R., Buda, A. J., Flynn, D. M., Blesse, S. A., Shin, A. M., Curtis, M. J., and Lefer, D. J. (1996). Nitric oxide attenuates neutrophil-mediated myocardial contractile dysfunction after ischemia and reperfusion. *Circ. Res.* **78,** 65–72.

Pryor, W. A., and Squadritto, G. L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide and superoxide. *Am. J. Physiol.* **268,** L699–L722.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1991). Modulation of platelet aggregation by an L-arginine-nitric oxide pathway. *Trends Pharmacol. Sci.* **12,** 87–88.

Rosenblum, W. I., Nelson, G. H., and Shimizu, T. (1992). L-Arginine suffusion restores response to acetylcholine in brain arterioles with damaged endothelium. *Am. J. Physiol.* **262,** H961–H964.

Rubanyi, G. M., and Vanhoutte, P. M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.* **250,** H822–H827.

Scalia, R., Pearlman, S., Campbell, B., and Lefer, A. M. (1996). Time course of endothelial dysfunction and neutrophil adherence and infiltration during murine traumatic shock. *Shock* **6,** 177–182.

Scalia, R., Hayward, R., Armstead, V. E., Minchenko, A. G., and Lefer, A. M. (1999). Effect of recombinant soluble P-selectin glycoprotein ligand-1 on leukocyte-endothelium interaction in vivo: Role in rat traumatic shock. *Circ. Res.* **84,** 93–102.

Schulz, R., and Wambolt, R. (1995). Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia–reperfusion injury. *Cardiovasc. Res.* **30,** 432–439.

Siegfried, M. R., Ma, X.-L., and Lefer, A. M. (1992a). Splanchnic vascular endothelial dysfunction in rat endotoxemia: Role of superoxide radicals. *Eur. J. Pharmacol.* **212,** 171–176.

Siegfried, M. R., Erhardt, J., Rider, T., Ma, X.-L., and Lefer, A. M. (1992b). Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia–reperfusion. *J. Pharmacol. Exp. Ther.* **260,** 668–675.

Siegfried, M. R., Carey, C., Ma, X.-L., and Lefer, A. M. (1992c). Beneficial effects of SPM-5185, a cysteine-containing NO donor in myocardial ischemia–reperfusion. *Am. J. Physiol.* **263,** H771–H777.

Stamler, J. S., Jaraki, O., Osbourne, J., Simon, D. I., Keaney Jr., J. F., Vita, J., Singel, C. R., Valeri, C. R., and Loscalzo, J. (1992). Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7674–7677.

Stewart, D. J., Pohl, U., and Bassenge, E. (1988). Free radicals inhibit endothelium-dependent dilation in the coronary resistance bed. *Am. J. Physiol.* **255,** H765–H769.

Szabo, C., Zingarelli, B., and Salzman, A. L. (1996). Role of poly-ADP ribosyltransferase activation in the vascular contractile and energetic failure elicited by exogenous nitric oxide and peroxynitrite. *Circ. Res.* **78,** 1051–1063.

Tiefenbacher, C. P., Chilian, W. M., Mitchell, M., and DeFily, D. V. (1996). Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. *Circulation* **94,** 1423–1429.

Tsao, P. S., and Lefer, A. M. (1990). Time course and mechanism of endothelial dysfunction in isolated ischemic and hypoxic perfused rat hearts. *Am. J. Physiol.* **259,** H1660–H1666.

Tsao, P. S., and Lefer, A. M. (1991). Recovery of endothelial function following myocardial ischemia and reperfusion in rats. *J. Vasc. Med. Biol.* **3,** 5–10.

Tsao, P. S., Aoki, N., Lefer, D. J., Johnson, G., and Lefer, A. M. (1990). Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation* **82,** 1402–1412.

Tsao, P. S., Ma, X.-L., and Lefer, A. M. (1992). Activated neutrophils aggravate endothelial dysfunction after reperfusion of the ischemic feline myocardium. *Am. Heart J.* **123,** 1464–1471.

Van Benthuysen, K. M., McMurtry, I. F., and Horowitz, L. D. (1987). Reperfusion after acute coronary occlusion in dogs impairs endothelial dependent relaxation to acetylcholine and augments contractile reactivity in vitro. *J. Clin. Invest.* **79,** 265–274.

von der Leyen, H. E., Gibbons, G., Morishita, R., Lewis, N. P., Zhang, L., Nakajima, M., Kaneda, Y., Cooke, J., and Dzau, V. J. (1995). Gene therapy inhibiting neointimal vascular lesion: In vivo transfer of endothelial cell nitric oxide synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1137–1141.

Wang, P., Zheng, F. B., and Chaudry, I. H. (1993). Endothelial cell dysfunction occurs very early following trauma-hemorrhage and persists despite fluid resuscitation. *Am. J. Physiol.* **265,** H973–H979.

Wang, P., Zheng, F., and Chaudry, I. H. (1995). Endothelium-dependent relaxation is depressed at the macro- and microcirculatory levels during sepsis. *Am. J. Physiol.* **269,** R988–R994.

Weyrich, A. S., Ma, X.-L., and Lefer, A. M. (1992). The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation* **86,** 279–288.

Weyrich, A. S., Ma, X.-L., Lefer, D. J., Albertine, K. H., and Lefer, A. M. (1993). In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J. Clin. Invest.* **91,** 2620–2629.

Weyrich, A. S., Buerke, M., Albertine, K. H., and Lefer, A. M. (1995). Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of the ischemic feline myocardium. *J. Leukocyte Biol.* **57,** 45–55.

Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987). Observation of free radical generation in the post-ischemic heart. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 1404–1407.

CHAPTER 41

Role of Nitric Oxide in Myocardial Ischemia–Reperfusion Injury

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NITRIC OXIDE (NO) HAS A PLETHORA OF PHYSIOLOGICAL EFFECTS IN NUMEROUS CELL TYPES AND TISSUES OF THE BODY. NO ATTENUATES THE ACTIVATION OF NEUTROPHILS [POLYMORPHONUCLEAR LEUKOCYTES (PMNS)] AND THE PRODUCTION OF SUPEROXIDE ANIONS, THE PROCESS OF DEGRANULATION, AND THE ADHERENCE OF PMNS TO THE VASCULAR ENDOTHELIUM. THE INTERACTION BETWEEN PMNS AND THE CORONARY VASCULAR ENDOTHELIUM IS A CRITICAL FACTOR IN TRIGGERING AND PERPETUATING THE INFLAMMATORY-LIKE RESPONSES CHARACTERISTIC OF MYOCARDIAL ISCHEMIA–REPERFUSION, WHICH CULMINATE IN ENDOTHELIAL INJURY, MICROVASCULAR DEFECTS AND IMPAIRED TISSUE BLOOD FLOW, AND INFARCTION. NO ATTENUATES THE UPREGULATION OF ADHESION MOLECULES DURING BOTH THE ACUTE RESPONSE AND THE LONG-TERM RESPONSES TO MYOCARDIAL REPERFUSION MEDIATED BY TRANSCRIPTION AND PROTEIN SYNTHESIS. THESE ATTRIBUTES HAVE GENERATED GREAT INTEREST IN NO AS A THERAPEUTIC AGENT AGAINST NONSURGICAL AND SURGICAL MYOCARDIAL ISCHEMIA–REPERFUSION INJURY. ENDOGENOUSLY PRODUCED NO APPEARS TO PARTICIPATE IN AN INHERENT MODULATION OF POSTISCHEMIC INJURY. AMPLIFICATION OF ENDOGENOUSLY GENERATED NO WITH THE PRECURSOR L-ARGININE AND INCREASED TISSUE LEVELS WITH PARENTERAL ADMINISTRATION OF VARIOUS CLASSES OF NO DONOR AGENTS HAVE BEEN USED TO EFFECTIVELY REDUCE NEUTROPHIL–ENDOTHELIUM INTERACTIONS, THE ACCUMULATION OF NEUTROPHILS IN REPERFUSED MYOCARDIUM, ENDOTHELIAL DYSFUNCTION, AND INFARCTION. NO THERAPY HAS ALSO BEEN USED AS AN ADJUNCT TO CARDIOPROTECTIVE STRATEGIES IN CARDIAC SURGERY, WHERE ISCHEMIA–REPERFUSION IS LIKELY A MORE COMPLEX PROCESS. HOWEVER, EXCESS CONCENTRATIONS OF NO, OR NO IN THE ABSENCE OF CERTAIN THIOLS THAT OTHERWISE CONVERT BY-PRODUCTS OF NO (SUCH AS PEROXYNITRITE) TO MOLECULES WITH NO-LIKE CHARACTERISTICS, HAS BEEN ASSOCIATED WITH INCREASED TISSUE INJURY. THE USE OF NO IN TARGETING MYOCARDIAL ISCHEMIC–REPERFUSION INJURY WILL LIKELY INCREASE AS MOLECULAR MECHANISMS OF ITS ACTIONS ARE IDENTIFIED.

Introduction

Nitric oxide (NO), also named nitrogen monoxide, is a unique molecule characterized by diametrically opposed chemical attributes and physiological actions on both cells and whole organs. NO is very labile in physiological systems, its existence averaging less than a second in human blood. It is rapidly bound to the heme moieties on hemoglo-

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bin and guanylyl cyclase—one of its major physiological effector molecules—and is rapidly transformed to the metabolites nitrate, nitrite, and peroxynitrite. However, the physiological actions of NO go beyond its short *in vivo* presence. These physiological actions include potent vasodilation, inhibition of adhesion molecule-dependent neutrophil–endothelial cell interactions, modulation of myocardial function and metabolism, and attenuation of gene expression of inflammatory mediators and their transcription signals (i.e., via nuclear factor κB, NF-κB).

The consequences of the physiological actions of NO may have lasting effects, despite the short life span of NO in blood and tissues. The attenuation of postischemic injury, particularly myocardial infarction and coronary vascular endothelial dysfunction, are among the consequences of the actions of NO that have a long-range impact on the health and life span of the patient. Under some circumstances, however, NO is associated with the pathogenesis of tissue injury and dysfunction, which seems to be in contrast to its potent cardioprotective attributes. Other opposing effects are exemplified by the role of NO in mediating shock, in contrast to its cardioprotective role in ischemia–reperfusion injury. Hence, NO has been cast as both (a) a pathological mediator of hypotension and cardiac dysfunction in septic shock or hypoxia–reoxygenation and (b) a potent therapeutic agent in ischemia–reperfusion and inflammation.

Finally, NO may also have opposing physiological actions depending on its biological environment. Studies show that NO may injure cells and tissue in an acellular (*in vitro*) environment, while exerting protective effects in *in vivo* blood environments. Concepts that unify this rather schizophrenic profile of NO have not been confirmed; therefore, this dichotomy of action is unresolved. Understandably, this plurality of physiological effects has made NO a topic of considerable debate and controversy, particularly regarding its role as a therapeutic agent.

This chapter focuses on the physiological actions of NO in the setting of myocardial ischemia and reperfusion encountered as a primary disease state, and as a special condition that exists during the unique circumstances presented by cardiac surgery using cardiopulmonary bypass. Ischemia–reperfusion injury not only targets myocytes, which are the contractile units of the heart, but also targets the coronary vasculature at the conduit level (arteries and veins) and at the microvascular level (arteriole, capillaries, venules). Furthermore, injury to the coronary vasculature is intimately involved in the pathogenesis (triggering) and propagation (amplification) of ischemia–reperfusion injury, whether the injury is expressed as necrosis, contractile dysfunction, or apoptosis. This chapter will describe the pathological effects of ischemia and reperfusion on the various cells that comprise the myocardium, and it discusses how NO interacts with other mechanisms triggered by ischemia and reperfusion that would potentially lead to reversible or irreversible tissue injury without its presence.

The Pathophysiology of Ischemia–Reperfusion Injury in the Heart

Ischemia

Ischemia is defined as an imbalance in the energy supply to the heart relative to its demand. Since the heart is primarily an aerobic organ with little biochemical capacity for anaerobic metabolism, the major metabolic processes are driven by oxygen. The myocardium extracts approximately 70% or more of the arterial oxygen content under normal conditions. With a physiological upper limit of 90–95% extraction, there is little oxygen extraction reserve on which to call. An ischemic condition exists if oxygen supplied by coronary blood flow is insufficient relative to the oxygen demands at the ambient level of cardiac work. A mismatch between the oxygen delivered to the myocardium and the oxygen required to sustain a given level of work, known as the oxygen supply demand status, is created either by decreasing the coronary blood flow (supply ischemia) or by increasing the work of the heart to a level beyond that which can be supported by the amount of oxygen delivered to the myocardium. The primary factors that determine the severity of ischemia in the myocardium include (a) the quantity of collateral blood flow available from other coronary arteries supplying the myocardium undergoing ischemia, (b) the ambient oxygen demands of the myocardium, and (c) the duration of ischemia. Owing to the aerobic nature of the myocardium, physiological processes requiring energy in the form of ATP, and ultimately the ability to maintain cell viability, are attenuated in proportion to the degree of the oxygen supply/demand mismatch. Hence, during ischemia, contractile dysfunction rapidly decreases (or fails to increase in the case of demand ischemia), the intracellular ionic balance is disrupted, and cell viability is threatened. *In vivo,* cell necrosis occurs after approximately 40 min of ischemia when followed by reperfusion with unmodified blood (Reimer and Jennings, 1979). Myocardium first begins to undergo necrosis in the subendocardium because the oxygen supply/demand mismatch is greatest in this region, and necrosis progresses in a ''wavefront'' pattern toward the subepicardium as the duration of ischemia increases. It should be noted that this temporal pattern of necrosis was observed in reperfused hearts and therefore represents combined ischemic–reperfusion injury rather than ischemic injury alone. This pattern is not apparent in hearts that are not reperfused or are reperfused with adjunctive cardioprotective agents administered systemically or intracoronarily.

Reperfusion

Although reperfusion of the myocardium is the definitive treatment for ischemia, and is the ultimate goal of both nonsurgical (coronary artery angioplasty) and surgical (coronary artery bypass surgery) revascularization procedures, reperfusion carries with it the potential for extending postis-

chemic injury to myocytes, coronary vascular endothelium, and the microvasculature (Ambrosio and Chiariello, 1991; Vaage and Valen, 1993). Reperfusion injury can be defined as the pathology that is extended, accelerated, or expressed *de novo* beyond that observed during ischemia alone, resulting from events occurring *after* restoration of blood flow. Although there has been some debate whether reperfusion injury, in fact, exists (Kloner, 1993; Opie, 1991; Przyklenk, 1997; Robicsek and Schaper, 1997), a consensus is being drawn that reperfusion is indeed a significant component of the picture of postischemic myocardial injury (Hudson, 1994) in experimental models and in humans.

It is our belief that ischemia makes the heart vulnerable to reperfusion injury after the normal blood supply has been reestablished, and that a substantial degree of injury occurs after reperfusion. In support of the concept that a significant portion of the final postischemic injury is sustained during reperfusion, several investigators (enumerated throughout this chapter) have reported an immediate recovery of contractility, a significant reduction in infarct size, an attenuation of endothelial injury, and a maintenance of ultrastructural integrity of the myocardium if the conditions of reperfusion and the composition of the reperfusate are modified before normal blood supply is reestablished. This ''modified reperfusion'' can take the form of (a) cardioprotective agents administered either systemically before reperfusion or by intracoronary infusion at the onset of reperfusion or (b) blood cardioplegia used in cardiac surgery in which agents that modify blood components (i.e., calcium concentration, osmolality) or target components of reperfusion injury are administered during the period of cardioplegia arrest, before the previously ischemic myocardium is exposed to normal blood.

Mechanisms of Myocardial Reperfusion Injury

Among the more important mechanisms of myocardial reperfusion injury in the heart are (a) calcium influx and loss of normal intracellular calcium homeostasis, (b) oxygen radical generation, (c) neutrophil activation, release of neutrophil-derived products, and adhesion to the endothelium, and (d) production of microvascular injury characterized by interstitial edema, impaired blood flow (''no-reflow''), and defective autoregulation. These mechanisms of injury do not operate independently, nor are they mutually exclusive. However, reperfusion injury has been shown to be a malleable process that can be attenuated by applying a number of therapies targeting these various mechanisms of injury. NO is one of these important cardioprotective therapies that has emerged in the 1990s.

Neutrophils have been implicated as a player in the pathophysiology underlying nonsurgical as well as surgical ischemic–reperfusion injury. The mechanisms of neutrophil-mediated injury include (a) generation of oxygen free radicals and related oxidants [hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), hypochlorous acid (HOCl), (b) degranulation to release myeloperoxidase that produce HOCl, as well as proteases or other enzymes that degrade membranous structures and components of the extracellular matrix, (c) the release of arachidonic acid metabolites [phospholipase A_2, leukotriene B_4 (LTB_4)] and other proinflammatory mediators such as platelet activating factor (PAF) and tumor necrosis factor α (TNF-α). These mechanisms also represent the primary targets toward which antineutrophil therapy is directed. Although superoxide anions, hydrogen peroxide, and hydroxyl radicals generated by neutrophils are important reactive oxygen species that mediate tissue damage, HOCl is the predominant cytotoxic oxidant derived from neutrophils. Its cytotoxicity results from production of powerfully oxidizing chloramines. In addition to causing direct injury to myocardium, these reactive oxygen species provide leukotactic signals by stimulating the generation of complement, by inducing expression of P-selectin on endothelium, and by inducing surface expression of PAF on endothelium.

The Coronary Vascular Endothelium, Nitric Oxide, and the Effects of Ischemia–Reperfusion Injury

Normal Physiology of Endothelium-Derived Nitric Oxide

NO is generated by endothelial-specific NO synthase (or eNOS) primarily localized in vascular endothelium, but localized also in cardiac myocytes. NO is a potent vasodilator, and it has been termed the endogenous nitrovasodilator because of its role in regulating tissue blood flow and vascular resistance. eNOS is a calcium/calmodulin-sensitive enzyme activity that is regulated by physiological concentrations of Ca^{2+}. Ca^{2+} increases the binding of calmodulin to eNOS, thereby initiating the electron flow necessary to convert the guanidino nitrogen of L-arginine to NO and L-citrulline in the presence of molecular oxygen. Tetrahydrobiopterin (TB_4) is a cofactor that facilitates the flow of electrons from NADPH to the heme moiety of eNOS. In the absence of TB_4 and L-arginine, eNOS can generate superoxide anions from the oxygenase and reductase domains of the heme enzyme.

Under basal conditions, the coronary vascular endothelium tonically releases NO (Guo *et al.,* 1996; Ma *et al.,* 1993; Engelman *et al.,* 1995a) in concentrations ranging from 0.1 to 1 n*M.* This basal release of NO is regulated by vascular shear stress (Buga *et al.,* 1991; Cooke *et al.,* 1990; Nerem *et al.,* 1993), by the presence of physiological agonists such as acetylcholine and bradykinin, and by the availability of the substrates L-arginine and oxygen. Vascular shear stress and associated deformation of the endothelium, both of which accompany pulsatile perfusion, activate eNOS (Stewart *et al.,* 1994; Smith and Canty, 1993). Higher shear stresses created by high blood flow in vessels stimulates eNOS activity to release greater quantities of NO, whereas low shear stress created in low-flow situations reduces the release of NO.

A number of agonists specifically stimulate the release of NO from the vascular endothelium. Acetylcholine and bradykinin trigger an increase in intracellular calcium through receptor-mediated mechanisms which, in turn, stimulates eNOS activity and the release of NO in a concentration-dependent manner. Ca^{2+} ionophores such as A23187 will also stimulate eNOS activity and the release of NO, but by receptor-independent mechanisms, which helps to differentiate receptor-dependent from receptor-independent pathways of signal transduction. This information can be used to distinguish whether receptor mechanisms or the enzyme system itself has been impaired by ischemia–reperfusion injury or other interventions. The NO released by these mechanisms induces vasorelaxation by diffusion to the vascular smooth muscle. The resultant concentration-dependent vasodilator (relaxation) responses to receptor-dependent and receptor-independent stimulators of eNOS, mediated largely by NO, form the basis of bioassay systems designed to quantify the functional state of vascular tissue *in vitro* and *in vivo.*

The generation of NO by vascular endothelium is inhibited by a number of pharmacologic agents. Guanidino-substituted L-arginine analogs such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) are effective inhibitors of eNOS activity, thereby reducing the production and release of NO into the luminal (area of blood flow) and abluminal (perivascular interstitial) compartments. These inhibitors of eNOS are often used in both *in vitro* and *in vivo* experiments to confirm the involvement of NO in physiological situations. For example, vascular ring preparations (Fig. 1) are used to measure vascular tension in small (2–3 mm) segments of vessels excised from the heart and other organs and then placed in chambers containing physiological buffer solutions. This *in vitro* preparation may include NO synthase inhibitors in the buffer to attenuate suspected NO-induced vasorelaxation responses to endothelial-specific agonist stimulators of nitric oxide synthase activity, such as acetylcholine or bradykinin, to distinguish the action of NO from that of other endogenous vasodilator substances such as prostacyclin or adenosine. Hence, these NOS inhibitors are used to ''chemically denudate'' vascular tissue of endothelium, again to describe the role of NO derived from the vascular endothelium.

The NOS inhibitors can also be used in organ chamber bioassay systems to qualitatively estimate the basal release of NO. Because NO is rapidly degraded in biological and *in vitro* environments, it is difficult to measure directly by electrode systems or colorometric methods. However, the concentration-dependent vasorelaxation induced by NO in blood vessels *in vitro* can be used to advantage to provide a useful bioassay by which to indirectly estimate the production of NO by vascular endothelium. A normal coronary artery with significant basal release of NO will accordingly

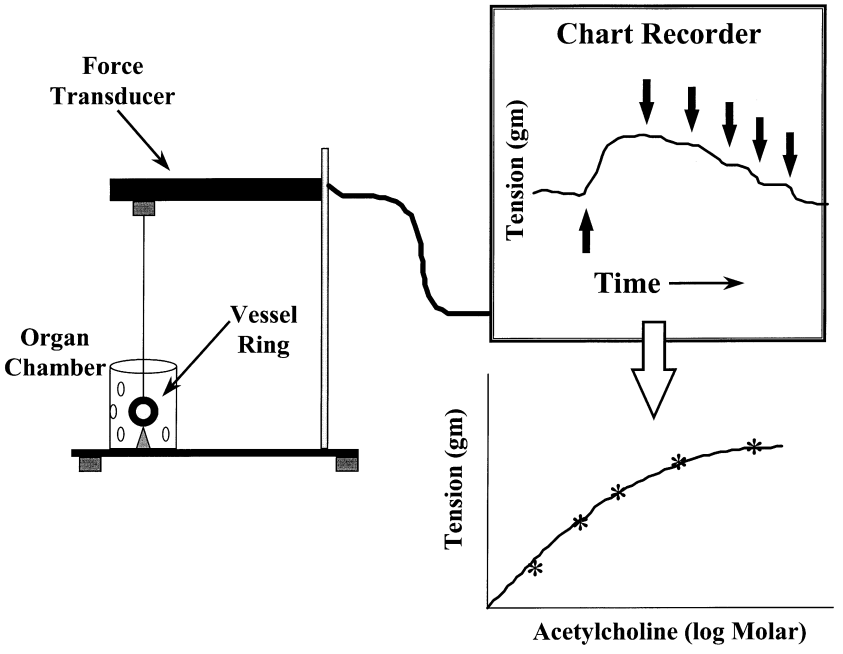


Figure 1 Diagram of the organ chamber technique for measuring endothelial function in segments of coronary artery and vein. The test vessel is placed in an organ chamber filled with a nutritive buffer at 37°C, and it is connected to a force transducer that measures the force generated by the vessel as it contracts or relaxes. Vessels are preconstricted with vasoconstrictor agents to give a background tension from which relaxation can be achieved. The force transducer is connected to a chart recorder or computer to display force changes. Test agonist stimulators of nitric oxide synthase or smooth muscle relaxing agents are added in incremental concentrations (arrows), which causes progressive relaxation. The percent relaxation from the preconstricted force is analyzed using the force–concentration plot shown in the bottom right panel.

demonstrate a vasoconstrictor response that is proportional to the NO-induced vasodilator tone, which is withdrawn by application of NO synthase inhibitors. However, coronary artery endothelium that has sustained injury from stresses such as exposure to superoxide radicals or ischemia–reperfusion will demonstrate substantially attenuated constrictor responses to application of NO synthase inhibitors, which is indicative of an impaired basal release of NO secondary to endothelial injury. Therefore, both the vasorelaxation responses to agonist stimulators of NO and the vasoconstrictor responses to inhibitors of eNOS have been used to indirectly estimate the capacity of vascular endothelium to release NO.

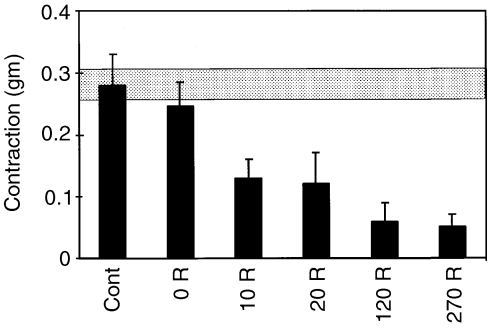


Figure 2 Basal endothelial function described by vasocontraction responses to inhibitors of nitric oxide synthase. In a feline model of ischemia –reperfusion, the left anterior descendary (LAD) coronary artery was occluded for 90 min and reperfused for a total of 270 min. At the various time points indicated on the abscissa, the coronary arteries were excised, cut into rings 2–3 mm in width, and mounted in organ chambers to measure tension. After preconstriction to a target tension, the nitric oxide inhibitor is added to the bath. Coronary artery rings will subsequently constrict in accordance with the withdrawal of NO-dependent vasodilator tone. Cont, normal control (no ischemia); R, minutes of reperfusion. Data adapted from Ma *et al.,* 1993.

Ischemia–Reperfusion Injury Leads to Endothelial Cell Dysfunction

Ischemia and reperfusion attenuate endothelial cell function. This dysfunction is expressed as attenuated basal release of NO (reduced constrictor responses to eNOS inhibitors), reduced vasodilator responses to agonist stimulators of eNOS, and a loss of its ability to prevent neutrophil adherence to the endothelial surface of coronary arteries and veins. In the absence of reperfusion, postischemic endothelial dysfunction is minimal after short-term ischemia (60–90 min) (Ma *et al.,* 1993; Tsao and Lefer, 1990), but it may be expressed after more prolonged periods of ischemia (>90 min) (Dignan *et al.,* 1992). Hence, if a period of either regional ischemia (coronary occlusion) or global ischemia were imposed and the coronary arteries excised before the onset of reperfusion, endothelial function would appear to be near normal. However, in the presence of reperfusion, endothelial dysfunction is expressed within minutes after the start of reflow, and it persists for hours (A. M. Lefer *et al.,* 1991; Tsao *et al.,* 1990) to days (Kaeffer *et al.,* 1996) after reperfusion. This dysfunction is described as a decrease in both basal and agonist-stimulated NO release and vasorelaxation and in adherence of neutrophils to the endothelium. These physiological responses are attenuated in reperfused coronary vessels roughly in proportion to the degree of injury (Dignan *et al.,* 1992; Nakanishi *et al.,* 1994; Tsao *et al.,* 1990; Tsao and Lefer, 1990).

BASAL ENDOTHELIAL FUNCTION

Under normal circumstances, the tonic or basal release of NO causes a continuous degree of vasodilation, the extent of which is dependent on the amount of NO released. This basal NO can be measured using direct methods such as NO-sensitive electrodes or methods based on the Griess reaction and similar colorimetric methods. In addition, the amount of NO release can also be estimated indirectly by observing the change in basal vascular tone when inhibitors of NO synthase activity are administered, as discussed earlier. Nonmetabolized analogs of L-arginine such as L-NAME or L-nitroarginine (L-NA) inhibit NO synthase activity and reduce appreciably the amount of NO released basally (and with agonist stimulators of NO synthase). When these NO synthase inhibitors are administered *in vitro,* the extent of vasoconstriction secondary to withdrawal of NO-induced vasodilator tone gives the investigator a bioassay barometer of this basal NO release. Similarly, when inhibitors are given *in vivo,* the extent of increase in local vascular resistance or the degree of hypertension observed after NO synthase inhibitor administration provides information as to the basal vasodilator tone.

In ischemia–reperfusion, tonic NO release by the coronary vascular endothelium measured directly using *in vitro* techniques is reduced in hearts subjected to ischemia–reperfusion (Engleman *et al.,* 1995a; Guo *et al.,* 1996). Commensurate with this attenuated tonic vasodilator tone, the vasocontraction observed when NO synthase inhibitors are administered *in vitro* in organ chambers is reduced owing to loss of tonic NO-dependent vasorelaxation (Ma *et al.,* 1993). This loss of basal NO-induced vasodilator tone is shown in Fig. 2. Note in Fig. 2 that in normal coronary arteries with healthy endothelium, the administration of an NO synthase inhibitor (L-NAME) to the organ chamber bath caused a brisk vasocontraction. Interestingly, after 90 min of ischemia without reperfusion, this vasocontraction is still essentially the same as in the normal coronary artery. However, with increasing durations of reperfusion, the vasocontraction is progressively lost, suggesting a progressive loss of basally released NO during reperfusion.

In addition to impaired vasocontraction responses to NO synthase inhibitors, basal endothelial function can also be assessed by quantifying the adherence of unstimulated neutrophils on the surface of test coronary arteries or veins. This method takes advantage of the potent antineutrophil effects of NO that is tonically released while segments of vessels are incubated in organ chambers or culture dishes. The adherence of unstimulated neutrophils is prevented by the tonic release of nitric oxide in normal vessels (Fig. 3). After 90

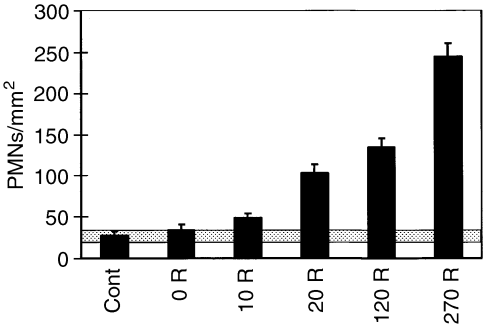


Figure 3 Adherence of unstimulated neutrophils to coronary artery endothelial surface is used as another measure of basal endothelial function. In this case, the LAD coronary artery in a feline model of ischemia–reperfusion was occluded for 90 min and reperfused for a total of 270 min. At the various time points indicated on the abscissa, the coronary arteries were excised, cut into segments and incubated with unstimulated fluorescent labeled neutrophils, and visualized under epifluorescence microscopy. Cont, normal control (no ischemia); R, minutes of reperfusion. Adapted from Ma *et al.* (1993).

min of ischemia, this ability to ward off adherence of unstimulated neutrophils to the endothelial surface is still intact. However, after reperfusion, the ability to prevent neutrophil adherence is increasing compromised with time. Therefore, both the adherence of unstimulated neutrophils to coronary artery endothelium and the loss of basally released nitric oxide (indirectly measured by vasocontraction responses to inhibitors of nitric oxide synthase) have been shown to progressively increase with reperfusion time, but were normal after ischemia alone (Ma *et al.,* 1993).

STIMULATED ENDOTHELIAL FUNCTION

NO synthase activity is stimulated by agonists such as acetylcholine, bradykinin, and substance P in a concentration-dependent manner. To quantify this ability of NO synthase to release NO under agonist stimulation in a bioassay system, coronary vessels (arteries or veins) are cut into segments and mounted in organ chambers, as shown in Fig. 1, and preconstricted with a constrictor agent such as norepinephrine or the thromboxane A_2 mimetic U46619. Sequential addition of an agonist stimulator of NO synthase, such as acetylcholine, in incremental concentrations will elicit increasing degrees of vasorelaxation until the normal vessel relaxes completely. When coronary arteries excised from hearts subjected to regional ischemia without reperfusion are analyzed in this way, relaxation responses to acetylcholine are essentially complete (top left tracing of Fig. 4). This relaxation response to acetylcholine is NO induced, as it can be completely blocked by inhibitors of NO synthase. However, the vasorelaxation is increasingly attenuated after reperfusion, starting as early as 2.5 min. These observations suggest that endothelial dysfunction after ischemia occurs principally during reperfusion, and that this ''reperfusion injury'' is a dynamic process that worsens with time of reperfusion. Attenuated endothelial vasodilator responses to intracoronary

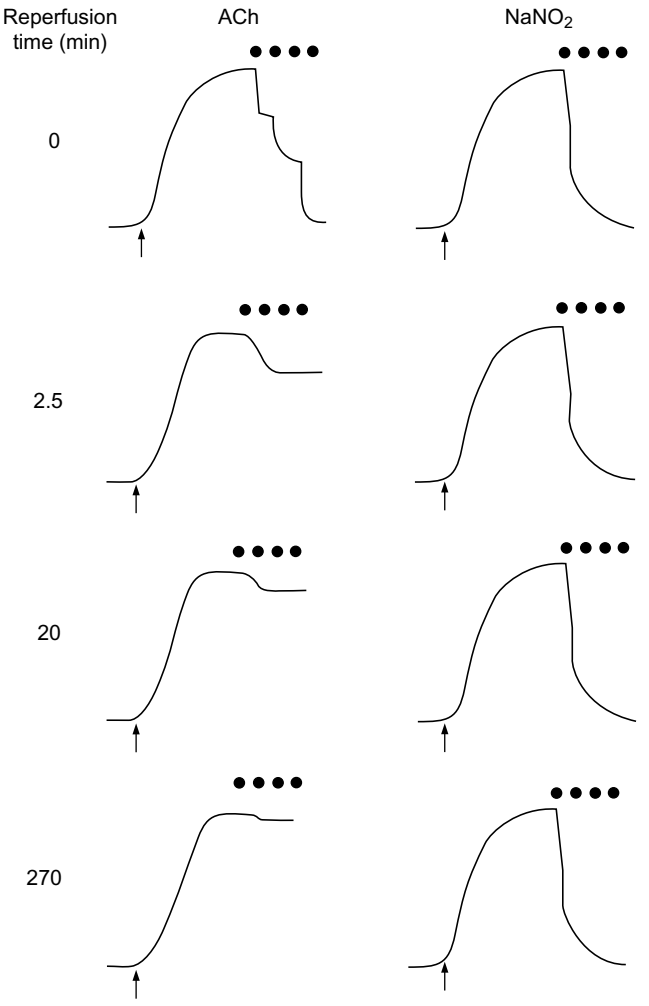


Figure 4 Vasodilator responses of coronary arteries to acetylcholine (left) and the NO donor agent acidified $NaNO_2$ (right) after ischemia and increasing periods of reperfusion. The dots over each tracing indicate application of respective drug. From Lefer and Scalia (1999), with permission.

acetylcholine infusions have also been reported *in vivo* (Sobey *et al.,* 1990) after coronary artery occlusion and reperfusion, and they are consistent with the *in vitro* findings using excised coronary vessels in organ chambers. This endothelial dysfunction after reperfusion is similar to the vasodilator responses to acetylcholine observed in coronary arteries with mechanical denudation of the endothelium. Morphologically, the coronary artery after 1 hour of ischemia appears normal with intact endothelium (VanBenthuysen *et al.,* 1987). After reperfusion, the endothelium remained intact despite the significant loss of vasodilator responses to acetylcholine (VanBenthuysen *et al.,* 1987). Therefore, endothelial dysfunction may involve a defect in the stimulation of eNOS or a biochemical defect in its activity, rather than solely a morphologic injury to the endothelial cell. Endothelial dysfunction, expressed as attenuated relaxation responses of the coronary vascular endothelium to ADP and increased adherence of unstimulated neutrophils, is also observed in coro-

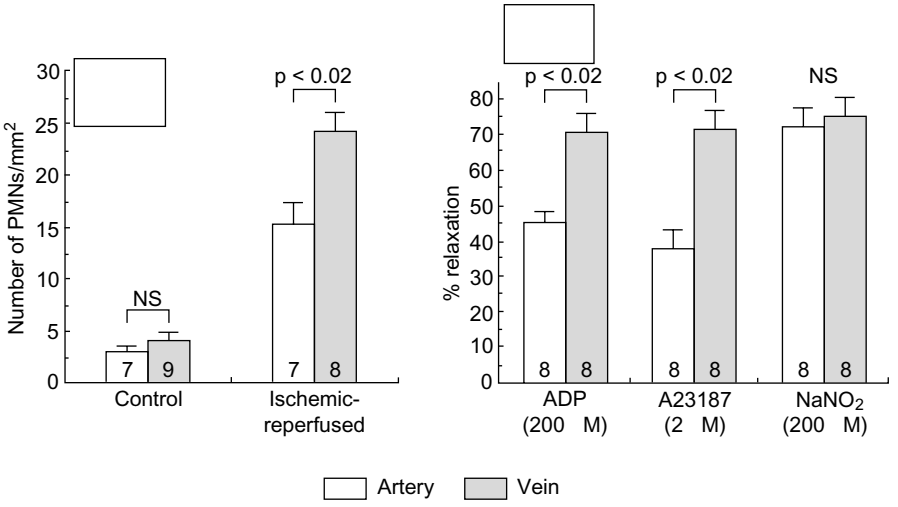


Figure 5 Endothelial dysfunction in coronary veins after 60-min occlusion of the paired coronary artery followed by 120 min of reperfusion. (Left) Adherence of unstimulated canine neutrophils to nonischemic (control) and ischemic-reperfused coronary veins. (Right) vasorelaxation responses of postischemic canine coronary veins to ADP (endothelium-specific stimulator), the calcium ionophore A23187 (endothelial receptor-independent stimulator), and acidified (pH 7.2) $NaNO_2$ (smooth muscle dilator). From Lefer *et al.* (1992), with permission.

nary veins (Fig. 5) (D. J. Lefer *et al.,* 1992). This observation is important because venules in the ischemic–reperfused myocardium may be the site of neutrophil adherence, transendothelial migration into the myocardial tissue, and subsequent interaction with myocytes. Loss of the antineutrophil characteristics of coronary venous endothelium potentially promotes neutrophil interaction.

Endothelial dysfunction similar to that seen in regional ischemia–reperfusion is observed in models of global ischemia. Tsao and Lefer (1990) subjected isolated perfused rat hearts to 20 min of global ischemia by stopping the perfusion of Krebs–Henseleit buffer to the heart. Endothelial function was assessed by the decrease in vascular resistance and perfusion pressure in response to acetylcholine or nitroglycerin when myocardial perfusion flow rate was held constant. Reperfusion with oxygenated (95% O_2 + 5% CO_2) Krebs–Henseleit buffer was accompanied by endothelial dysfunction expressed as a decrease in vasodilator (coronary perfusion pressure) response to acetylcholine. Smooth muscle responses to nitroglycerin were not affected. Similar to the time course of endothelial dysfunction described above for regional ischemia, the extent of endothelial dysfunction was exacerbated with increasing duration of reperfusion in this global ischemic model. The endothelial dysfunction may have been caused by oxygen radicals since other sources of reactive oxygen species (i.e., neutrophils) were absent. Indeed, Tsao and Lefer (1990) noted a significant increase in superoxide radical production specifically from the endothelium, measured by chemiluminescence, in the first moments of reperfusion. Extensive studies have shown that superoxide radicals participate in the pathophysiology of endothelial dysfunction (Lefer and Lefer, 1991) and other end points such as necrosis (Lucchesi, 1994) and contractile dysfunction (Bolli, 1988, 1991). Potential sources of superoxide radicals in this neutrophil-free environment include mitochondria, xanthine oxidase, and NAD(P)H oxidase.

The Role of Neutrophils in Mediating Postischemic Endothelial Dysfunction, Contractile Dysfunction, and Necrosis

Neutrophils have been found to be important mediators of *in vivo* reperfusion injury of the vascular endothelium, with significant consequences to the pathogenesis of necrosis (infarct size), defective regulation of blood flow and its transmural distribution, tissue edema, and contractile dysfunction. Neutrophil adherence to the coronary endothelium induces functional injury (A. M. Lefer *et al.,* 1993; Sato *et al.,* 1996, 1997a). As discussed later, NO is a very potent inhibitor of neutrophil events. Hence, a discussion of neutrophils in the pathogenesis of myocardial ischemia–reperfusion injury is germane to understanding the role of endothelial injury in NO-related therapy in attenuating this injury.

The coronary vascular endothelium plays a critical role in the pathogenesis of reperfusion injury in the myocardium (Boyle *et al.,* 1996; A. M. Lefer *et al.,* 1991, 1993; D. J. Lefer *et al.,* 1992). During the early moments of reperfusion, neutrophils are recruited to the endothelial surface in a requisite interaction between the two cell types. This interaction is mediated by a highly specific and well-orchestrated sequence of events involving the upregulation of adhesion molecules on both the endothelium and neutrophils, as summarized in Fig. 6. The initial event is characterized by skipping or ''rolling'' of the neutrophils along the endothelial surface, a process mediated by interaction between

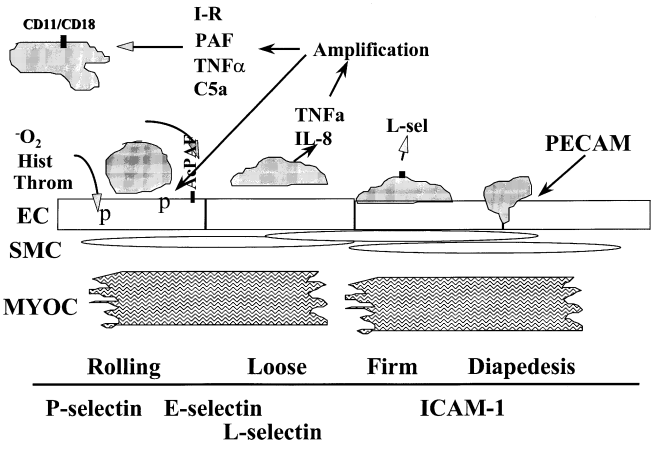


Figure 6 Diagram of neutrophil–endothelial cell interactions during reperfusion with regard to phases of adherence and the principal adhesion molecules involved on the bottom of the figure. Endothelium (EC) can be activated by ischemia–reperfusion (I-R) as well, or products thereof such as superoxide anions (O_2^-) and thrombin (Throm), causing the surface expression of P-selectin (P) from preformed Weibel–Palade bodies. This is the endothelial ''trigger'' phase. Neutrophils are activated by platelet activating factor (PAF), ischemia–reperfusion (I-R), the complement fragment C5a, or other stimulators. Activation of EC and neutrophils attracts the neutrophils toward the EC, to which the neutrophils first ''roll'' [mediated by P-selectin and L-selectin or by P-selectin glycoprotein ligand-1 (PSGL-1)] and later firmly adhere [mediated by intracellular adhesion molecule-1 (ICAM-1) on the endothelium and CD11/CD18 on the neutrophil]. Amplification of the inflammatory response occurs when activated endothelium and neutrophils release oxidants and proinflammatory mediators, which recruits more neutrophils to the site of injury. PECAM, platelet–endothelial cell adhesion molecule; SMC, smooth muscle cells; Hist, histamine; AcPAF, acylated platelet activating factor.

P-selectin on the endothelium and sialylated glycoprotein on the neutrophil, most likely the sialomucin P-selectin glycoprotein ligand-1 (PSGL-1) (McEver and Cummings, 1997; Moore *et al.,* 1995) or sialyl Lewis^x. This initial interaction is an obligatory step necessary for distal firm adherence and transendothelial migration into the myocardial parenchyma and their physiological sequelae (no-reflow, necrosis) (Davenpeck *et al.,* 1994a; Geng *et al.,* 1990; Jerome *et al.,* 1994; Kubes *et al.,* 1995). E-selectin on the endothelium may also interact with a sialylated glycoprotein (sialyl Lewis^x) to facilitate neutrophil rolling. After this initial loose tethering of neutrophils to the vascular endothelium mediated by P-selectin, firm adherence is facilitated by interaction of intracellular adhesion molecule-1 (ICAM-1) on the endothelium with its counterligand CD11b/CD18 on neutrophils. ICAM-1 is constitutively expressed on the endothelial surface, but is upregulated after 2 to 4 hours of reperfusion by cytokines.

After this cascade has led to firm adherence on the endothelium, the neutrophils emigrate through the endothelial layer (transendothelial migration) and accumulate in the interstitial compartment of the ischemic–reperfused myocardium. The transendothelial migration, like the adherence process itself, occurs primarily after the onset of reperfusion, but at a later stage (~4 hours) (Dreyer *et al.,* 1991a; Entman *et al.,* 1991; A. M. Lefer *et al.,* 1993). Dreyer *et al.* (1991a) have shown that the rate of neutrophil accumulation within the reperfused area occurs most rapidly within the first hour of reperfusion, which may represent primarily intravascular neutrophils adherent to the endothelium rather than interstitial neutrophils. Lefer *et al.* (1991) have reported a similar time course using tissue myeloperoxidase activity as a marker of neutrophil accumulation. Furthermore, Lefer *et al.* (1993) have shown that this accumulation of neutrophils in ischemic tissue is preceded first by adherence of neutrophils to the coronary vascular endothelium, followed by accumulation in the area at risk most likely secondary to transendothelial migration. The adherence of neutrophils is closely associated with the time course of endothelial dysfunction in both surgical (Byrne *et al.,* 1992; D. J. Lefer *et al.,* 1993a; Nakanishi *et al.,* 1995; Sato *et al.,* 1995a; Wilson *et al.,* 1993) and nonsurgical ischemia–reperfusion models.

The critical factor that allows neutrophils to interact with the endothelium is the stimulation of the endothelium during early reperfusion to express certain adhesion molecules. These early events act as the endothelial ''trigger'' of the inflammatory cascade. First, the endothelium releases a burst of superoxide radicals (Tsao and Lefer, 1990; Zweier *et al.,* 1987) in the first few minutes of reperfusion. This burst of superoxide radicals not only activates the endothelium and stimulates the expression of adhesion molecules involved in the neutrophil–endothelial cell cascade (McIntyre *et al.,* 1995), but also directly quenches NO through a biradical reaction (Gryglewski *et al.,* 1986; Rubanyi and Vanhoutte, 1986), leading to a decrease in the periendothelial concentration of NO. Second, the endothelium is activated by inflammatory mediators such as TNF-α, interleukin 6 (IL-6), thrombin, histamine, LTB_4, and complement fragments released either locally or into the systemic circulation. Complement fragments such as C3a and C5a are found in the lymph drained from the area at risk during reperfusion (Dreyer *et al.,* 1989), and they are produced locally in the area at risk (Pinckard *et al.,* 1980). Third, the interaction between neutrophils and endothelium stimulates the neutrophils directly, thereby triggering a release of reactive oxygen species (superoxide anion, hydrogen peroxide, hydroxyl radical, hypochlorous acid) and inflammatory mediators. This second phase is an amplification stage that causes further endothelial injury and increased recruitment of neutrophils to the site of injury subsequent to the initial triggering event.

Neutrophils have been shown to directly cause injury to basal and stimulated function of the endothelium. Administration of thrombin to a coincubate of neutrophils and coronary artery rings with healthy endothelium in organ chambers causes contraction of the artery, not only because of neutralization of NO by superoxide anion, but also because of impaired release of nitric oxide (Fig. 7A). Thrombin selectively stimulates the endothelium by triggering the surface expression of P-selectin, but it has no direct effect on neutrophil activation. The degree of vasocontraction (an estimate of basal NO release) is paralleled by the number of

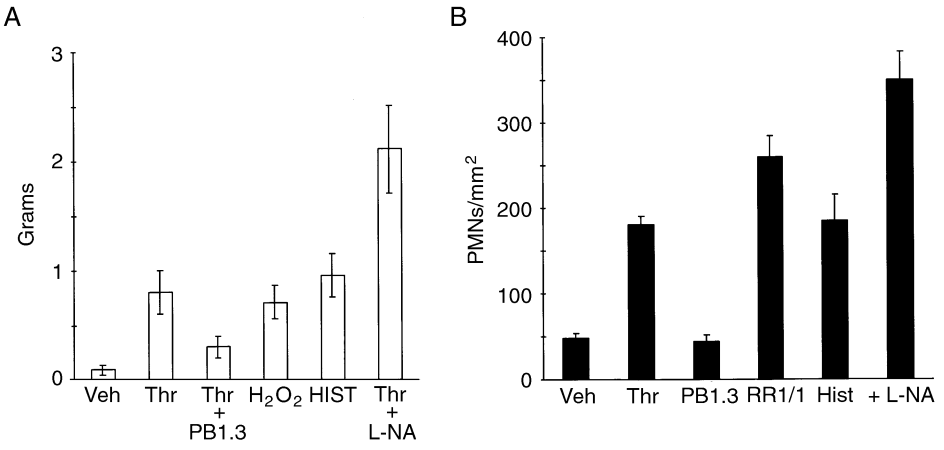


Figure 7 Neutrophil-induced vasocontraction and adherence to canine coronary artery segments. (A) Vasocontraction is measured in grams tension in organ chambers. Thrombin (2 U/ml) increases the vasocontraction response induced by unstimulated neutrophils when they are added to the bath. This vasocontraction is reversed by P-selectin antibody PB1.3, suggesting that adherence of neutrophils to endothelium is requisite for the induction of contraction. Other stimulators such as H_2O_2 and histamine stimulate the endothelium to increase adherence of unstimulated neutrophils, again resulting in vasocontraction. Blockade of basal NO release with L-NA increases neutrophil-induced vasocontraction, suggesting that this tonically released NO has important antineutrophil effects. (B) Adherence of neutrophils to thrombin-stimulated canine coronary artery endothelium. The similarity to the pattern in A suggests that adherence of neutrophils is a necessary step in producing the vasocontraction. Reprinted from *Cardiovascular Research,* Volume 43, J. E. Jordan, Z.-Q. Zhao, and J. Vinten-Johansen, The role of neutrophils in myocardial ischemia–reperfusion injury, pp. 860–878, Copyright 1999, with permission from Elsevier Science.

neutrophils adherent to the coronary artery endothelium (Fig. 7B). In addition, the vasocontraction responses could be attenuated by an antibody to P-selectin, PB1.3, demonstrating the dependence of P-selectin-mediated interaction on subsequent events leading to vascular endothelial injury. Furthermore, the degree of vasocontraction after coincubation of neutrophils with endothelium is accentuated by the NO synthase inhibitor L-nitroarginine (L-NA), again associated with an increase in neutrophil adherence.

Figure 8 shows endothelial responses to acetylcholine (endothelium-dependent stimulator) and sodium nitroprusside (endothelium-independent agonist) after incubation of unstimulated neutrophils with coronary thrombin-stimulated arterial rings. Thrombin treatment in the absence of neutro-

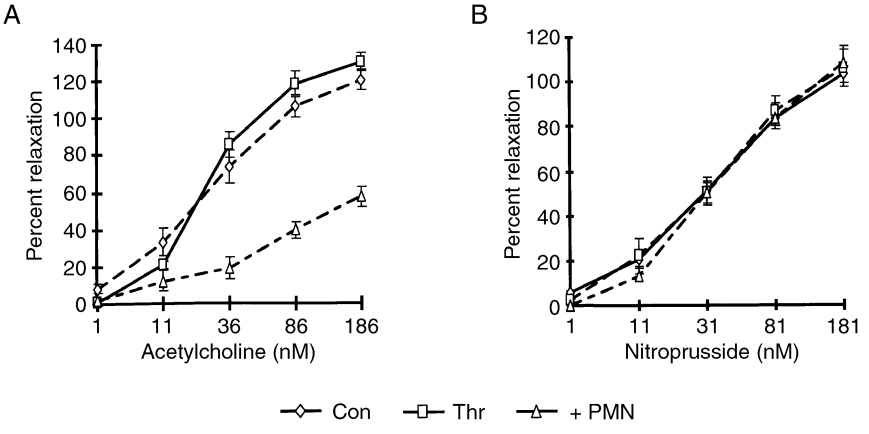


Figure 8 Vasorelaxation response to incremental concentrations of acetylcholine (A) and sodium nitroprusside (B) in thrombin-stimulated canine coronary artery rings coincubated with unstimulated neutrophils. The coronary arteries were preconstricted with the thromboxane A2 mimetic U46619. Con, unstimulated coronary arteries; Thr, thrombin stimulated coronary arteries without neutrophils; + PMN = incubation with unstimulated neutrophils. Reprinted from *Cardiovascular Research,* Volume 43, J. E. Jordan, Z.-Q. Zhao, and J. Vinten-Johansen, The role of neutrophils in myocardial ischemia–reperfusion injury, pp. 860–878, copyright 1999, with permission from Elsevier Science.

phils induces no discernible alterations in agonist-stimulated vasorelaxation responses. In contrast, coincubation of neutrophils with thrombin-stimulated coronary artery endothelium is associated with a significant decrease in the concentration–response vasorelaxation curve, with a characteristic decrease in maximal response to the highest concentration of acetylcholine used. The vasorelaxation responses to the endothelium-dependent, receptor-independent agent A23187 (not shown) demonstrated similar attenuated vasorelaxation responses, suggesting that the defect was distal to the muscarinic receptor. In contrast, vasorelaxation responses to the direct smooth muscle dilator sodium nitroprusside were complete in all treatments. Taken together, these data suggest that neutrophils induce endothelium-specific damage to receptor-dependent and receptor independent vasodilator function.

Endothelial Injury and Infarction

The coronary vascular endothelium is essentially the ''front line'' defense system for the myocardium. The vascular endothelium is strategically located between the intravascular (blood) compartment, with its neutrophils and circulating inflammatory mediators and the underlying myocardial parenchyma. Accordingly, NO is released into the critical locations necessary to interdict the strategic participants in the inflammatory cascade of ischemia–reperfusion injury. NO diffuses into the abluminal side in the proximity of the vascular smooth muscle, into the interstitial compartment where it is in close proximity to myocytes, and into the intravascular compartment where it exerts important physiological effects on neutrophils, endothelium, and platelets. As we discussed earlier, the endothelium is a critical factor in the neutrophil component of pathophysiological responses to ischemia–reperfusion. In addition, it is well known experimentally and clinically that ischemia–reperfusion causes injury to structures other than the endothelium. The myocyte is an obvious target of ischemia–reperfusion injury, but other targets of injury include the microvasculature and the genetic apparatus. The various manifestations of ischemia–reperfusion injury in the heart are discussed next.

ENDOTHELIAL INJURY

Endothelial injury occurs within minutes of reperfusion, and it is thought to be a critical factor in triggering and amplifying injury to the myocyte in terms of viability (necrosis) and function (contraction). Hence, there is a close association between the endothelium and myocyte in models of irreversible ischemia–reperfusion injury. However, in less severe models of ischemia causing myocardial stunning in which necrosis does not occur, therapeutic strategies toward reducing endothelial dysfunction may not predict the benefits to postischemic contractile function. In a model of 30-min left anterior descending (LAD) occlusion causing contractile dysfunction (Bufkin *et al.,* 1998), Thourani *et al.* (1999) observed that ischemic preconditioning reduced endothelial dysfunction (vasoreactive responses to acetylcholine) but failed to improve postischemic systolic or diastolic function in the involved myocardium. More relevant to cardiac surgery using cardioplegia, Mizuno *et al.* (1997) reported that 20 min of normothermic global ischemia followed by unsupplemented blood cardioplegia was associated with no significant postischemic systolic (pressure–volume relations and Starling function curves) or diastolic dysfunction. However, there was significant endothelial dysfunction. Hence, endothelial dysfunction can occur in the absence of either contractile dysfunction or necrosis in nonlethal models of ischemia–reperfusion injury. In the study by Mizuno *et al.* (1997), 2 m*M* L-arginine in the blood cardioplegia solution, a concentration sufficient to increase transcardiac nitrate/nitrite production suggestive of increased NO production, reduced postischemic endothelial dysfunction, which was reversible with the NO synthase inhibitor L-NAME. Therefore, endothelial dysfunction may occur in the absence of contractile dysfunction in models of nonlethal injury, and recovery of endothelial function (with NO therapy or other cardioprotective therapies) does not always imply recovery of contractile function.

MYOCYTE NECROSIS

That ischemia–reperfusion injury causes myocardial necrosis of myocytes is well known. Necrosis is defined as ''accidental'' cell death involving a failure to generate high-energy phosphate fuel in the form of ATP, cell swelling secondary to impaired function of the ionic pumps of the cell and consequent gain in intracellular water, intracellular accumulation of calcium, and a terminal disintegration of the cell membrane with release of cell contents into the pericellular region. The latter event provides a leukotactic signal that precipitates an inflammatory response by neutrophils and macrophages, which are in turn recruited to the site of injury to ''mop up'' cell debris. These neutrophils and macrophages are programmed to attack myocytes that have been ''marked'' for destruction by the expression of certain adhesion molecules on myocytes, which act as ligands allowing attachment of the inflammatory cells. Myocytes express certain adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and endothelial–leukocyte adhesion molecule-1 (ELAM-1), that allow neutrophils to attach by CD18-dependent mechanisms (Bullough *et al.,* 1996; Smith *et al.,* 1991; Youker *et al.,* 1992) and stimulate a burst of oxygen radicals which then damages or kills the myocyte (Entman *et al.,* 1992). The expression of adhesion molecules on the cell surface of myocytes is facilitated by exposure of the cells to circulating cytokines such as IL-6, TNF-α, and complement fragments. In addition, the release of chemokines such as macrophage inflammatory protein-2 (MIP-2) and monocyte chemotactic protein (MCP) (Massey *et al.,* 1995) directly stimulate the recruitment of leukocytes to sites of ischemia–reperfusion injury. Hence, neutrophil activation at the endothelial surface ultimately leads to the movement through the endothelium (endothelial transmigration) into the interstitial compartment, facilitated by chemotactic stimuli released by myocytes, where direct contact to

the myocytes leads to damage or cell death. However, sublethal damage or frank necrosis to myocytes does not depend wholly on direct contact with inflammatory cells. Cytokines and oxygen radicals released from neutrophils at the endothelium may lead to necrosis by directly affecting underlying myocytes or by inducing severe injury to the microvasculature, thereby precipitating a no-reflow state with subsequent secondary ischemia.

Nitric Oxide Therapy to Reduce Ischemia–Reperfusion Injury

Direct Scavenging of Superoxide Radicals by NO

Both NO and superoxide anions are radical species. Both species of radicals can react with biological tissues to generate products of oxidation. However, these reactive species can neutralize each other in a biradical reaction with a 1:1 stoichiometry that is essentially diffusion limited. The reaction proceeds at a rate of $6.7 \times 10^9\ M^{-1}\ s^{-1}$, which exceeds the reaction rate between superoxide anions and superoxide dismutase by a factor of approximately three. In fact, the reaction of NO with superoxide radicals outcompetes the endogenous superoxide dismutases present in the tissue. In addition, the reaction is essentially irreversible due to its highly exothermic nature, with the release of 22 kcal/M. Because of the equimolar stoichiometry of the reaction, either substrate can be rate limiting, depending on its availability, but conversely an increase in one substrate can overwhelm and quench the physiological effects of the other (Rubanyi and Vanhoutte, 1986). Hence, either superoxide radical or NO is a factor in determining the *in vivo* concentration and physiological actions of the other radical species. Under normal conditions, the production of superoxide radicals is limited, although NO is produced tonically at relatively higher (low nanomolar) concentrations (Kelm and Schrader, 1990). However, the production of both superoxide and NO is greatest during early reperfusion following a period of ischemia, and both molecules are placed within the same proximity in and adjacent to the vascular wall, making their interaction and coneutralization likely. On the one hand, this biradical neutralization reaction reduces the concentration of a potentially harmful oxidant derived from several sources, including activated neutrophils and activated endothelium. On the other hand, this reaction reduces the biological availability of NO and attenuates its physiological effects (Gryglewski *et al.,* 1986; Rubanyi *et al.,* 1989). The administration of superoxide dismutase to vascular ring preparations stimulated by inflammatory mediators will produce increased tonic vasodilator tone as a result of increased availability of NO. Peroxynitrite ($ONOO^-$, the major reaction product of this biradical reaction between NO and superoxide radicals, has potent oxidant actions and is more stable than either of its two reactants. The important physiological effects of $ONOO^-$ impacting on salvage or injury induced to the vascular wall and the myocardium will be discussed later.

Effects of NO on the Coronary Vascular Endothelium

The ability of nitric oxide to affect multiple points (neutrophil activation, endothelial activation) in the neutrophil–endothelial cell adhesion process makes it a potent therapeutic agent in the treatment of the inflammatory component of ischemia–reperfusion injury leading to necrosis and contractile dysfunction. At nanomolar concentrations that do not produce vasodilation, NO attenuates the expression of certain key adhesion molecules, notably E-selectin and ICAM-1 on the endothelium and CD11/CD18 on the neutrophil (De-Caterina *et al.,* 1995; Ohashi *et al.,* 1997). In addition, NO inhibits superoxide anion production from the endothelium by direct action in the NADPH oxidase system (Clancy *et al.,* 1992). By acting on the expression of adhesion molecules on endothelial cells and neutrophils, NO inhibits the proximal steps in the pathogenesis of postischemic injury, thereby truncating subsequent pathways distal to the point of interdiction.

As discussed earlier, the expression of selectin (P-selectin) and members of the immunoglobulin superfamily [ICAM-1, vascular cell adhesion molecule (VCAM-1)] is increased after ischemia (Weyrich *et al.,* 1995), which has been associated with increased adhesion of neutrophils to the endothelium, an impaired release of NO by the vascular endothelium, and increased tissue damage after myocardial (Sluiter *et al.,* 1993; Weyrich *et al.,* 1993) or intestinal (Carden *et al.,* 1993) ischemia–reperfusion and other inflammatory-type conditions (Foreman *et al.,* 1994). Kubes *et al.* (1991) reported that inhibition of NO synthase activity with L-NAME increased PMN adherence to feline mesenteric postcapillary venules, measured by intravital microscopy, suggesting that endogenous NO modulates neutrophil–endothelial cell interactions. Because P-selectin is upregulated during reperfusion in association with a decrease in NO production by the endothelium, a regulatory connection between NO and the mechanism of P-selectin surface expression on the vascular endothelium was suggested.

In the heart, Weyrich *et al.* (1993) showed that P-selectin was increased within minutes after the onset of reperfusion, and that immunoneutralization of this adhesion molecule with a monoclonal antibody attenuated postischemic injury. A subsequent study by Davenpeck *et al.* (1994b) demonstrated that superfusion of the feline mesenteric microcirculation with L-NAME attenuated NO generation and increased PMN adherence to the venular endothelial surface in conjunction with an increased surface expression of P-selectin. These physiological responses were reversed by the coadministration of L-arginine and a cGMP analog (8-bromo-cGMP), thereby implicating NO as the active molecule. Importantly, NO inhibits the surface expression of these key adhesion molecules. Conversely, blockade of NO production with L-NAME or other NO synthase inhibitors increases the surface expression of these adhesion molecules. Hence, NO may be involved in regulating the expression of adhesion molecules and the subsequent reduction in adhesion molecule-dependent tissue injury in response to

ischemia–reperfusion. The loss of NO generation secondary to reperfusion injury may be a major factor governing the increased surface expression of adhesion molecules on endothelium and neutrophils at this time.

The mechanism of adhesion molecule downregulation by NO has been investigated. Armstead *et al.* (1997) demonstrated that NO donor agents or L-arginine attenuated the expression of P-selectin protein and mRNA in cultured human iliac artery and vein endothelial cells. Conversely, the NOS inhibitor L-NAME increased the expression of P-selectin on these endothelial cells. Furthermore, DeCaterina *et al.* (1995) reported that NO donors downregulated the expression of VCAM protein on endothelium as well as mRNA for both ICAM-1 and VCAM. Attenuation of the transcription and translation of these adhesion molecules was linked to a decrease in TNF-α-stimulated activation of NF-κB, a transcription factor that promotes or signals the synthesis of adhesion molecules by turning on the target gene after translocation of the nuclear factor from the cytosol to the nucleus. This same decrease in NF-κB activity was not present in endothelium exposed to cGMP, glutathione, or nitrite (DeCaterina *et al.,* 1995), implying a direct action by NO. In summary, these data suggest that NO attenuates not only the surface expression of these adhesion molecules, but also the signaling processes involved in the transcription and translation of new adhesion molecule proteins. This important action of NO has the potential to decrease the adherence of neutrophils to endothelium and their ultimate accumulation in the myocardium following ischemia and reperfusion in the early phases (minutes) as well as over the longer term requiring *de novo* synthesis.

Therapy to Increase Endogenous Nitric Oxide

Endogenous NO participates in the tonic modulation of various postischemic physiological processes including electrophysiological abnormalities and myocardial injury (i.e., infarction). In view of the important modulation of postischemic injury by NO, tonically released NO may provide endogenous cardioprotection.

In vivo, Pabla and Curtis (1996) reported that the reduction of endogenous NO generation by L-NAME in isolated perfused rabbit hearts increased the incidence of reperfusion-induced ventricular fibrillation, which was reversible by coinfusion with L-arginine, thereby ascribing a beneficial electrophysiological effect to NO. Furthermore, the study by Williams *et al.* (1995) using an *in vivo* rabbit model demonstrated that the systemic administration of the eNOS inhibitor L-NA either before ischemia or just prior to reperfusion increased infarct size (51 ± 2% and 49 ± 3% of area at risk, respectively, compared to 27 ± 2% of area at risk in control). In agreement with the study of Williams *et al.* (1995), other studies have reported a reduction in the time to ischemic contracture in globally ischemic hearts (Pabla and Curtis, 1996), a decrease in postischemic contractile recovery (Pabla *et al.,* 1996), and an increase in infarct size (Hoshida *et al.,* 1995) with NOS inhibition, suggestive of withdrawal of NO-mediated cardioprotection. Reductions in contractile recovery and infarct size were associated with a concomitant increase in neutrophil accumulation. These effects on arrhythmias, contractile dysfunction, and infarction were reversed by coinfusion of L-arginine. These data suggest a cardioprotective role for tonically released NO that, when NO is withdrawn, exacerbates postischemic injury.

In contrast to these reports of beneficial effects of endogenous NO, a number of studies have shown that NO actually increases postischemic arrhythmias, contractile dysfunction, and infarction. This notion that NO increases postischemic injury, by extension, would suggest that blockade of NOS activity would reduce postischemic damage. Accordingly, Woolfson *et al.* (1995a) reported that the L-arginine analog L-NAME reduced infarct size in an *in situ* rabbit model, which seemed to support a deleterious role for NO. In addition, Schultz and Wambolt (1995) demonstrated that the cardioprotective effects resulting from NOS inhibition with L-NAME was abolished by coadministration of L-arginine but not by D-arginine. In contrast, Sun and Wainwright (1997) reported that neither the NOS inhibitor L-NAME nor the NO donor agent C87-3754 altered the incidence and severity of ventricular arrhythmias during a 30-min period of coronary artery occlusion in anesthetized rats. Hence, these data would suggest that endogenous NO is deleterious in myocardial ischemia–reperfusion.

The discrepancy between the reported beneficial effects versus the deleterious effects of endogenous NO on postischemic arrhythmias and infarct size is not fully resolved, but it may involve several factors. The differences in observed effects may be due, in part, to the choice of L-arginine analog. Methylated analogs demonstrate muscarinic-like antagonistic effects not observed with nonmethylated analogs. In addition, L-NAME and other NO synthase inhibitors may induce severe vasoconstriction and ischemia when administered in high concentrations, so that more severe ischemia occurs with these inhibitors, or vasoconstriction prevents full reperfusion and a persistent ischemia which would increase the degree of injury. The reduction in postischemic damage reported with NOS inhibition may be due to a compensatory release of the cardioprotective autacoid adenosine due to myocardial ischemia (Patel *et al.,* 1993a; Woolfson *et al.,* 1995b). In the study by Patel *et al.* (1993b), ischemia induced by NOS inhibition (and presumably to reduced NO release) is supported by an increase in lactate release (Patel *et al.,* (1993b). Therefore, the cardioprotection may have been related to the effects of adenosine (Patel *et al.,* 1993b) released by ischemic myocardium rather than to the reduced NO levels (which were not measured in this study).

Endogenous NO can be therapeutically augmented by administering the precursor for NO, L-arginine. Although L-arginine appears in the blood in sufficient concentrations to saturate NO synthase, supplemental L-arginine has been reported to increase NO release (directly or indirectly measured) by the coronary vascular endothelium (Engelman *et al.,* 1995a; Guo *et al.,* 1996; Palmer *et al.,* 1988). In isolated rat aortic endothelial cells, 1 m*M* L-arginine increased NO

generation by approximately 40% above basal levels (Guo *et al.,* 1996). The nonmetabolized enantiomer D-arginine, at the same concentration, did not increase tonically released NO. Hence, the provision of L-arginine stimulates a concentration-dependent production of NO.

The cardioprotection from reperfusion injury observed with L-arginine may be derived from the potent antineutrophil effects of NO. L-Arginine was reported by Sato *et al.* (1996) to significantly inhibit neutrophil adherence to coronary artery endothelium when stimulated with platelet activating factor (PAF). PAF increased the adherence to endothelium fourfold, whereas 10 m*M* L-arginine attenuated this increased adherence to control levels (Fig. 9). L-NA reversed the inhibitory effects of L-arginine, whereas equimolar concentrations of D-arginine failed to reduce neutrophil adherence, both interventions indicating an NO-mediated mechanism of attenuated neutrophil adherence. Interestingly, L-arginine had no direct effect on neutrophil generation of superoxide radicals when stimulated by PAF, suggesting that the effects of L-arginine were on the adherence process, rather than other processes involved in the inflammatory-like responses (i.e., stimulation of P-selectin expression on endothelium). The concentration of L-arginine found to be effective in reducing neutrophil-mediated effects on the coronary vascular endothelium was between 3 and 10 m*M* (Nakanishi *et al.,* 1992; Sato *et al.,* 1995b; Weyrich *et al.,* 1992), whereas greater concentrations of L-arginine have been associated with myocardial or endothelial injury.

The above *in vitro* data suggested that L-arginine may be useful in limiting endothelial dysfunction and infarction secondary to ischemia–reperfusion *in vivo.* In models of regional ischemia, intravenous (Weyrich *et al.,* 1992) or intracoronary (Nakanishi *et al.,* 1992) supplementation with L-arginine at the time of reperfusion significantly decreased both postischemic coronary artery endothelial dysfunction and infarct size. In a study by Weyrich *et al.* (1992), using a feline model of 90 min of LAD occlusion and 270 min of reperfusion, the intravenous infusion of L-arginine significantly reduced infarct size, which was associated with a decrease in neutrophil accumulation in the area at risk and with a reduction in endothelial function in the ischemic–reperfused coronary artery. In neither of the cited studies (Nakanishi *et al.,* 1992; Weyrich *et al.,* 1992) was there a significant hemodynamic effect (hypotension) of L-arginine infusion. In the study by Nakanishi *et al.* (1992), 10 m*M* L-arginine was infused in the LAD coronary artery at the onset of reperfusion. Infarct size in the L-arginine group was reduced from 35 ± 2% of the area at risk to 18 ± 3% (Fig. 10A), whereas infarct size was actually increased in a group receiving equimolar concentrations of D-arginine (infarct size 49 ± 5% of area at risk), confirming that the infarct-sparing effect of L-arginine likely occurred by the L-arginine–NO synthase pathway, without a significant metabolic or other effect of L-arginine unrelated to NO. Consistent with the antineutrophil effect of endogenously released NO, there was less neutrophil accumulation in the area at risk with L-arginine treatment at reperfusion than in vehicle or D-arginine treated groups (Fig. 10B). Neither postischemic regional contractile function nor postischemic myocardial blood flow was improved with L-arginine treatment in this study. However, postischemic endothelial vasorelaxation responses to acetylcholine were significantly better in the L-arginine-treated group compared to both the vehicle group and the D-arginine group (Fig. 10C). Hence, the cardioprotection observed with L-arginine is due to increased NO release and to an NO-related reduction in neutrophil-mediated injury.

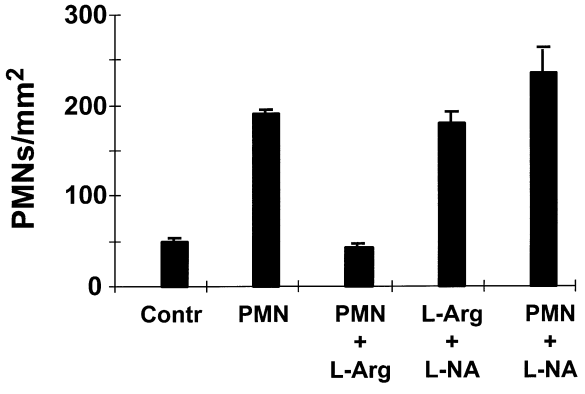


Figure 9 The effects of L-arginine on adherence of fluorescently labeled canine neutrophils to normal (control) or thrombin-stimulated coronary artery segments detected by epifluorescence microscopy. Stimulation with 2 U/ml of thrombin increased neutrophil (PMN) adherence fourfold over unstimulated coronary artery endothelium. L-Arginine (10-m*M*) decreased adherence to control levels, which was reversed by the nitric oxide synthase inhibitor L-NA. L-NA alone tended to increase neutrophil adherence above thrombin stimulation alone (PMN group) by inhibition of basal NO release.

The relatively small infarct-sparing effect of L-arginine reported in the study by Nakanishi *et al.* (1992) raised the question of whether maximal cardioprotection was achieved with the dose of intracoronary L-arginine used, or whether ischemia limited the ability of the endothelium to generate NO in the presence of enhanced concentrations of L-arginine. Damaged endothelium has a defect either in receptor signaling mechanisms of stimulating eNOS or in the activity of the enzyme system itself (Giraldez *et al.,* 1997). Hence, the responses to enhanced concentrations of L-arginine may likewise be limited, thereby reducing the concentration-dependent generation and release of NO. In addition, too high a concentration of L-arginine may be accompanied by increased neutrophil-mediated damage and tissue injury. Further investigation is needed on the association between dose of L-arginine used and the limitation imposed by endothelial injury on the extent of cardioprotection achieved by precursor therapy.

Exogenous NO Therapy

The potential limitation of damaged coronary endothelium to generate cardioprotective quantities of NO may potentially be overcome by the exogenous administration of

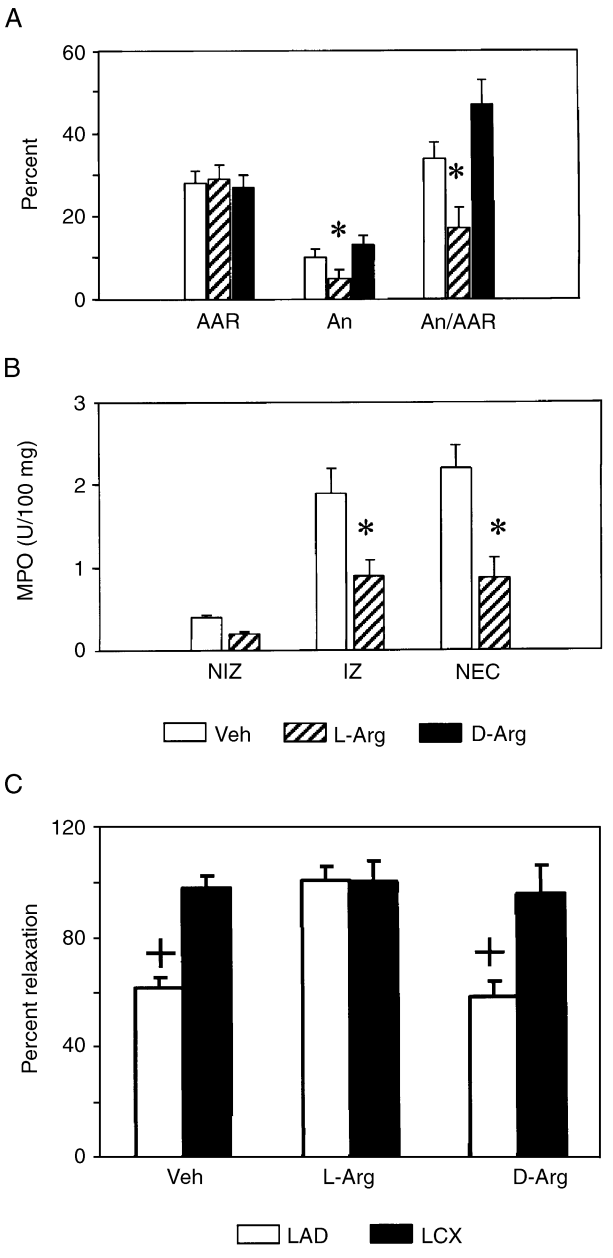


Figure 10 Intracoronary L-arginine during reperfusion on infarct size (A), neutrophil accumulation measured by tissue myeloperoxidase activity (B), and relaxation responses of ischemic–reperfused (60 min followed by 270 min, respectively) LAD coronary artery to acetylcholine (C). Veh, vehicle-treated group; LCX, left circumflex coronary artery; +, $p < 0.05$ versus Lcx responses in A; *, $p < 0.05$ versus Veh in B and C. From Vinten-Johansen *et al.* (1999), with permission.

agents that donate NO *in vivo*. The exogenous approach to NO therapy using NO donors administered only at the onset of reperfusion was first reported by Johnson *et al.* (1990) in a feline model of regional myocardial ischemia–reperfusion (90-min LAD occlusion followed by 4.5 hours of reperfusion) using subvasodilator concentrations of intravenous acidified (pH 7.2) $NaNO_2$ (12.5–50 m*M*/kg/hour) as an NO donor agent or authentic nitric oxide gas dissolved in saline to achieve local concentrations approximating 2–10 n*M* (Johnson *et al.,* 1991). Both forms of NO therapy decreased infarct size by approximately 75%, concomitant with a similar reduction in plasma creatine kinase activity. The reduced infarct size was associated with decreased neutrophil accumulation in the area at risk measured by tissue myeloperoxidase activity. These data suggest that NO therapy introduced at the time of reperfusion reduced myocardial infarction through an antineutrophil mechanism. Subsequent studies have demonstrated similar cardioprotective effects of various classes of organic nitrates that release NO readily (D. J. Lefer *et al.,* 1993b; Pabla *et al.,* 1996; Siegfried *et al.,* 1992a) and of nitrosylated molecules (Delyani *et al.,* 1996) in models of *in vivo* coronary occlusion and reperfusion. The organic NO donor agents include the sydnonimines, the cysteine-containing donors, and the nonoates. The organic donors release NO either spontaneously or after bioconversion of the parent molecule. Nitroglycerin is the classic prototype NO donor agent, but it is a poor NO donor because bioconversion is required by a cysteine-containing enzyme that is partially depleted in the microvasculature after ischemia–reperfusion. In addition, tolerance to the classic nitrates develops with prolonged exposure to the agent. Other organic NO donor agents, such as the early prototype sydnonimines [e.g., 1,3-morpholina-syndnonimine hydrochloride (SIN-1)], release superoxide concomitantly with NO, thereby providing the substrates and the close proximity required to generate $ONOO^-$ via a biradical reaction.

A cysteine-containing organic donor compound, SPM-5185 [(*N*-(3-hydroxy-pivaloyl)-*S*-(*N*'-acetylalanoyl)-L-cysteine ethyl ester] (Schwarz Pharma, Monheim, Germany), readily releases NO spontaneously (D. J. Lefer *et al.,* 1993b,c). This NO donor agent was tested in a canine model of LAD occlusion and reperfusion in which infarct size, neutrophil accumulation, and postischemic endothelial function in ischemic–reperfused and nonischemic coronary arteries were measured. SPM-5185 was administered by intracoronary infusion (500 n*M*) during reperfusion after 1-hour LAD occlusion. SPM-5185 reduced infarct size from 47.5 ± 9% to 14.5 ± 4% of the area at risk, which was paralleled by a similar decrease in plasma creatine kinase activity. In addition, the infarct reduction was associated with a 58% reduction in neutrophil accumulation (tissue myeloperoxidase activity) in the area at risk (D. J. Lefer *et al.,* 1993c). The nonactive form of the compound, SPM-5267, had no effect on any of the physiological end points measured. Similar cardioprotection has been achieved with other NO donor agents introduced by intracoronary infusion at the onset of reperfusion (Pabla *et al.,* 1995).

In addition to the intracoronary route of delivery, the intravenous route has also been shown to be effective. Siegfried *et al.* (1992a,b) administered SPM-5185 (Siegfried *et al.,* 1992b) or the other organic donor agents SIN-1 and C87-3754 (Siegfried *et al.,* 1992a) intravenously in a feline model of LAD occlusion and reperfusion with similar degrees of infarct reduction and attenuation of postischemic endothelial dysfunction in the occluded–reperfused LAD coronary artery. In contrast to L-arginine, these NO donor agents have

been reported to directly attenuate superoxide radical production (measured by cytochrome *c* reduction) and attenuate adherence of neutrophils to coronary artery endothelium.

Whether the cardioprotection afforded by NO therapy is predominantly due to inhibition of neutrophil-mediated damage was studied by Pabla *et al.* (1996) These investigators used the isolated perfused rat heart model in which the heart was perfused in the presence or absence of human neutrophils for a short period during the early moments of reperfusion. These investigators did not show any significant improvement in postischemic contractile function (left ventricular developed pressure) with NO donor agents in hearts perfused without neutrophils, but some reduction was observed in perfusate creatine kinase activity. However, a greater reduction in creatine kinase activity was seen in the hearts perfused with neutrophil-supplemented buffer in which NO donor agents were added. Therefore, NO seems to inhibit neutrophil-mediated injury leading to infarction and endothelial dysfunction, but it may not prevent contractile dysfunction.

To summarize, NO exerts potent antineutrophil effects by attenuating superoxide radical generation and by inhibiting adherence to coronary vascular endothelium. In addition, NO attenuates the expression of adhesion molecules on the surface of the endothelium. These effects inhibit the inflammatory interaction between neutrophils and coronary endothelial cells. Accordingly, NO attenuates the distal consequences of ischemic–reperfusion injury (infarction, endothelial dysfunction, microvascular blood flow defects) in association with reduction in the accumulation of neutrophils in the myocardial area at risk.

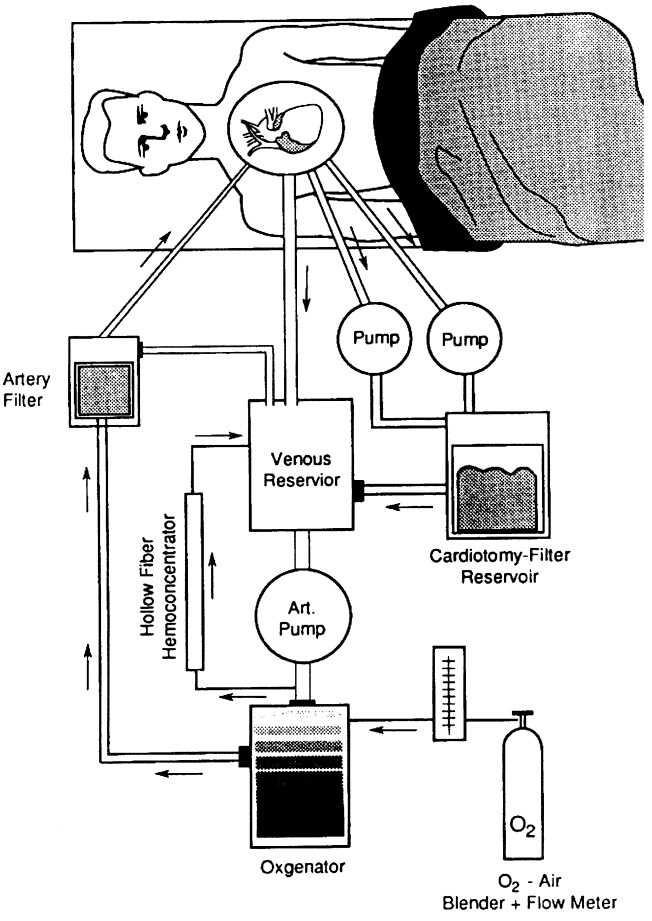


Figure 11 Schematic diagram of cardiopulmonary bypass circuitry. From Casthely P. A. (1991). The anatomy of cardiopulmonary bypass. *In* "Cardiopulmonary Bypass: Physiology, Related Complications and Pharmacology" (P. A. Casthely and D. Bregman, eds.), pp. 23–35. Futura Publ., Mount Kisco, New York.

Ischemia–Reperfusion Injury, the Vascular Endothelium, and Nitric Oxide in Cardiac Surgery

Cardiac Surgery as a Unique Model of Ischemia and Reperfusion

Cardiac surgery is a unique model of ischemia–reperfusion injury, and it has significant differences from its nonsurgical counterpart that impact on the application of NO-related therapy. Although cardiac surgery shares a common denominator of the cellular mechanisms of the postischemic inflammatory cascade and key mediators of parenchymal and endothelial cell injury with its nonsurgical counterpart (i.e., oxygen radicals, neutrophils), the frequency of ischemia and reperfusion events as well as the biological environment (bypass tubing, oxygenators, use of cardioplegic solutions, etc.) encountered in the surgical setting have an important impact on the pathophysiological processes leading to injury once the target vessel is reperfused. In conventional cardiac surgery, the patient is placed on the cardiopulmonary bypass (heart–lung machine), which consists of an oxygenator that exchanges oxygen and carbon dioxide with venous blood, with the arterialized blood being returned to the arterial system of the patient (Fig. 11). After cardiopulmonary bypass is initiated, and the heart and lungs are essentially "out of the loop" in maintaining the circulation; the myocardium is perfused by blood from the arterial system. During cardiac surgery, the aorta is cross-clamped, thereby separating the heart from the circulating blood and rendering the heart ischemic, and a chemical cardioplegia (cardio = heart, plegia = paralyzed, immobile) solution is delivered selectively to the heart to initiate elective cardiac arrest. This quiescent heart not only provides the surgical team with a bloodless field and quiet heart in which surgical precision is facilitated, but it also protects the heart from intraoperative ischemic injury by cooling the heart and delivering various cardioprotective agents (Buckberg, 1987, 1995; Vinten-Johansen and Cordell, 1992; Vinten-Johansen and Hammon, 1993).

Cardiopulmonary bypass is not innocuous, however, because the blood and the patient are exposed to surfaces (i.e., plastic tubing, plastic oxygenator) that are foreign in biological terms. These foreign surfaces are recognized as such by the immune system, which responds accordingly with an inflammatory response characterized by release of complement fragments C3a and C5a and of cytokines such as IL-6

and TNF-α. These inflammatory mediators in turn mobilize a systemic inflammatory response in which neutrophils are activated, generate superoxide radicals and other cytokines, and adhere to vascular endothelium; the response is amplified by the recruitment of other inflammatory cells to sites of endothelial activation. This systemic response to cardiopulmonary bypass is similar to the inflammatory-like response engaged in ischemic–reperfusion injury, and it may, in fact, synergize with the inflammatory processes initiated by myocardial ischemia–reperfusion. Cardiopulmonary bypass, independent of myocardial ischemia, activates the complement cascade (Chenowith *et al.,* 1981; Videm *et al.,* 1990) with the subsequent activation of neutrophils (Faymonville *et al.,* 1991) and their adherence-triggered release of cytotoxic products, all of which directly or indirectly (through recruitment of inflammatory cells) contribute to tissue injury in the ischemic–reperfused myocardium (Faymonville *et al.,* 1991; Lucchesi and Mullane, 1986). Therefore, the extracorporeal techniques used in cardiac surgery may themselves amplify the inflammatory responses initiated by myocardial (and other organ) ischemia–reperfusion injury, and thereby augment the total degree of injury (Boyle *et al.,* 1997). However, the cardioprotective techniques used in cardiac surgery, particularly cardioplegia solutions and their various additives, as well as hypothermia, reduce other pathological components of ischemic–reperfusion injury, potentially attenuating postsurgical injury.

Cardioplegia solutions are either of an acellular (crystalloid) or blood-based formulation, and they are delivered only to the heart after cardiopulmonary bypass is initiated. A cross-clamp placed on the aorta effectively separates the heart from the rest of the circulation, allowing delivery of the cardioplegia solution selectively to the heart. The solution is designed to (1) arrest the heart using high potassium concentrations (~20 mEq/liter) or hyperpolarizing agents (Cohen *et al.,* 1993; de Jong *et al.,* 1990) in order to present the surgeon with a motionless heart and thereby facilitate surgical precision and reduce energy demands, (2) cool the heart to various levels of hypothermia in order to further reduce energy demands and hence protect the heart during periods when the heart is not receiving a nutritional supply of blood or cardioplegia, (3) provide a vehicle for the delivery of various drugs to reduce ischemic injury and metabolically resuscitate the heart, and (4) act as a buffer before the heart is reperfused with normal systemic blood (modified reperfusate) in order to reduce reperfusion injury.

To summarize, the differences between the nonsurgical model and the surgical model of ischemia–reperfusion include the following: (1) multiple encounters with both ischemia and reperfusion, which add a dimension of complexity that is absent from nonsurgical models; (2) the accentuated roles of neutrophils and of the inflammatory mediators such as complement, tumor necrosis factors (TNF-α), and interleukins that are activated secondary to extracorporeal equipment (plastic tubing, cardiotomy reservoirs, oxygenators, suckers, etc.) used in "on-pump" heart surgery, which amplify the inflammatory response involving these components; and (3) surgery-specific techniques such as hypothermia and cardioplegia that alter the mechanisms of both ischemia and reperfusion in complex and sometimes unpredictable ways.

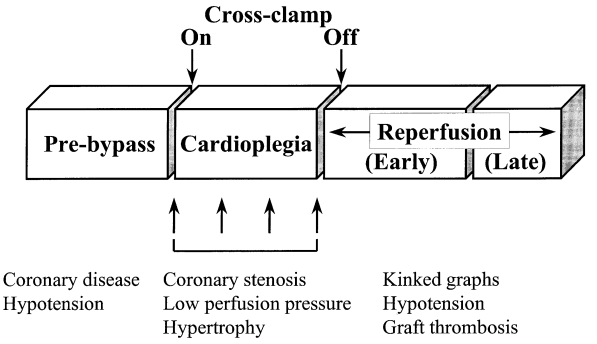


Figure 12 Points where potential ischemic injury can occur during a routine cardiac operation. The three "windows" of potential injury are (1) before bypass, (2) during the delivery of intermittent cardioplegia, and (3) after initiation of reperfusion by removal of the aortic cross-clamp. The arrows indicate the delivery of blood cardioplegia.

During cardiac surgery, ischemic injury to the myocardium can be encountered at three major time points, as summarized in Fig. 12: (1) pre-bypass ischemia that is encountered prior to the initiation of cardiopulmonary bypass or the delivery of cardioplegia solution (i.e., "unprotected" ischemia), and is related to attenuated perfusion to the myocardium secondary to coronary artery occlusions or extreme hypotension; (2) "protected" ischemia that occurs during the cardiac surgery using chemical cardioplegia delivered intermittently for several minutes followed by a period of no cardioplegia delivery; and (3) ischemia after the heart is reperfused, when closure of the coronary artery grafts or extreme hypotension limits blood flow to the heart. Reperfusion injury can be encountered (a) after resuscitation from prebypass hypotension or arrest, (b) during the infusions of cardioplegia (perfusion injury) dependent on the composition and delivery pressure of the cardioplegia solution, and (c) after removal of the aortic cross-clamp when the myocardium is perfused by native unmodified blood. Reperfusion injury can occur acutely (<4 hours after reperfusion) and during later phases of reperfusion during the first 4 to 6 hours after removal of the aortic cross-clamp. Oxygen radicals and neutrophil–endothelial cell interactions feature prominently in the acute phase of reperfusion injury (Dreyer *et al.,* 1991a,b; Zhao *et al.,* 1998; Boyle *et al.,* 1996).

Endothelial Injury in Cardiac Surgery

Patients undergoing cardiac surgery for valve repair or replacement, correction of anomalies of the great vessels, coronary artery bypass grafting, etc., often have underlying diseases such as hyperlipidemia, hypertension, and atherosclerosis. Endothelial dysfunction plays a major role in the pathological profile of these medical conditions in that the generation of NO has been attenuated as a result of the pri-

mary or secondary disease, or it is a part of the pathogenesis of the disease. This preoperative endothelial dysfunction has an impact on the postoperative outcome and management of the patient. Acute spasm of arterial grafts, acute loss of patency of any biological vascular conduit, and long-term patency are all factors that may be influenced by the preoperative health of the endothelium and its responses to cardiopulmonary bypass and/or surgical revascularization procedures. Loss of full graft patency or vascular coagulapathies may precipitate acute cardiac failure or other acute or long-term complications. The health of the endothelium also governs to some extent the degree of reperfusion injury that occurs as a consequence of surgical revascularization. Therefore, strategies to mitigate vascular dysfunction and protect the heart and other organs can use the concepts of nitric oxide to improve postoperative outcome over a broad range of disease presentations.

In the previous section on nonsurgical ischemia–reperfusion injury, the effects of ischemia and subsequent reperfusion on endothelial function and other end points were discussed. In cardiac surgery, however, intraoperative ischemia may superimpose on preoperative ischemia and any contributing endothelial dysfunction. In addition, hypothermia and cardioplegia solutions of various formulations and modalities of delivery may alter the response of the heart to ischemia and reperfusion. Figure 13 shows coronary artery endothelial function assayed as vasodilator responses to acetylcholine after 45 min of global normothermic ischemia, with or without reperfusion, or after multidose, hypothermic potassium blood cardioplegia for an additional hour of ''protected'' ischemia with or without reperfusion (Nakanishi *et al.,* 1994). Note from Fig. 13 that endothelial-dependent responses to acetylcholine were normal after the 45-min period of ischemia without reperfusion, but that dysfunction was expressed after reperfusion. Interestingly, the additional hour of hypothermic cardioplegia was not associated with a decrease in endothelial responses to acetylcholine, despite the intermittent nature of delivery, likely due to the protective attributes of the cardioplegia solution (hypothermia to 10°C myocardial temperature, buffering of acidosis, metabolic substrates in blood, increased oncotic properties). However, when the hearts were reperfused with normal systemic blood by removing the aortic cross-clamp, endothelial responses showed significant dysfunctional vasorelaxation responses to acetylcholine. A similar response profile was observed for the endothelial receptor-independent agent A23187, suggesting that there was a defect in the NO synthase activity. Smooth muscle relaxation responses to the NO donor agent acidified (pH 2.0) $NaNO_2$ were comparable to the control group, indicating normal function of the vascular smooth muscle. Endothelial dysfunction after global ischemia–reperfusion was also associated with morphological abnormalities in endothelial structure, including partial or complete detachment of endothelial cells from the basement membrane, hypervesiculation of endothelial cells present, and neutrophil attachments. These data suggested that the period of cardioplegic arrest with blood cardioplegia solution did not cause additional endothelial dysfunction despite the hour of cold arrest, but that endothelial dysfunction occurred during reperfusion with normal blood, similar to that observed with normothermic ischemia (without cardioplegia) followed by reperfusion. This observation is consistent with endothelial dysfunction observed with regional ischemia discussed earlier. Hence, strategies are necessary to address this postcardioplegia reperfusion injury.

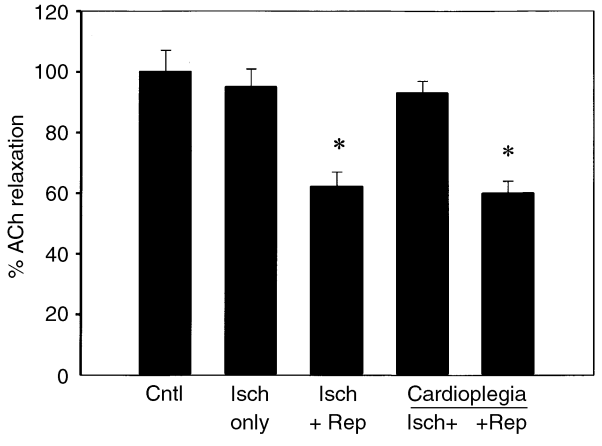


Figure 13 Defects in endothelial-specific vasorelaxation responses to acetylcholine after 30 minutes of normothermic global ischemia, after global ischemia plus reperfusion, after global ischemia followed by 60 min cardioplegic arrest with multidose hypothermic blood cardioplegia, and after global ischemia and blood cardioplegia followed by normal blood reperfusion.

Cardioprotective Effects of Nitric Oxide in Cardiac Surgery

As in the use of NO-related therapy in the treatment of myocardial ischemia–reperfusion injury, strategies for delivering NO therapy fall into two broad categories: (1) modulation of endogenous NO by addition of NO precursors or agents that stimulate native NO production by NO synthase and (2) administration of authentic NO or NO donor agents. These therapeutics may be applied systemically (appropriate for pre-bypass or off-pump applications) or as an adjunct to the cardioplegia solution, whereby higher concentrations of the agents can be achieved than are tolerated systemically.

Enhancing Endogenous Nitric Oxide Release

Increasing endogenous NO can be achieved by supplementing the cardioplegia solution with its precursor L-arginine. The ideal effect of this therapy is to enhance recovery of both the endothelium and the myocyte, the latter in terms of contractile function as well as viability. In surgical models using antecedent ischemia (regional or global) with subsequent delivery of blood cardioplegia and reperfusion, blood cardioplegia supplemented with L-arginine demonstrated significant benefit over unsupplemented blood cardioplegia, consistent with the potent anti-inflammatory effects

of NO. In a canine model of 90-min left anterior descending coronary artery ligation followed by cardioplegic arrest using blood cardioplegia, Sato *et al.* (1995a) reported that in the group in which blood cardioplegia was supplemented with 10 m*M* L-arginine (with a simultaneous intravenous infusion of 4 mg/kg/min starting at release of the cross-clamp), there was a 33% increase in postischemic systolic contractile function in the ischemic–reperfused area, a significant increase in regional compliance, and a 30% reduction in infarct size, compared to the group without L-arginine-supplemented blood cardioplegia. These beneficial effects of L-arginine-enhanced blood cardioplegia were associated with a significant decrease in neutrophil accumulation in the reperfused region compared to the unsupplemented group. In addition, coronary arteries taken from the ischemic–reperfused segment demonstrated better endothelial function compared to the unsupplemented blood cardioplegia group (Fig. 14). The beneficial effects of L-arginine were reversed by the NO synthase inhibitor L-NA administered intravenously before delivery of L-arginine-enhanced blood cardioplegia.

In a model of 20-min global normothermic ischemia, arrest with blood cardioplegia, followed by reperfusion, Mizuno *et al.* (1997) reported no postcardioplegia systolic or diastolic dysfunction, but they did not observe significant coronary artery endothelial dysfunction. This endothelial dysfunction was reversed by 2 m*M* L-arginine used as additive to the blood cardioplegia solution. The improved endothelial function was associated with greater transcardiac nitrate/nitrite concentration in the cardioplegia affluent, suggesting that the adjunct L-arginine indeed increased the release of NO during delivery of the solution. These results have been corroborated by other studies in which cardioplegia solutions were supplemented with L-arginine (Engleman *et al.,* 1995b; Hiramatsu *et al.,* 1995a). Interestingly, 3 m*M* L-arginine given after the delivery of a crystalloid cardioplegia solution was reported by Engleman *et al.* (1996) to be detrimental. This observation may be related to the generation of the deleterious peroxynitrite by-product of the NO and superoxide neutralization reaction or to some unrecognized direct action of NO.

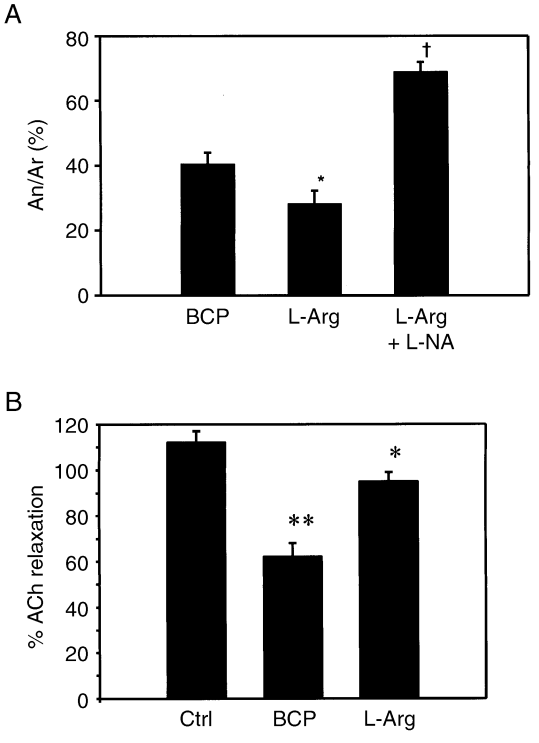


Figure 14 Effect of L-arginine-enhanced blood cardioplegia (BCP) on infarct size (A) and postischemic endothelial-specific coronary artery vasorelaxation responses to acetylcholine (B). Redrawn from Sato *et al.* (1995a).

Other studies have reported cardioprotection by supplemental L-arginine in cardiac surgery models. Hiramatsu *et al.* (1995a,b) administered L-arginine during the early phase of reperfusion in a blood perfused isolated heart preparation; this protocol intentionally targeted reperfusion events mediated by neutrophils. This study demonstrated better recovery of postcardioplegia systolic function compared to unsupplemented cardiopleiga. A similar benefit from L-arginine, administered at reperfusion, was also reported by Armani *et al.* (1995) in a cell-free perfusate system, suggesting that NO derived from L-arginine may exert beneficial effects independent of its antineutrophil effects, that is, quenching of superoxide anions. This is consistent with data reported by Pabla *et al.* (1996) showing a direct, but smaller, beneficial effect on recovery of contractile function independent of neutrophils compared to neutrophil-supplemented perfusate.

Exogenous Delivery of Nitric Oxide Donors

The studies of Nakanishi *et al.* (1992) and Sato *et al.* (1996) exposed several potential limitations in the therapeutic efficacy of the L-arginine-enhanced endogenous NO therapy in which the desired increased generation of NO from the coronary vascular endothelium is dependent on normal endothelial function. However, it has been demonstrated that ischemia and reperfusion impair NO generation by the endothelium (Sato *et al.,* 1997b). This potential limitation in the endogenous generation of nitric oxide may be overcome by administering nitric oxide donor agents. In a study by Nakanishi *et al.* (1995) using a canine model of cardiopulmonary bypass and cardioplegia, hearts were subjected to 30 min of normothermic ischemia to render the myocardium vulnerable to reperfusion injury. The ischemia was followed by 1-hour cardioplegia (4°C multidose blood cardioplegia, 4:1 blood:crystalloid ratio). Hearts received either unsupplemented blood cardioplegia (BCP) or blood cardioplegia supplemented with 10 μmol/liter SPM-5185 (BCP + SPM). After an hour of cardioplegic arrest, the heart was weaned from cardiopulmonary bypass and reperfused for a total of 30 min off-pump. Postischemic left ventricular function, assessed by end-systolic pressure–volume relations (impedance catheter), was reduced by 53.7% of preischemic values in the unsupplemented blood cardioplegia group (Fig. 15A). In contrast, there was nearly complete postischemic functional recovery in the high-dose SPM-enhanced blood

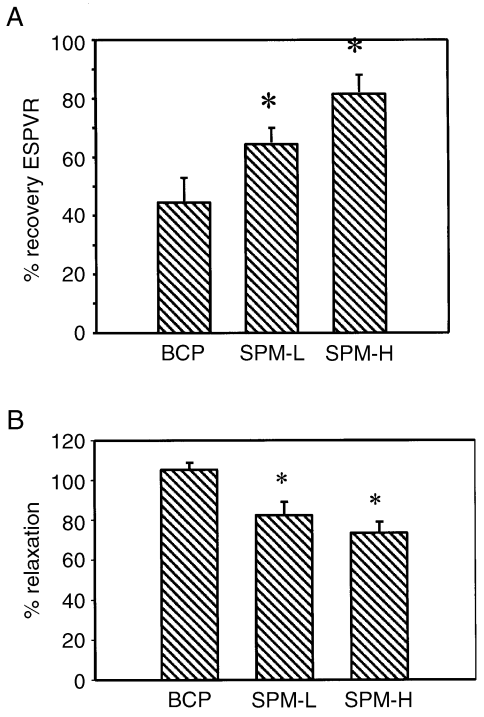


Figure 15 Cardioprotective effects of the NO donor agent SPM-5185 in blood cardioplegia on recovery of left ventricular function assessed by the end-systolic pressure–volume relationship as a percentage of baseline slope of the linear relationship (A) and on the maximum vasorelaxation responses of postischemic coronary arteries derived from incremental concentrations of acetylcholine (B). *$p < 0.05$ vs BCP.

cardioplegia group, with intermediate results in the 1 μmol/liter SPM-5185 group. This improved functional recovery in the SPM-5185 blood cardioplegia group was associated with significantly reduced myeloperoxidase (MPO) activity (1.27 ± 0.45 U/100 mg tissue) in postischemic myocardium (used as an index of neutrophil accumulation), in contrast to the greater MPO activity in the unsupplemented blood cardioplegia group (3.36 ± 0.58 U/100 mg tissue). In postischemic coronary arteries isolated from these hearts, endothelium-dependent maximal relaxation responses to acetylcholine was attenuated by 27% in the unsupplemented blood cardioplegia group. However, endothelial responses recovered to approximately 100% in the high-dose SPM enhanced blood cardioplegia group (Fig. 15B). Therefore, this study demonstrated that addition of an NO donor agent in blood cardioplegia improved postischemic ventricular performance and postcardioplegia endothelial function in ischemically injured hearts, possibly by attenuating neutrophil-mediated damage. These data are consistent with findings in regionally ischemic–reperfused models in which NO donor agents were administered before the onset of reperfusion.

NO Effects on Mechanical Function of the Heart Relevant to Heart Surgery

Nitric oxide has been suggested to have inhibitory effects on cardiac systolic function, while increasing diastolic mechanics and thereby improving diastolic function. However, there is controversy over the effects of NO on the inotropic state and contractile function of the heart. Finkel *et al.* (1992) first reported a negative inotropic effect of cytokines (TNF-α) in papillary muscle preparations, which they attributed to the effects of NO. This observation would have relevance to procedures using cardiopulmonary bypass, because cytokines (Kawamura *et al.,* 1993; Steinberg *et al.,* 1993) as well as complement (Chenoweth, 1983; Davies *et al.,* 1991; Kirklin *et al.,* 1983) are elevated during bypass by exposure of blood to foreign surfaces and the triggering of cytokine and complement fragment release by ischemia–reperfusion. In support of stimulated release of NO during bypass, transmyocardial NO levels as well as NOS activity have been observed to be elevated after release of the cross-clamp during cardiac surgery (Hattler *et al.,* 1994), a time when cardiac functional depression is often observed. However, other studies have reported no negative inotropic effects of physiological concentrations of NO (Hasebe *et al.,* 1993; Weyrich *et al.,* 1994). The disparate observations may be related to the amount of NO released, which may be higher (micromolar range) with cytokine stimulation (i.e., of iNOS) compared to either endogenously released NO (0.1–1 n*M* in coronary circulation) (Kelm and Schrader, 1990) or therapeutic concentrations of NO donor agents (approximately 500 n*M*). Higher concentrations of NO released by endotoxin stimulation may mediate cardiac depression during the later stages of septic shock. However, in surgically or nonsurgically reperfused postischemic hearts, any direct negative inotropic actions of NO may be overridden by the net cardioprotective effects of NO secondary to a reduction of neutrophil- and oxidant-mediated injury, resulting in better postischemic function (Hiramatsu *et al.,* 1995a,c; D. J. Lefer *et al.,* 1993a; Nakanishi *et al.,* 1995; Sato *et al.,* 1995c).

Peroxynitrite: The Product of Interaction between NO and Superoxide Anions

Nitric oxide and superoxide anions undergo a spontaneous and very rapid biradical, reaction forming the peroxynitrite anion. Peroxynitrite ($ONOO^-$) is formed at the near diffusion-limited rate of 5×10^9 to 6.7×10^9 $M^{-1}s^{-1}$ (Koppenol, 1998; Ma *et al.,* 1997a), which is three times greater than the reaction between superoxide radical and superoxide dismutase. This suggests that accumulation of superoxide radical levels may preferentially neutralize NO, reducing its bioavailability and thereby attenuating its physiological and cardioprotective functions. The reaction between these two radical species is potentially good and bad; a potentially deleterious anion is eliminated, but at the same time a potentially beneficial and cardioprotective NO molecule is eliminated. $ONOO^-$ is a strong oxidant capable of forming intermediates with hydroxyl-like actions that produce biological tissue damage by hydroxylating aromatic compounds. In addition, $ONOO^-$ induces cellular injury by

causing lipid peroxidation (Radi *et al.,* 1991), by inducing DNA fragmentation similar to that produced by apoptosis (Lin *et al.,* 1997a,b), by modifying proteins and plasma lipids (Van Der Vliet *et al.,* 1994), by depleting important plasma antioxidants such as glutathione and cysteine, and by nitrating proteins leading to cellular and organ dysfunction (Lopez *et al.,* 1997; Ma *et al.,* 1997a). Because both NO and superoxide radicals are produced during the early moments of reperfusion, the formation of peroxynitrite may be greatly increased at this important time. Studies demonstrate that $ONOO^-$ is indeed a potential mechanism causing cellular injury in pathological conditions such as ischemia–reperfusion, inflammation, hypercholesterolemia, and angiotensin-induced hypertension (Lopez *et al.,* 1997; Ma *et al.,* 1997a,b; Nossuli *et al.,* 1998; Radi *et al.,* 1991; Xia *et al.,* 1996; Yasmin *et al.,* 1997).

$ONOO^-$ may play a pivotal role in surgical and nonsurgical ischemic reperfusion injury. On the one hand, the production of $ONOO^-$ may cause tissue damage by the mechanisms enumerated above. On the other hand, the nitrosylated breakdown products of $ONOO^-$ may not only prevent toxic buildup of the anion, but they would generate potentially cardioprotective nitrates such as nitrosoglutathione. These diametrically opposed actions and fates of $ONOO^-$ may resolve the equally opposing data regarding the cardioprotection or injury produced by NO. Whether $ONOO^-$ generates deleterious by-products or whether it generates nitrosylated intermediates with cardioprotective actions (or authentic NO itself) is dependent on the biological environment, specifically whether the anion is present in a crystalloid environment or a blood environment. This has particular relevance to cardiac surgery in which the formulation of cardioplegia used provides a blood or crystalloid environment. *In vitro* studies conducted in crystalloid solutions demonstrate increased myocardial contractile dysfunction, increased infarct size, and direct endothelial toxicity via lipid peroxidation and DNA injury (Ma *et al.,* 1997a). In contrast, studies of $ONOO^-$ conducted in a blood environment have shown benefits similar to those described above with authentic NO or NO donor agents.

Nossuli *et al.* (1998) reported a $>50\%$ reduction of infarct size and significant reduction in postischemic coronary artery endothelial dysfunction (vasorelaxation responses to acetylcholine) compared to a vehicle group in a feline model of 90 min of LAD occlusion and 270 min of reperfusion when 2 μM authentic peroxynitrite was infused during reperfusion. However, in a follow-up study, Nossuli *et al.* (1998) showed that higher concentrations (20 μM) of peroxynitrite were associated with increased infarct size and greater postischemic endothelial dysfunction. The actions of peroxynitrite in these two different environments may depend on the concentration of the anion and also on the presence of thiol-containing molecules such as glutathione, albumin, and cysteine, all of which are normal constituents of blood and are absent from crystalloid buffer solutions. Reaction of these compounds with peroxynitrite converts them to nitrosothiols such as nitrosoglutathione, which have been found to mimic the physiological actions of NO, particularly the inhibitory effects on neutrophil–endothelial cell interactions (D. J. Lefer *et al.,* 1997).

This dichotomy in the physiological actions of peroxynitrite may impact on the decision to use NO in crystalloid cardioplegia solutions. In fact, the deleterious actions of L-arginine in crystalloid cardioplegia reported by Engelman *et al.* (1995a) may be related in part to the toxic buildup of peroxynitrite. Whether NO is converted to $ONOO^-$ with subsequent negative consequences has an important impact on the surgical strategies of cardioprotection, namely, whether crystalloid or blood cardioplegia is used with NO-related therapeutics. This question was addressed in a study reported by Ronson *et al.* (1998). In this study, canine hearts *in vivo* were placed on cardiopulmonary bypass and subjected to 30 min of normothermic ischemia, followed by 1 hour of arrest using multidose hypothermic cardioplegia of either a crystalloid formulation (Plegisol) or 4:1 blood crystalloid: cardioplegia representing a crystalloid environment or a blood environment, respectively. Each formulation was subdivided into two groups: one with no supplemental $ONOO^-$ and the other with 5 μM authentic peroxynitrite. After the cardioplegia period, the hearts were reperfused for 2 hours. Ronson *et al.* (1998) found that in the crystalloid cardioplegia ''environment,'' $ONOO^-$ was associated with significant decreases in postcardioplegia functional recovery and increased neutrophil accumulation compared to its counterpart without peroxynitrite. Conversely, peroxynitrite in the blood cardioplegia group increased functional recovery and decreased neutrophil accumulation compared to the blood cardioplegia group without peroxynitrite. Immunohistochemistry for nitrotyrosine, the ''footprint'' of the actions of peroxynitrite in tissue, confirmed that peroxynitrite was delivered at the tissue level. The results from this study are consistent with the sensitivity of $ONOO^-$ to detoxification agents, and they may have clinical implications for NO additives used in crystalloid cardioplegic solutions with respect to myocardial protection, in that appropriate adjunct detoxifying agents may be necessary to detoxify $ONOO^-$ generated by NO. A follow-up study by Nakamura *et al.* (1999) showed that glutathione added to this crystalloid cardioplegia solution containing peroxynitrite attenuates the deleterious effects of the anion (Fig. 16). $ONOO^-$ in crystalloid cardioplegia was associated with endothelial dysfunction, which was completely reversed with 500 mM glutathione. Analysis of the cardioplegia effluent suggested that the $ONOO^-$ was being converted by glutathione to nitrosoglutathione, and was thereby being effectively detoxified.

Summary

Nitric oxide is a pluripotent radical molecule whose physiological actions impact multiple disease processes such as atherosclerosis, hypercholesterolemia, circulatory shock, and ischemia–reperfusion injury in the pulmonary, cardiovascular, and other organ systems. The efficacy of NO in

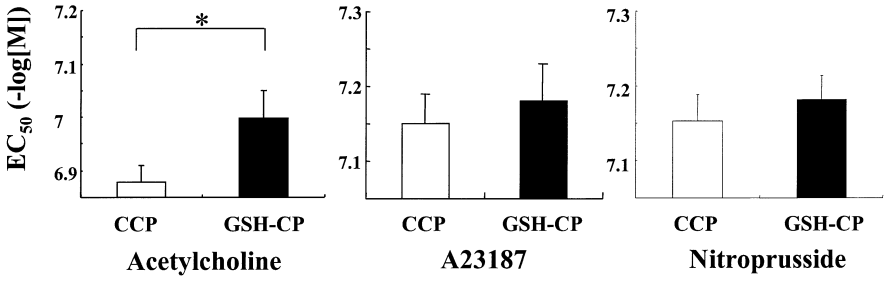


Figure 16 EC_{50} values for vascular relaxation responses to incremental concentrations of acetylcholine (left panel), the calcium ionophore A23187 (center panel) and the smooth muscle relaxant sodium nitroprusside (right panel) in the group with 5 μM $ONOO^-$-containing crystalloid cardioplegia (CCP) and a group in which 500 mM glutathione (GSH) was added to $ONOO^-$-containing crystalloid cardioplegia (CCP + GSH). Maximal endothelial relaxations to acetylcholine were greater, and the EC_{50} lower, in the GSH-containing group. $*p < 0.05$ versus group without glutathione.

attenuating ischemia–reperfusion injury in the cardiology and cardiac surgery settings derives from its inhibitory actions at a critical proximal point in the inflammatory cascade involving adhesion molecule-mediated interaction between neutrophils and endothelial cells with downstream effects on clinically relevant end points. NO therapy has been shown to reduce the following: infarct size, neutrophil activation and superoxide anion generation, the surface expression of adhesion molecules on the endothelium, the adherence of neutrophils to endothelium and subsequent accumulation in the reperfused area at risk, postischemic endothelial dysfunction, microvascular defects, and in some cases contractile dysfunction. NO may directly neutralize superoxide radicals, thereby inhibiting oxidant-mediated injury, but with the potential price of generating peroxynitrite unless thiol-containing agents are available to ''detoxify'' the anion. The attenuation of adhesion molecule protein synthesis at the molecular level by interfering with NF-κB gene signaling has implications for long-term effects as well as acute effects on ischemia–reperfusion injury. Therapeutic agents are available that target the critical events in ischemia–reperfusion injury at subvasodilator concentrations, which is important clinically for the avoidance of hypotension in patients with the potential for hemodynamic instability of cardiac and vascular origin.

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References

Ambrosio, G., and Chiariello, M. (1991). Myocardial reperfusion injury: Mechanisms and management—a review. [Review.] *Am. J. Med.* **91,** 86S–88S.

Amrani, M., Chester, A. H., Jayakumar, J., Schyns, C. J., and Yacoub, M. (1995). L-Arginine reverses low coronary reflow and enhances postischaemic recovery of cardiac mechanical function. *Cardiovasc. Res.* **30,** 200–204.

Armstead, V. E., Minchenko, A. G., Schuhl, R. A., Hayward, R., Nossuli, T. O., and Lefer, A. M. (1997). Regulation of P-selectin expression in human endothelial cells by nitric oxide. *Am. J. Physiol. Heart Circ. Physiol.* **42,** H740–H746.

Bolli, R. (1988). Oxygen-derived free radicals and postischemic myocardial dysfunction (''Stunned Myocardium'') reviews. *J. Am. Coll. Cardiol.* **12,** 239–249.

Bolli, R. (1991). Oxygen-derived free radicals and myocardial reperfusion injury: An overview. [Review.] *Cardiovasc. Drugs Ther.* **5**(Suppl. 2), 249–268.

Boyle, E. M., Pohlman, T. H., Cornejo, C. J., and Verrier, E. D. (1996). Endothelial cell injury in cardovascular surgery: Ischemia–reperfusion. *Ann. Thorac. Surg.* **62,** 1868–1875.

Boyle, E. M., Pohlman, T. H., Johnson, M. C., and Verrier, E. D. (1997). Endothelial cell injury in cardiovascular surgery: The systemic inflammatory response. *Ann. Thorac. Surg.* **63,** 277–284.

Buckberg, G. D. (1987). Strategies and logic of cardioplegic delivery to prevent, avoid, and reverse ischemic and reperfusion damage. *J. Thorac. Cardiovasc. Surg.* **93,** 127–139.

Buckberg, G. D. (1995). Update on current techniques of myocardial protection. [Review.] *Ann. Thorac. Surg.* **60,** 805–814.

Bufkin, B. L., Shearer, S. T., Vinten-Johansen, J., Duarte, I. G., Thourani, V. H., Nakamura, M., Zhao, Z.-Q., and Guyton, R. A. (1998). Preconditioning during simulated MIDCABG attenuates blood flow defects and neutrophil accumulation. *Ann. Thorac. Surg.* **66,** 726–732.

Buga, G. M., Gold, M. E., Fukuto, J. M., and Ignarro, L. J. (1991). Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension* **17,** 187–193.

Bullough, D. A., Magill, M. J., Mullane, K. M., and Firestein, G. S. (1996). Carbohydrate- and CD18-dependent neutrophil adhesion to cardiac myocytes: Effects of adenosine. *Cardiovasc. Res.* **32,** 328–334.

Byrne, J. G., Smith, W. J., Murphy, M. P., Couper, G. S., Appleyard, R. F., and Cohn, L. H. (1992). Complete prevention of myocardial stunning, contracture, low- reflow, and edema after heart transplantation by blocking neutrophil adhesion molecules during reperfusion. *J. Thorac. Cardiovasc. Surg.* **104,** 1589–1596.

Carden, D. L., Young, J. A., and Granger, D. N. (1993). Pulmonary microvascular injury after intestinal ischemia–reperfusion: Role of P-selectin. *J. Appl. Physiol.* **75,** 2529–2534.

Chenoweth, D. E. (1983). Complement activation during cardiopulmonary bypass. *In* ''Pathophysiology and Techniques of Cardiopulmonary Bypass'' (J. R. Utley and R. Betleski, eds.), pp. 49–60. Williams & Wilkins, Baltimore, Maryland.

Chenowith, D. E., Cooper, S. W., Hugli, T. E., Stewart, R. W., Blackstone, E. H., and Kirklin, J. W. (1981). Complement activation during cardiopulmonary bypass: Evidence for generation of C3a and C5a anaphylatoxins. *N. Engl. J. Med.* **304,** 497–503.

Clancy, R. M., Leszczynska-Piziak, J., and Abramson, S. B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.* **90,** 1116–1121.

Cohen, N. M., Wise, R. M., Wechsler, A. S., and Damiano, R. J., Jr. (1993). Elective cardiac arrest with a hyperpolarizing adenosine triphosphate-sensitive potassium channel opener. *J. Thorac. Cardiovasc. Surg.* **106,** 317–328.

Cooke, J. P., Stamler, J., Andon, N., Davies, P. F., McKinley, G., and Loscalzo, J. (1990). Flow stimulates endothelial cells to release a nitrovasodilator that is potentiated by reduced thiol. *Am. J. Physiol. Heart Circ. Physiol.* **259,** H804–H812.

Davenpeck, K. L., Gauthier, T. W., Albertine, K. H., and Lefer, A. M. (1994a). Role of P-selectin in microvascular leukocyte–endothelial interaction in splanchnic ischemia–reperfusion. *Am. J. Physiol.* **267,** H622–H630.

Davenpeck, K. L., Gauthier, T. W., and Lefer, A. M. (1994b). Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation [see comments]. *Gastroenterology* **107,** 1050–1058.

Davies, S. W., Bailey, J., Wedzicha, J. A., and Rudd, R. M. (1991). Complement activation and lung permeability during cardiopulmonary bypass. *Ann. Thorac. Surg.* **51,** 693–697.

DeCaterina, R., Libby, O., Peng, H., Thannickal, V., Rajavashisth, T., Gimbrone, M., Shin, W., and Liao, J. (1995). Nitric oxide decreases cytokine-induced endothelial activation. *J. Clin. Invest.* **96,** 60–68.

de Jong, J. W., van der Meer, P., van Loon, H., Owen, P., and Opie, L. H. (1990). Adenosine as adjunct to potassium cardioplegia: Effect on function, energy metabolism, and electrophysiology. *J. Thorac. Cardiovasc. Surg.* **100,** 445–454.

Delyani, J. A., Nossuli, T. O., Scalia, R., Thomas, G., Garvey, D. S., and Lefer, A. M. (1996). S-Nitrosylated tissue-type plasminogen activator protects against myocardial ischemia/reperfusion injury in cats: Role of the endothelium. *J. Pharmacol. Exp. Ther.* **279,** 1174–1180.

Dignan, R. J., Dyke, C. M., Abd-Elfattah, A. S., Lutz, H. A., Yeh, T., Jr., Lee, K. F., Parmar, J., and Wechsler, A. S. (1992). Coronary artery endothelial cell and smooth muscle dysfunction after global myocardial ischemia. *Ann. Thorac. Surg.* **53,** 311–317.

Dreyer, W. J., Smith, C. W., Michael, L. H., Rossen, R. D., Hughes, B. J., Entman, M. L., and Anderson, D. C. (1989). Canine neutrophil activation by cardiac lymph obtained during reperfusion of ischemic myocardium. *Circ. Res.* **65,** 1751–1762.

Dreyer, W. J., Michael, L. H., West, M. W., Smith, C. W., Rothlein, R., Rossen, R. D., Anderson, D. C., and Entman, M. L. (1991a). Neutrophil accumulation in ischemic canine myocardium: Insights into time course, distribution, and mechanism of localization during early reperfusion. *Circulation* **84,** 400–411.

Dreyer, W. J., Smith, C. W., and Entman, M. L. (1991b). Invited letter concerning: Neutrophil activation during cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* **102,** 318–320.

Engelman, D. T., Watanabe, M., Engleman, R. M., Rousou, J. A., Flack III, J. E., Deaton, D. W., and Das, D. K. (1995a). Constitutive nitric oxide release is impaired after ischemia and reperfusion. *J. Thorac. Cardiovasc. Surg.* **110,** 1047–1053.

Engelman, D. T., Watanabe, M., Maulik, N., Cordis, G. A., Engelman, M., Rousou, J. A., Flack III, J. E., Deaton, D. W., and Das, D. K. (1995b). L-Arginine reduces endothelial inflammation and myocardial stunning during ischemia/reperfusion. *Ann. Thorac. Surg.* **60,** 1275–1281.

Engelman, D. T., Watanabe, M., Maulik, N., Engelman, R. M., Rousou, J. A., Flack III, J. E., Deaton, D. W., and Das, D. K. (1996). Critical timing of nitric oxide supplementation in cardioplegic arrest and reperfusion. *Circulation* **94,** II-407–II-411

Entman, M. L., Michael, L., Rossen, R. D., Dreyer, W. J., Anderson, D. C., Taylor, A. A., and Smith, C. W. (1991). Inflammation in the course of early myocardial ischemia. *FASEB J.* **5,** 2529–2537.

Entman, M. L., Youker, K., Shoji, T., Kukielka, G., Shappell, S. B., Taylor, A. A., and Smith, C. W. (1992). Neutrophil induced oxidative injury of cardiac myocytes. *J. Clin. Invest.* **90,** 1335–1345.

Faymonville, M. E., Pincemail, J., Duchateau, J., Paulus, J. M., Adam, A., Deby-Dupont, G., Deby, C., Albert, A., Larbuisson, R., Limet, R., and Lamy, M. (1991). Myeloperoxidase and elastase as markers of leukocyte activation during cardiopulmonary bypass in humans. *J. Thorac. Cardiovasc. Surg.* **102,** 309–317.

Finkel, M. S., Oddis, C. V., Jacob, T. D., Watkins, S. C., Hattler, B. G., and Simmons, R. L. (1992). Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* **257,** 287–289.

Foreman, K. E., Vaporciyan, A. A., Bonish, B. K., Jones, M. L., Johnson, K. J., Glovsky, M. M., Eddy, S. M., and Ward, P. A. (1994). C5a-induced expression of P-selectin in endothelial cells. *J. Clin. Invest.* **94,** 1147–1155.

Geng, J.-G., Bevilacqua, M. P., Moore, K. L., McIntyre, T. M., Prescott, S. M., Kim, J. M., Bliss, G. A., Zimmerman, G. A., and McEver, R. P. (1990). Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* **343,** 757–760.

Giraldez, R. R., Panda, A., Xia, Y., Sanders, S. P., and Zweier, J. L. (1997). Decreased nitric-oxide synthase activity causes impaired endothelium-dependent relaxation in the postischemic heart. *J. Biol. Chem.* **272,** 21420–21426.

Gryglewski, R. J., Palmer, R. M. J., and Moncada, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. *Nature* **320,** 454–460.

Guo, J.-P., Murohara, T., Buerke, M., Scalial, R., and Lefer, A. M. (1996). Direct measurement of nitric oxide release from vascular endothelial cells. *J. Appl. Physiol.* **81,** 774–779.

Hasebe, N., Shen, Y.-T., and Vatner, S. F. (1993). Inhibition of endothelium-derived relaxing factor enhances myocardial stunning in conscious dogs. *Circulation* **88,** 2862–2871.

Hattler, B. G., Gorcsan, I. J., Shah, N., Oddis, C. V., Billiar, T. R., Simmons, R. L., and Finkel, M. S. (1994). A potential role for nitric oxide in myocardial stunning. *J. Cardiac Surg.* **9,** 425–429.

Hiramatsu, T., Forbess, J. M., Miura, T., and Mayer, J. E., Jr. (1995a). Effect of L-arginine cardioplegia on recovery of neonatal lamb hearts after 2 hours of cold ischemia. *Ann. Thorac. Surg.* **60,** 1187–1192.

Hiramatsu, T., Forbess, J. M., Miura, T., Nomura, F., and Mayer, J. E., Jr. (1995b). Additive effects of L-arginine infusion and leukocyte depletion on recovery after hypothermic ischemia in neonatal lamb hearts. *J. Thorac. Cardiovasc. Surg.* **110,** 172–179.

Hiramatsu, T., Forbess, J. M., Miura, T., and Mayer, J. E., Jr. (1995c). Effect of L-arginine and L-nitro-arginine methyl ester on recovery of neonatal lamb hearts after cold ischemia. Evidence for an important role of endothelial production of nitric oxide. *J. Thorac. Cardiovasc. Surg.* **109,** 81–87.

Hoshida, S., Yamashita, N., Igarashi, J., Nishida, M., Hori, M., Kamada, T., Kuzuya, T., and Tada, M. (1995). Nitric oxide synthase protects the heart against ischemia–reperfusion injury in rabbits. *J. Pharmacol. Exp. Ther.* **274,** 413–418.

Hudson, K. F. (1994). A phenomenon of paradox: Myocardial reperfusion injury. [Review.] *Heart Lung* **23,** 384–393; quiz, 394–396.

Jerome, S. N., Dore, M., Paulson, J. C., Smith, C. W., and Korthuis, R. J. (1994). P-selectin and ICAM-1-dependent adherence reactions: Role in the genesis of postischemic no-reflow. *Am. J. Physiol.* **266,** H1316–21.

Johnson III, G., Tsao, P. S., Mulloy, D., and Lefer, A. M. (1990). Cardioprotective effects of acidified sodium nitrite in myocardial ischemia with reperfusion. *J. Pharmacol. Exp. Ther.* **252,** 35–41.

Johnson III, G., Tsao, P. S., and Lefer, A. M. (1991). Cardioprotective effects of authentic nitric oxide in myocardial ischemia with reperfusion. *Crit. Care Med.* **19,** 244–252.

Jordan, J. E., Zhao, Z.-Q., and Vinten-Johansen, J. (1999). The role of neutrophils in myocardial ischemia–reperfusion injury. *Cardiovasc. Res.* **43,** 860–878.

Kaeffer, N., Richard, V., Francois, A., Lallemand, F., Henry, J.-P., and Thuillez, C. (1996). Preconditioning prevents chronic reperfusion-induced coronary endothelial dysfunction in rats. *Am. J. Physiol.* **271,** H842–H849.

Kawamura, T., Wakusawa, R., Okada, K., and Inada, S. (1993). Elevation of cytokines during open heart surgery with cardiopulmonary bypass:

Participation of interleukin 8 and 6 in reperfusion injury [see comments]. *Can. J. Anaesth.* **40,** 1016–1021.

Kelm, M., and Schrader, J. (1990). Control of coronary vascular tone by nitric oxide. *Circ. Res.* **66,** 1561–1575.

Kirklin, J. K., Westaby, S., Blackstone, E. H., Kirklin, J. W., Chenoweth, D. E., and Pacifico, A. D. (1983). Complement and the damaging effects of cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* **86,** 845–857.

Kloner, R. A. (1993). Does reperfusion injury exist in humans? [Review.] *J. Am. Coll. Cardiol.* **21,** 537–545.

Koppenol, W. H. (1998). The basic chemistry of nitrogen monoxide and peroxynitrite. [Review.] *Free Radicals Biol. Med.* **25,** 385–391.

Kubes, P., Suzuki, M., and Granger, N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci.* **88,** 4651–4655.

Kubes, P., Jutila, M., and Payne, D. (1995). Therapeutic potential of inhibiting leukocyte rolling in ischemia/reperfusion. *J. Clin. Invest.* **95,** 2510–2519.

Lefer, A. M., and Lefer, D. J. (1991). Endothelial dysfunction in myocardial ischemia and reperfusion: Role of oxygen-derived free radicals. *Steinkopff Verlaag Darmstadt,* 109–116.

Lefer, A. M., and Scalia, R. (1999). Nitric oxide in myocardial and splanchnic ischemia/reperfusion. *In* ''Pathophysiology and Clinical Applications of Nitric Oxide'' (G. M. Rubanyi, ed.), Part B, pp. 301–321. Harwood, Academic Publ., Amsterdam, The Netherlands.

Lefer, A. M., Tsao, P. S., Lefer, D. J., and Ma, X.-L. (1991). Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. *FASEB J.* **5,** 2029–2034.

Lefer, A. M., Ma, X.-L., Weyrich, A., and Lefer, D. J. (1993). Endothelial dysfunction and neutrophil adherence as critical events in the development of reperfusion injury. *Agents Actions Suppl.* **41,** 127–135.

Lefer, D. J., Nakanishi, K., Vinten-Johansen, J., Ma, X.-L., and Lefer, A. M. (1992). Cardiac venous endothelial dysfunction after myocardial ischemia and reperfusion in dogs. *Am. J. Physiol.* **263,** H850–H856.

Lefer, D. J., Nakanishi, K., and Vinten-Johansen, J. (1993a). Endothelial and myocardial cell protection by a cysteine-containing nitric oxide donor after myocardial ischemia and reperfusion. *J. Cardiovasc. Pharmacol.* **22**(Suppl. 7), S34–S43.

Lefer, D. J., Nakanishi, K., and Vinten-Johansen, J. (1993b). Endothelial and myocardial cell protection by a cysteine-containing nitric oxide donor after myocardial ischemia and reperfusion. *J. Cardiovasc. Pharmacol.* **22,** S34–S43.

Lefer, D. J., Nakanishi, K., Johnston, W. E., and Vinten-Johansen, J. (1993c). Antineutrophil and myocardial protection actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. *Circulation* **88,** 2337–2350.

Lefer, D. J., Scalia, R., Campbell, B., Nossuli, T. O., Hayward, R., Salamon, M., Grayson, J., and Lefer, A. M. (1997). Peroxynitrite inhibits leukocyte–endothelial cell interactions and protects against ischemia–reperfusion injury in rats. *J. Clin. Invest.* **99,** 684–691.

Lin, K.-T., Xue, J.-Y., Sun, F. F., and Wong, P. Y. K. (1997a). Reactive oxygen species participate in peroxynitrite-induced apoptosis in HL-60 cells. *Biochem. Biophys. Res. Commun.* **230,** 115–119.

Lin, K.-T., Xue, J.-Y., and Wong, P. Y. K. (1997b). Peroxynitrite. An apoptotic agent in Hl-60 cells. *In* ''Eicosanoids and Other Bioactive Lipids in Cancer Inflammation and Radiation Injury'' (Honn *et al.,* eds.), pp. 413–419. Plenum Press, New York.

Lopez, B. L., Liu, G.-L., Christopher, T. A., and Ma, X.-L. (1997). Peroxynitrite, the product of nitric oxide and superoxide, causes myocardial injury in the isolated perfused rat heart. *Coronary Artery Dis.* **8,** 149–153.

Lucchesi, B. R. (1994). Complement, neutrophils and free radicals: Mediators of reperfusion injury. [Review.] *Arzneim.-Forsch./Drug Res.* **44,** 420–432.

Lucchesi, B. R., and Mullane, K. M. (1986). Leukocytes and ischemia-induced myocardial injury. *Ann. Rev. Pharmacol. Toxicol.* **26,** 201–224.

Ma, X.-L., Weyrich, A. S., Lefer, D. J., and Lefer, A. M. (1993). Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ. Res.* **72,** 403–412.

Ma, X.-L., Lopez, B. L., Liu, G.-L., Christopher, T. A., and Ischiropoulos, H. (1997a). Peroxynitrite aggravates myocardial reperfusion injury in the isolated perfused rat heart. *Cardiovasc. Res.* **36,** 195–204.

Ma, X.-L., Lopez, B. L., Liu, G.-L., Christopher, T. A., Gao, F., Guo, Y., Feuerstein, G. Z., Ruffolo, R. R., Barone, F. C., and Yue, T.-L. (1997b). Hypercholesterolemia impairs a detoxification mechanism against peroxynitrite and renders the vascular tissue more susceptible to oxidative injury. *Circ. Res.* **80,** 894–901.

McEver, R. P., and Cummings, R. D. (1997). Role of PSGL-1 binding to selectins in leukocyte recruitment. *J. Clin. Invest.* **100,** 485–492.

McIntyre, T. M., Patel, K. D., Zimmerman, G. A., and Prescott, S. M. (1995). Oxygen radical-mediated leukocyte adherence. *In* ''Physiology and Pathophysiology of Leukocyte Adhesion'' (D. N. Granger and G. W. Schmid-Schönbein, eds.), pp. 261–277. Oxford Univ. Press, New York.

Massey, K. D., Strieter, R. M., Kunkel, S. L., Danforth, J. M., and Standiford, T. J. (1995). Cardiac myocytes release leukocyte-stimulating factors. *Am. J. Physiol. Heart Circ. Physiol.* **269,** H980–H987.

Mizuno, A., Baretti, R., Buckberg, G. D., Young, H. H., Vinten-Johansen, J., Ma, X.-L., and Ignarro, L. J. (1997). Endothelial stunning and myocyte recovery after reperfusion of jeopardized muscle: A role of L-arginine blood cardioplegia. *J. Thorac. Cardiovasc. Surg.* **113,** 379–389.

Moore, K. L., Patel, K. D., Bruehl, R. E., Fugang, L., Johnson, D. A., Lichenstein, H. S., Cummings, R. D., Bainton, D. F., and McEver, R. P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J. Cell Biol.* **128,** 661–671.

Nakamura, M., Thourani, V. H., Ronson, R. S., Velez, D., Ma, X.-L., Brown, L. A. S., Guyton, R. A., and Vinten-Johansen, J. (1999). Adjunct glutathione reverses endothelial damage from peroxynitrite, the by-product of nitric oxide degradation, in crystalloid cardioplegia. *Circulation* **100,** I-227.

Nakanishi, K., Vinten-Johansen, J., Lefer, D. J., Zhao, Z.-Q., Fowler III, W. C., McGee, D. S., and Johnston, W. E. (1992). Intracoronary L-arginine during reperfusion improves endothelial function and reduces infarct size. *Am. J. Physiol.* **263,** H1650–H1658.

Nakanishi, K., Zhao, Z.-Q., Vinten-Johansen, J., Lewis, J. C., McGee, D. S., and Hammon, J. W. J. (1994). Coronary artery endothelial dysfunction after ischemia, blood cardioplegia, and reperfusion. *Ann. Thorac. Surg.* **58,** 191–199.

Nakanishi, K., Zhao, Z.-Q., Vinten-Johansen, J., Hudspeth, D. A., McGee, D. S., and Hammon, J. W., Jr. (1995). Blood cardioplegia enhanced with nitric oxide donor SPM-5185 counteracts postischemic endothelial and ventricular dysfunction. *J. Thorac. Cardiovasc. Surg.* **109,** 1146–1154.

Nerem, R. M., Harrison, D. G., Taylor, W. R., and Alexander, R. W. (1993). Hemodynamics and vascular endothelial biology. [Review.] *J. Cardiovasc. Pharmacol.* **21**(Suppl. 1), S6–S10.

Nossuli, T. O., Hayward, R., Jensen, D., Scalia, R., and Lefer, A. M. (1998). Mechanisms of cardioprotection by peroxynitrite in myocardial ischemia and reperfusion injury. *Am. J. Physiol.* **275,** H509–H519.

Ohashi, Y., Kawashima, S., Hirata, K., Akita, H., and Yokoyama, M. (1997). Nitric oxide inhibits neutrophil adhesion to cytokine-activated cardiac myocytes. *Am. J. Physiol.* **272,** H2807–H2814.

Opie, L. H. (1991). Reperfusion injury—fad, fashion, or fact? *Cardiovasc. Drugs Ther.* **5**(Suppl. 2), 223–224.

Pabla, R., and Curtis, M. J. (1996). Effect of endogenous nitric oxide on cardiac systolic and diastolic function during ischemia and reperfusion in the rat isolated perfused heart. *J. Mol. Cell. Cardiol.* **28,** 2111–2121.

Pabla, R., Buda, A. J., Flynn, D. M., Salzberg, D. B., and Lefer, D. J. (1995). Intracoronary nitric oxide improves postischemic coronary blood flow and myocardial contractile function. *Am. J. Physiol.* **269,** H1113–H1121.

Pabla, R., Buda, A. J., Flynn, D. M., Blesse, S. A., Shin, A. M., Curtis, M. J., and Lefer, D. J. (1996). Nitric oxide attenuates neutrophil-mediated myocardial contractile dysfunction after ischemia and reperfusion. *Circ. Res.* **78,** 65–72.

Palmer, R. M. J., Rees, D. D., Ashton, D. S., and Moncada, S. (1988). L-Arginine is the physiological precursor for the formation of nitric

oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.* **153,** 1251–1256.

Patel, V. C., Woolfson, R. G., Neild, G. H., and Yellon, D. M. (1993a). Reduction of infarct size following nitric oxide inhibition is due to compensatory adenosine release. *Circulation* **88,** I–330 (Abstract).

Patel, V. C., Yellon, D. M., Singh, K. J., Neild, G. H., and Woolfson, R. G. (1993b). Inhibition of nitric oxide limits infarct size in the *in situ* rabbit heart. *Biochem. Biophys. Res. Commun.* **194,** 234–238.

Pinckard, R. N., O'Rourke, R. A., Crawford, M. H., Grover, F. S., McManus, L. M., Gaidoni, J. J., Storrs, S. B., and Olson, M. S. (1980). Complement localization and mediation of ischemic injury in baboon myocardium. *J. Clin. Invest.* **66,** 1050–1056.

Przyklenk, K. (1997). Lethal myocardial ''reperfusion injury'': The opinions of good men. *J. Thrombosis Thrombolysis* **4,** 5–6.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288,** 481–487.

Reimer, K. A., and Jennings, R. B. (1979). The ''wavefront phenomenon'' of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardial at risk) and collateral flow. *Lab. Invest.* **40,** 633–644.

Robicsek, F., and Schaper, J. (1997). Reperfusion injury: Fact or myth? *J. Cardiac Surg.* **12,** 133–137.

Ronson, R. S., Thourani, V. H., Ma, X.-L., Katzmark, S., Shearer, S. T., Zhao, Z.-Q., Nakamura, M., Guyton, R. A., and Vinten-Johansen, J. (1998). Peroxynitrite is beneficial in blood cardioplegia, but is deleterious in crystalloid cardioplegia *in vivo*. *Circulation* **98,** I-750 (Abstract).

Rubanyi, G. M. and Vanhoutte, P. M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.* **250,** H822–H827.

Rubanyi, G. M., Ho, E. H., Cantor, E. H., Lumma, W. C., and Parker-Botelho, L. H. (1989). Cytoprotective functions of nitric oxide: Inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* **181,** 1392–1397.

Sato, H., Zhao, Z.-Q., McGee, D. S., Williams, M. W., Hammon, J. W., Jr., and Vinten-Johansen, J. (1995a). Supplemental L-arginine during cardioplegic arrest and reperfusion avoids regional postischemic injury. *J. Thorac. Cardiovasc. Surg.* **110,** 302–314.

Sato, H., Zhao, Z.-Q., McGee, D. S., Williams, M. W., Hammon, J. W., Jr., and Vinten-Johansen, J. (1995b). Supplemental L-arginine during cardioplegic arrest and reperfusion avoids regional postischemic injury. *J. Thorac. Cardiovasc. Surg.* **110,** 302–314.

Sato, H., Zhao, Z.-Q., McGee, D. S., Williams, M. W., Vinten-Johansen, J., and Hammon, J. W., Jr. (1995c). Supplementation of blood cardioplegia with L-arginine avoids regional postischemic injury by NO pathway. *Ann. Thorac. Surg.* **60,** 871 (Abstract).

Sato, H., Zhao, Z.-Q., and Vinten-Johansen, J. (1996). L-Arginine inhibits neutrophil adherence and coronary artery dysfunction. *Cardiovasc. Res.* **31,** 63–72.

Sato, H., Zhao, Z.-Q., Jordan, J. E., Todd, J. C., Riley, R. D., Taft, C. S., Hammon, J. W., Jr., Li, P., Ma, X.-L., and Vinten-Johansen, J. (1997a). Basal nitric oxide expresses endogenous cardioprotection during reperfusion by inhibition of neutrophil-mediated damage after surgical revascularization. *J. Thoracic. Cardiovasc. Surg.* **113,** 399–409.

Sato, H., Zhao, Z.-Q., Jordan, J. E., Todd, J. C., Riley, R. D., Taft, C. S., Hammon, J. W., Jr., Li, P., Ma, X.-L., and Vinten-Johansen, J. (1997b). Basal nitric oxide expresses endogenous cardioprotection during reperfusion by inhibition of neutrophil-mediated damage after surgical revascularization. *J. Thorac. Cardiovasc. Surg.* **113,** 399–409.

Schulz, R., and Wambolt, R. (1995). Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia–reperfusion injury. *Cardiovasc. Res.* **30,** 432–439.

Siegfried, M. R., Erhardt, J., Rider, T., Ma, X.-L., and Lefer, A. M. (1992a). Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia–reperfusion. *J. Pharmacol. Exp. Ther.* **260,** 668–675.

Siegfried, M. R., Carey, C., Ma, X.-L., and Lefer, A. M. (1992b). Beneficial effects of SPM-5185, a cysteine-containing NO donor in myocardial ischemia–reperfusion. *Am. J. Physiol.* **263,** H771–H777.

Sluiter, W., Pietersma, A., Lamers, J. M. J., and Koster, J. F. (1993). Leukocyte adhesion molecules on the vascular endothelium: Their role in the pathogenesis of cardiovascular disease and the mechanisms underlying their expression. *J. Cardiovasc. Pharmacol.* **22**(Suppl. 4), S37–S44.

Smith, C. W., Entman, M. L., Lane, C. L., Beaudet, A. L., Ty, T. I., Youker, K., Hawkins, H. K., and Anderson, D. C. (1991). Adherence of neutrophils to canine cardiac myocytes in vitro is dependent on intercellular adhesion molecule-1. *J. Clin. Invest.* **88,** 1216–1223.

Smith, T. P., Jr., and Canty, J. M., Jr. (1993). Modulation of coronary autoregulatory responses by nitric oxide. Evidence for flow-dependent resistance adjustments in conscious dogs. *Circ. Res.* **73,** 232–240.

Sobey, C. G., Dusting, G. J., Grossman, H. J., and Woodman, O. L. (1990). Impaired vasodilation of epicardial coronary arteries and resistance vessels following myocardial ischemia and reperfusion in anesthetized dogs. *Coronary Artery Dis.* **1,** 363–374.

Steinberg, J. B., Kapelanski, D. P., Olson, J. D., and Weiler, J. M. (1993). Cytokine and complement levels in patients undergoing cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.* **106,** 1008–1016.

Stewart, J. M., Wang, J., and Hintze, T. H. (1994). Role of EDRF in the regulation of shear rate in large coronary arteries in conscious dogs. *J. Mol. Cell. Cardiol.* **26,** 1625–1633.

Sun, W., and Wainwright, C. L. (1997). The role of nitric oxide in modulating ischaemia-induced arrhythmias in rats. *J. Cardiovasc. Pharmacol.* **29,** 554–562.

Thourani, V. H., Nakamura, M., Duarte, I. G., Bufkin, B. L., Zhao, Z.-Q., Jordan, J. E., Shearer, S. T., Guyton, R. A., and Vinten-Johansen, J. (1999). Ischemic preconditioning attenuates postischemic coronary artery endothelial dysfunction in a model of minimally invasive direct coronary artery bypass. *J. Thorac. Cardiovasc. Surg.* **117,** 383–389.

Tsao, P. S., and Lefer, A. M. (1990). Time course and mechanism of endothelial dysfunction in isolated ischemic- and hypoxic-perfused rat hearts. *Am. J. Physiol.* **259,** H1660–H1666.

Tsao, P. S., Aoki, N., Lefer, D. J., Johnson III, G., and Lefer, A. M. (1990). Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation* **82,** 1402–1412.

Vaage, J., and Valen, G. (1993). Pathophysiology and mediators of ischemia–reperfusion injury with special reference to cardiac surgery. A review. [Review.] *Scand. J. Thorac. Cardiovasc. Surg. (Suppl.)* **41,** 1–18.

Van Der Vliet, A., Smith, D., O'Neill, C. A., Kaur, H., Darley-Usmar, V., Cross, C. E., and Halliwell, B. (1994). Interactions of peroxynitrite with human plasma and its constituents: Oxidative damage and antioxidant depletion. *Biochem. J.* **303,** 295–301.

VanBenthuysen, K. M., McMurtry, I. F., and Horwitz, L. D. (1987). Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity in vitro. *J. Clin. Invest.* **79,** 265–274.

Videm, V., Fosse, E., Mollnes, T. E., Garred, P., and Svennevig, J. L. (1990). Complement activation with bubble and membrane oxygenators in aortocoronary bypass grafting. *Ann. Thorac. Surg.* **50,** 387–391.

Vinten-Johansen, J., and Cordell, A. R. (1992). Experimental and clinical aspects of myocardial preservation. *In* ''Cardiac Surgery: State of the Art Reviews'' (W. Myers, ed.), pp. 315–349. Hanley & Belfus, Philadelphia, Pennsylvania.

Vinten-Johansen, J., and Hammon, J. W., Jr. (1993). Myocardial protection during cardiac surgery. *In* ''Cardiopulmonary Bypass: Principles and Practice'' (G. P. Gravlee, R. F. Davis, and J. R. Utley, eds.), pp. 155–206. Williams & Wilkins, New York.

Vinten-Johansen, J., Zhao, Z.-Q., Nakamura, M., Jordan, J. E., Ronson, R. S., Thourani, V. H., and Guyton, R. A. (1999). Nitric oxide and the vascular endothelium in myocardial ischemia–reperfusion injury. *In* ''Heart in Stress'' (Anonymous), pp. 354–370. Annals of the New York Academy of Sciences, New York.

Weyrich, A. S., Ma, X.-L., and Lefer, A. M. (1992). The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation* **86,** 279–288.

Weyrich, A. S., Ma, X.-L., Lefer, D. J., Albertine, K. H., and Lefer, A. M. (1993). In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J. Clin. Invest.* **91,** 2620–2629.

Weyrich, A. S., Ma, X.-L., Buerke, M., Murohara, T., Armstead, V. E., Lefer, A. M., Nicolas, J. M., Thomas, A. P., Lefer, D. J., and Vinten-Johansen, J. (1994). Physiological concentrations of nitric oxide do not elicit an acute negative inotropic effect in unstimulated cardiac muscle. *Circ. Res.* **75,** 692–700.

Weyrich, A. S., Buerke, M., Albertine, K. H., and Lefer, A. M. (1995). Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of the ischemic feline myocardium. *J. Leukocyte Biol.* **57,** 45–55.

Williams, M. W., Taft, C. S., Ramnauth, S., Zhao, Z.-Q., and Vinten-Johansen, J. (1995). Endogenous nitric oxide (NO) protects against ischaemia–reperfusion injury in the rabbit. *Cardiovasc. Res.* **30,** 79–86.

Wilson, I., Gillinov, A. M., Curtis, W. E., DiNatale, J., Burch, R. M., Gardner, T. J., and Cameron, D. E. (1993). Inhibition of neutrophil adherence improves postischemic ventricular performance of the neonatal heart. *Circulation* **88,** II372–9.

Woolfson, R. G., Patel, V. C., Neild, G. H., and Yellon, D. M. (1995a). Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation* **91,** 1545–1551.

Woolfson, R. G., Patel, V. C., Neild, G. H., and Yellon, D. M. (1995b). Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation* **91,** 1545–1551.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6770–6774.

Yasmin, W., Strynadka, K. D., and Schulz, R. (1997). Generation of peroxynitrite contributes to ischemia–reperfusion injury in isolated rat hearts. *Cardiovasc. Res.* **33,** 422–432.

Youker, K., Smith, C. W., Anderson, D. C., Miller, D., Michael, L. H., Rossen, R. D., and Entman, M. L. (1992). Neutrophil adherence to isolated adult cardiac myocytes. Induction by cardiac lymph collected during ischemia and reperfusion. *J. Clin. Invest.* **89,** 602–609.

Zhao, Z.-Q., Nakamura, M., Wang, N.-P., Wilcox, J. N., Katzmark, S., Guyton, R. A., and Vinten-Johansen, J. (1998). Infarct extension and dynamic coronary endothelial dysfunction in the late reperfusion phase. *Circulation* **98,** I-796 (Abstract).

Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987). Direct measurement of free radicals generated following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci.* **84,** 1404–1407.

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CHAPTER 42

Use of Mutant Mice to Elucidate Neuroprotective and Neurotoxic Actions of Nitric Oxide in Cerebral Ischemia

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THE ROLE OF NITRIC OXIDE (NO) IN ISCHEMIC PHYSIOPATHOLOGY HAS GENERATED CONSIDERABLE CONTROVERSY BECAUSE NO MAY POSITIVELY IMPACT THE OUTCOME OF ISCHEMIA AS A POWERFUL VASODILATOR AND INHIBITOR OF BOTH PLATELET AGGREGATION AND PLATELET AND LEUKOCYTE ADHESION. NO ALSO PROMOTES TISSUE INJURY BY GENERATION OF OXYGEN RADICALS. USING NEURONAL NOS (NNOS) AND ENDOTHELIAL NOS (ENOS) KNOCKOUT MICE, WE DOCUMENTED THE DUAL ROLE OF NO IN CEREBRAL INFARCTION. NNOS KNOCKOUTS DEVELOPED 38% SMALLER INFARCTS THAN WILD TYPE AFTER PERMANENT MIDDLE CEREBRAL ARTERY (MCA) OCCLUSION. INFARCTS WERE 69% SMALLER IN NNOS KNOCKOUTS SUBJECTED TO TRANSIENT FOCAL ISCHEMIA, WITH LESS BRAIN EDEMA. BASAL CGMP LEVELS WERE LOWER IN MUTANTS AND DID NOT ENHANCE DURING ISCHEMIA CONTRARY TO THE ROBUST INCREASE IN WILD-TYPE MICE. NEUROLOGICAL DEFICITS ALSO WERE LESS. ADMINISTERING NITRO-L-ARGININE ELIMINATED THE RESISTANCE OF NNOS KNOCKOUTS TO FOCAL ISCHEMIA, PRESUMABLY BY INHIBITION OF ENOS, AND ITS NEGATIVE IMPACT ON CEREBRAL BLOOD FLOW, WBC, AND PLATELET FUNCTION. IN LINE WITH THIS EVIDENCE, LARGER INFARCTS DEVELOPED IN ENOS KNOCKOUT MICE AFTER MCA OCCLUSION. ENOS MUTANTS DISPLAYED MORE PRONOUNCED RCBF REDUCTIONS AFTER MCA OCCLUSION AND EXHIBITED LOWER RCBFS AT REDUCED PERFUSION PRESSURES DURING CONTROLLED HEMORRHAGIC HYPOTENSION. DYNAMIC CT SCANNING DEMONSTRATED THAT THE HEMODYNAMIC PENUMBRA WAS SIGNIFICANTLY SMALLER AND CORE LARGER IN ENOS MUTANTS COMPARED TO WILD-TYPE MICE. UNLIKE THE NNOS MUTANT, L-NA DECREASED INFARCT SIZE IN THE ENOS MUTANT, POSSIBLY BY INHIBITING NNOS. DNA DAMAGE WAS ALSO LESS IN NNOS KNOCKOUT ANIMALS, AS INDICATED BY FEWER POLY(ADP)RIBOSE POSITIVE CELLS COMPARED TO THEIR LITTERMATE CONTROLS. NUMBERS OF APOPTOTIC CELLS WERE SIGNIFICANTLY LOWER IN NNOS MUTANTS, SUGGESTING THAT EXCESS AMOUNTS OF NO GENERATED BY NNOS DURING CEREBRAL ISCHEMIA CAUSES CELL DEATH BY APOPTOSIS AS WELL AS NECROSIS. STRATEGIES AIMED AT UPREGULATING VASCULAR NO SYNTHESIS AND/OR INHIBITING PARENCHYMAL NO GENERATION ARE PROMISING TREATMENTS IN ACUTE ISCHEMIC INJURY.

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Introduction

The role of nitric oxide (NO) in ischemic pathophysiology has generated considerable controversy because NO positively impacts the outcome of ischemia (e.g., as a powerful vasodilator and inhibitor of platelet aggregation, platelet adhesion, and leukocyte adhesion) but also promotes tissue injury (by generating peroxynitrite, a major mediator of cytotoxicity) (Dalkara and Moskowitz, 1994, 1997; Table I). To add to the complexity, NO protects tissues by scavenging superoxide (Niu *et al.,* 1994). Similarly, NO has been shown in part to mediate *N*-methyl-D-aspartate (NMDA) toxicity, but, on the other hand, its cationic redox form blocks NMDA receptor activation (Dawson *et al.,* 1991, 1996; Lipton *et al.,* 1993; Ayata *et al.,* 1997). More interestingly, peroxynitrite in small amounts protects against myocardial ischemia–reperfusion injury (Nossuli *et al.,* 1998). Moreover, NO donor agents administered during reperfusion protected pancreas (Benz *et al.,* 1998), liver (Ohmori *et al.,* 1998), and hind limb (Johnson *et al.,* 1998), whereas nitric oxide synthase (NOS) inhibitors protected against mesenteric ischemia (Takada *et al.,* 1998; Cuzzocrea *et al.,* 1998).

Possibly as a result of these opposing actions of NO, studies using NOS inhibitors or NO donor agents have produced conflicting results. The predominant effect may depend on the protocol and tissue type. For instance, the beneficial effects of neuronal NOS (nNOS) inhibition may be masked by drug-induced decreases in ischemic blood flow due to endothelial NOS (eNOS) inhibition. In ischemic brain, where neurons generate NO, the problem is equally complex. L-Nitroarginine methyl ester (L-NAME) or L-N^G-nitroarginine administration increases or decreases tissue injury in models of ischemia following middle cerebral artery (MCA) occlusion (for review, see Dalkara and Moskowitz, 1994). We have documented the detrimental role of neuronal NO and the protective effect of endothelial-derived NO on the development of cerebral infarction using mutant mice that do not express the gene encoding the neuronal or endothelial NOS isoform (Huang *et al.,* 1994, 1996; Table II). This approach helped to elucidate the relative importance of the two or more opposing actions of NO generated by the isoforms during ischemia and reperfusion. The evidence will be reviewed below.

Table I Positive and Negative Impacts of NO on Cerebral Ischemia

Positive	Negative
Enhancement of rCBF[a]	Disruption of cellular metabolism
Inhibition of platelet aggregation	Inhibition of DNA synthesis
Inhibition of platelet and neurophil adhesion	Triggering Haber–Weiss reaction by releasing intracellular iron
Scavenging superoxide	Peroxynitrite formation
Inhibition of NMDA[b] current (NO^+ only)	DNA damage
	Activation of PARP[c]
	Induction of apoptosis

[a] rCBF, regional cerebral blood flow.
[b] NMDA, *N*-methyl-D-aspartate.
[c] PARP, poly(ADP-ribose) polymerase.

Table II Effect of Nitro-L-arginine on Infarct Size 24 Hours after Permanent MCA Occlusion in the Wild-type (SV-129) and Mutant Mouse Lacking Expression of Neuronal or Endothelial Isoforms of NOS

	Infarct size[a]		
		Mutants	
Treatment	Wild type	nNOS (−)	eNOS (−)
Vehicle	L	S	VL
After nitro-L-arginine	nc	L[b]	L[b]

[a] L, large infarct; S, small infarct; VL, very large infarct; nc, no change.
[b] $p < 0.05$ as compared to vehicle.

NO-Mediated Toxicity

NOS-containing neurons comprise 1–2% of all the neurons in the cerebral cortex, corpus striatum, and hippocampus; however, they extensively branch among neighboring neurons (Bredt *et al.,* 1991; Fischer and Kuljis, 1994). This configuration led to the idea that NOS neurons can kill bordering cells when they produce NO in excessive amounts (Bredt *et al.,* 1991; Dawson and Dawson, 1995). Interestingly, these neurons themselves are resistant to various insults, including NMDA toxicity, cell death in Huntington's disease, and ischemia (Koh and Choi, 1988; Uemura *et al.,* 1990; Gonzalez-Zulueta *et al.,* 1998).

NO may cause cytotoxicity by several mechanisms, including disruption of cellular metabolism, inhibition of DNA synthesis, and damage to DNA structure (Dawson and Dawson, 1995; Liu and Hotchkiss, 1995). DNA damage leads to activation of a DNA repairing enzyme, poly(ADP-ribose) polymerase (PARP), which consumes high amounts of ATP and NAD^+ and may facilitate collapse of energy metabolism and cell death (J. Zhang *et al.,* 1994). In addition to promoting necrotic cell death, NO may cause death by an apoptotic process (Albina *et al.,* 1993; Ankarcrona *et al.,* 1994; Estevez *et al.,* 1995; Lin *et al.,* 1995). Formation of peroxynitrite ($ONOO^-$) by interaction of NO with superoxide anion appears to be a major cell death mediator (Beckman and Koppenol, 1996; Beckman *et al.,* 1996; Kamii *et al.,* 1996). In a superoxide-rich environment, NO may also release intracellular iron, thereby triggering the Haber–Weiss reaction and formation of reactive oxygen species (Gross and Wolin, 1995).

NO and Cerebral Ischemia

Neurons, perivascular nerves, and cerebrovascular endothelium may generate NO during cerebral ischemia. It is likely that constitutive NOS activity increases during ischemia owing to a rise in intracellular Ca^{2+}. Constitutive NOS is activated by intracellular Ca^{2+} concentrations slightly above the resting level and is maximally stimulated at 0.5 μ*M* (Knowles *et al.,* 1989), suggesting that it is fully active during focal ischemia, in which much higher Ca^{2+} levels are attained in neurons (10–100 μ*M*). This hypothesis is supported by a striking increase in cortical NO levels from approximately 10 n*M* to 2.2 μ*M* within 3 to 24 min after MCA occlusion (Malinski *et al.,* 1993). With use of the same porphyrinic microsensor technique, a rapid increase in nitric oxide was measured in femoral artery wall subjected to ischemia (from 50 to 450 nmol/liter) (Huk *et al.,* 1998). Brain nitrite (stable NO metabolite) and cGMP (a product of NO action) levels also rise within the first half hour of MCA occlusion (Kader *et al.,* 1993; Huang *et al.,* 1994). These increases are effectively blocked by prior L-N^G-nitroarginine administration, indicating enhanced NOS activity. Similar increases in NO (Olesen *et al.,* 1997) and in nitrate/nitrite levels (Togashi *et al.,* 1998; Rao *et al.,* 1998) have also been reported in global ischemia.

Constitutive NOS activity decreases shortly after its activation at the onset of ischemia (Kader *et al.,* 1993; Malinski *et al.,* 1993; Huk *et al.,* 1998). Depression of constitutive NOS activity was reported to continue up to 10 days following ischemia (Grandati *et al.,* 1997). It is possible that NOS activity may differ between the core and penumbra. Ohta *et al.* (1997) found a brief NO burst in areas where regional cerebral blood flow (rCBF) dropped to 9% of the basal level but a sustained enhancement in NO synthesis in areas where rCBF was 16%. Higher nitrotyrosine formation was detected in the penumbra than in the core region (Fukuyama *et al.,* 1998). It has been reported that nNOS generates NO in proportion to the O_2 concentration over an extremely wide range (Abu-Soud *et al.,* 1996). This feature enables nNOS to couple its rate of NO synthesis to the O_2 concentration. The nNOS isoform has an estimated K_mO_2 of ~400 μ*M* (saturation at ~800 μ*M*). In the absence of L-arginine, estimated K_mO_2 is ≤40 μ*M* (saturation at ~100 μ*M*). Under conditions of arginine or tetrahydrobiopterin deficiency, NOS generates superoxide, which may directly contribute to toxicity or react with NO to form peroxynitrite (Abu-Soud *et al.,* 1996; Xia *et al.,* 1996. 3-Nitrotyrosine staining, reflecting peroxynitrite formation, was identified in ischemic tissue by immunohistochemistry (Eliasson *et al.,* 1999). A late but sustained increase in NO levels may also occur due to expression of inducible NOS within microglia, invading inflammatory cells, and cerebrovascular endothelium 24–72 hours after the induction of MCA occlusion (Iadecola *et al.,* 1995; Galea *et al.,* 1998).

During the immediate period following ischemia, NO production may improve blood flow and be neuroprotective. Indeed, infusion of L-arginine dilates pial vessels via an NO-dependent mechanism, increases rCBF in normal as well as in ischemic brain, and reduces infarct size (Morikawa *et al.,* 1994). L-Arginine leads to electrocorticogram recovery if blood flow enhancement exceeds the functional flow threshold of approximately 30% of preischemic levels (Dalkara *et al.,* 1994). Importantly, L-arginine infusion did not augment blood flow in eNOS −/− mice. However, this favorable action of L-arginine is not sustained during ischemia, owing to rapid inactivation of eNOS (Dalkara *et al.,* 1994; Kirsch *et al.,* 1997). Intracarotid administration of NO donor agents, on the other hand, leads to blood flow increases within the ischemic tissue for at least 1 hour after arterial occlusion and a decrease in infarct size in models of focal ischemia (F. Zhang *et al.,* 1994). In addition to its blood flow-enhancing effect, endothelial NO production may also improve microcirculation by reducing platelet aggregation and leukocyte adhesion (Niu *et al.,* 1994; Gidday *et al.,* 1998). Sodium nitroprusside was shown to improve rCBF in ischemic brain areas and inhibit platelet aggregation and adhesion molecule expression in stroke patients (Butterworth *et al.,* 1998). Selective eNOS upregulation might also provide a useful treatment strategy to enhance vascular NO production. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been shown to selectively upregulate endothelial NOS with an action independent of their cholesterol lowering effect, and they have been shown to confer protection in rodent stroke models (Endres *et al.,* 1998a). Supporting this idea, a clinical trial (CARE study) demonstrated that stroke incidence decreased by 31% in 4159 subjects treated chronically with pravastatin (Sacks *et al.,* 1996). Interestingly, a point mutation in the eNOS gene has been reported to be associated with coronary artery spasm (Yoshimura *et al.,* 1998).

Overproduction of NO by neurons during the early stages of ischemia may be detrimental to neuronal survival by accelerating cellular metabolism and/or damaging DNA. Peroxynitrite may be a major mediator of cell death (Beckman *et al.,* 1996; Kamii *et al.,* 1996; Endres *et al.,* 1998b; Forman *et al.,* 1998). A second wave of NO and peroxynitrite generation may take place during reperfusion if constitutive NOS is reactivated (Fukuyama *et al.,* 1998; Higuchi *et al.,* 1998). However, NO formation may also benefit the microcirculation during reperfusion. The net outcome, determined in part by duration and depth of ischemia, is impacted by NOS activity, L-arginine depletion, superoxide production, and formation of platelet and neutrophil aggregates.

Knockout Mice

Research since the 1980s has clearly documented the importance of genetically engineered mice as a promising tool for elucidating the importance of a targeted gene. Several mutant strains were generated in the 1990s (Thomas, 1995; Rubin and Barsh, 1996) by disruption of one (sometimes

two) of an estimated 50,000–100,000 mouse genes by homologous reconstitution (Fässler *et al.,* 1995). The majority of available mutants have ''null'' (or loss-of-function) alleles of the gene. Null mutants have been developed to study the role of proteins during development, in adult life, and in disease states. Surprisingly, many knockouts exhibit a normal phenotype, most likely owing to redundancy of genes compensating for the deleted gene product (e.g., Huang *et al.,* 1993). However, increasing evidence indicates that, despite an apparently normal phenotype, knockout animals may display conspicuous abnormalities in unanticipated tissues (e.g., Huang *et al.,* 1993) and respond differently than wild-type animals to various manipulations (e.g., Irikura *et al.,* 1995). They have been especially useful in confirming the pharmacological activity of drugs whose selectivity is in question. For example, basic fibroblast growth factor (bFGF) has been demonstrated to protect the brain against ischemia (Koketsu *et al.,* 1994; Fisher *et al.,* 1995; Jiang *et al.,* 1996). However, its mode of action was not clear because part of this protective effect might be due to its vasodilatory action and part might be due to trophic action, as suggested by the considerable neuroprotection obtained with its intracerebroventricular administration. Knockout mice that do not express the gene for eNOS helped to elucidate this controversy; they did not show cerebrovasodilation in response to bFGF administration, yet they developed significantly smaller infarcts when subjected to MCA occlusion (Huang *et al.,* 1997).

Neuronal NO Synthase Mutant Mice

Targeted disruption of the gene encoding nNOS was achieved using homologous recombination by substitution of a neomycin resistance gene for the first protein coding exon (Huang *et al.,* 1993). The mutant mouse develops normally, and the endothelium of nNOS knockouts expresses eNOS immunoreactivity (Irikura *et al.,* 1995). In nNOS mutants, neuronal NOS expression and NADPH diaphorase staining were markedly deficient, and *in vitro* NOS activity was significantly reduced (Huang *et al.,* 1993). Interestingly, alternative splice variants have been reported that generate NO *in vitro* but lack the PDZ-containing domain and potential coupling to the NMDA receptor (Brenman *et al.,* 1996; Eliasson *et al.,* 1997a). However, the expressed splice variants do not compensate sufficiently, as NOS activity in nNOS −/− brains is severely reduced (<5–8% of normal). Consistent with this, low basal levels of L-N^G-[^{3}H] nitroarginine binding (Hara *et al.,* 1997) and brain cGMP levels were found in mutants, and no cGMP enhancement was observed during ischemia or hypercapnia, contrary to the robust increases in wild-type mice (Huang *et al.,* 1994; Irikura *et al.,* 1995). Consistent results were also obtained by NMR spectroscopy, as NO adducts were undetectable during cerebral ischemia as measured in nNOS null mice (Mullins *et al.,* unpublished observations). Regional cerebral blood flow responses to hypercapnia (Irikura *et al.,* 1995) and to whisker stimulation (Ma *et al.,* 1996) and pial arterial dilation to topical acetylcholine (ACh) superfusion (Meng *et al.,* 1996) were not significantly different from wild-type mice, although these were markedly reduced after administering NOS inhibitors.

Neuronal NO Synthase Mutant Mice and Cerebral Ischemia

The deficiency in neuronal NO production is associated with increased resistance to cerebral ischemia. The nNOS knockouts develop infarcts 38% smaller than the wild-type mice when subjected to 24-hour permanent MCA occlusion (Huang *et al.,* 1994). Infarct size is also reduced in mutants 3 to 4 days after permanent MCA occlusion. Neurological deficits are less in nNOS knockout mice. Because reductions in regional cerebral blood are similar within homologous ischemic regions after MCA occlusion in both groups, the observed group differences were attributed to the consequence of neuronal NOS deletion in brain tissue and not to hemodynamic differences between strains.

Infarcts were 69% smaller in nNOS knockouts than in wild-type mice in animals subjected to 3 hours of ischemia and 24 hours of reperfusion (Hara *et al.,* 1996). Brain protection was greater than after permanent occlusion, possibly due to greater superoxide anion production during reversible occlusion (Chan *et al.,* 1993; Yang *et al.,* 1994; Chan, 1996). Interestingly, quantitative L-N^G-[^{3}H]nitroarginine autoradiography demonstrated a significant increase (50–250%) in the density of L-N^G-[^{3}H]nitroarginine binding sites (B_{max}), but not the dissociation constant (K_d), during transient focal ischemia and first 3 hours of reperfusion (Hara *et al.,* 1997). nNOS mRNA was also increased as detected by reverse transcription–polymerase chain reaction. As noted earlier, L-N^G-[^{3}H]nitroarginine binding to nNOS protein was very low in nNOS mutant mice, and only a very small increase was observed after ischemia or NMDA excitotoxicity.

nNOS mutants are also more resistant to global ischemia (Panahian *et al.,* 1996). Fewer dead hippocampal neurons were counted 3 days after transient global ischemia induced by bilateral common carotid plus basilar artery occlusion for 5 or 10 min. Not only were more hippocampal cells viable, but overall morbidity, weight loss, and neurological outcome were better in the mutant strain.

Endothelial NO Synthase Mutant Mice and Cerebral Ischemia

Consistent with the evidence just given, larger infarcts developed in eNOS knockout mice after 24-hour permanent MCA occlusion (Huang *et al.,* 1996). Deletion of eNOS rendered these mutants hypertensive (Huang *et al.,* 1995). However, hypertension per se did not account for the increased susceptibility of eNOS mutants, because infarct size did not

decrease after blood pressure was reduced by hydralazine (Huang *et al.,* 1996). eNOS mutants developed more pronounced rCBF reductions in corresponding brain regions after MCA occlusion and exhibited proportionally lower rCBFs at reduced perfusion pressures during controlled hemorrhagic hypotension (Huang *et al.,* 1996). Dynamic computerized tomography (CT) scanning demonstrated that areas of hemodynamic penumbra were significantly smaller and the core relatively larger in eNOS mutants (Lo *et al.,* 1996). Hence, the susceptibility of eNOS mutants to ischemic injury may be due to its diminished capacity to adapt to reduced perfusion pressure (i.e., dilate) at the margins of an ischemic lesion. This, coupled with enhanced platelet and neutrophil adhesion, renders eNOS mutants more susceptible to injury. Consistent with this notion, L-N^G-nitroarginine administration increased infarct size in the nNOS knockout mouse, presumably due to inhibition of the constitutively expressed eNOS isoform (Huang *et al.,* 1996). Neuroprotection was obtained in ischemic injury after administering selective nNOS inhibitors, 7-nitroindazole (Yoshida *et al.,* 1994), ARL17477 (Zhang *et al.,* 1996), or 1-(2-trifluoromethylphenyl) imidazole (Escott *et al.,* 1998).

Poly(ADP-Ribose) Polymerase Activation and NO in Cerebral Ischemia

As noted earlier, one candidate pathway for NO-mediated neuronal injury is DNA damage leading to the obligatory activation of the nuclear enzyme PARP. PARP is activated in ischemic brain within minutes after reperfusion following MCA occlusion, as evidenced by poly(ADP-ribose) immunohistochemistry. Poly(ADP-ribose)-positive cells show signs of early ischemic damage. NAD^+ levels as shown by *in situ* histochemistry become depleted in the MCA territory (Endres *et al.,* 1997). Inhibition of PARP activation or disruption of the PARP gene (PARP knockouts) confers protection after brain ischemia (Endres *et al.,* 1997; Eliasson *et al.,* 1997b). Consistent with this protection, poly(ADP)ribose formation is inhibited and NAD^+ levels are significantly higher in these animals, demonstrating an energy-preserving neuroprotective mechanism. Total DNA damage, upstream to PARP activation in the cascade, is not affected by PARP inhibition or gene deleteion (Endres *et al.,* 1997).

We further established the importance of NO to the PARP activation pathway *in vivo* by evaluating poly(ADP-ribose) formation in nNOS knockout animals. Following a 2-hour MCA occlusion and reperfusion, nNOS knockout animals exhibited strikingly less poly(ADP-ribose)-positive cells compared to their littermate controls (Endres *et al.,* 1998b). Moreover, the density of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling)-positive cells is decreased after ischemia in nNOS knockout mice, indicating less DNA damage (see also next paragraph). Thus, decreased DNA damage and subsequent PARP activation may confer resistance to ischemic brain damage in nNOS knockout animals. Endres *et al.* (1998b) also tested in glioma cells whether NO or peroxynitrite was the actual trigger of PARP activation. Peroxynitrite, but not various NO donor agents, activated PARP and suppressed cellular viability in a PARP-dependent fashion.

NO-Induced Apoptotic Cell Death

At least two mechanistically distinct forms of neuronal death have been identified. Severely injured neurons that do not immediately die by swelling and lysis may ultimately undergo apoptosis. Apoptotic neuronal death contributes to infarct formation in cerebral ischemia (Li *et al.,* 1995; Charriaut-Marlangue *et al.,* 1996; MacManus and Linnik, 1997). NMDA, or concurrent generation of nitric oxide and superoxide, can cause both necrosis and apoptosis, depending on the severity of the insult and resulting mitochondrial dysfunction (Bonfoco *et al.,* 1995; Ankarcrona *et al.,* 1995). Both NO and $ONOO^-$ have been linked to apoptosis (Ankarcrona *et al.,* 1994; Estevez *et al.,* 1995; Keller *et al.,* 1998). Because blockade of neuronal NO synthesis proves to be neuroprotective in focal and global cerebral ischemia and NMDA toxicity *in vivo,* it is important to understand the extent to which NO-induced apoptosis contributes to neuronal death under these pathological conditions.

We compared the number of apoptotic cells in wild-type and nNOS mutant mice 6, 24, and 72 hours after permanent MCA occlusion by filament. TUNEL-positive neurons were detected at 6 hours and continued to increase by 72 hours. These cells were detected in both penumbra and core regions, but most were located on the inner boundary zone of the infarct. Numbers of apoptotic cells as well as their densities were significantly lower in nNOS mutants, suggesting a selective decrease in the number of TUNEL-positive cells. Such findings support the notion that NO and its reaction products promote apoptosis as a mechanism of cytotoxicity, as suggested by studies reporting apoptotic cell death after application of NO or peroxynitrite (Ratan *et al.,* 1994; Estevez *et al.,* 1995; Bonfoco *et al.,* 1995; Palluy and Rigaud, 1996; for review, see Nicotera *et al.,* 1995). Precisely how the development of apoptosis in ischemia relates to caspase activation (Namura *et al.,* 1998) and cleavage of PARP remains for further study.

References

Abu-Soud, H. M., Rousseau, D. L., and Stuehr, D. J. (1996). Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. *J. Chem. Biol.* **271,** 32515–32518.

Albina, J. E., Cui, S., Mateo, R. B., and Reichner, J. S. (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150,** 5080–5085.

Ankarcrona, M., Dypbukt, J. M., Brune, B., and Nicotera, P. (1994). Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Exp. Cell Res.* **213,** 172–177.

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. (1995). Glutamate-induced neuronal

death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15,** 961–973.

Ayata, C., Ayata, G., Hara, H., Matthews, R. T., Beal, M. F., Ferrante, R. J., Endres, M., Kim, A., Christie, R. H., Waeber, C., Huang, P. L., Hyman, B. T., and Moskowitz, M. A. (1997). Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. *J. Neurosci.* **17,** 6908–6917.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide and peroxynitrite: the good, the bad and ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Beckman, J. S., Ye, Y. Z., Chen, J., and Conger, K. A. (1996). The interaction of nitric oxide with oxygen radicals and scavengers in cerebral ischemic injury. *Adv. Neurol.* **71,** 339–350.

Benz, S., Schnabel, R., Weber, H., Pfeffer, F., Wiesner, R., von Breitenbuch, P., Nizze, H., Schareck, W., and Hopt, U. T. (1998). The nitric oxide donor sodium nitroprusside is protective in ischemia/reperfusion injury of the pancreas. *Transplantation* **66,** 994–999.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., and Lipton, S. A. (1995). Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with *N*-methy-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7162–7166.

Bredt, D. S., Glatt, E. C., Hwang, P. M., Fotuhi, M., Dawson, T. M., and Snyder, S. H. (1991). Nitric oxide synthase protein and mRNA discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* **7,** 615–624.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and a1-syntrophin mediated by PDZ domains. *Cell* **84,** 757–767.

Butterworth, R. J., Cluckie, A., Jackson, S. H., Buxton-Thomas, M., and Bath, P. M. (1998). Pathophysiological assessment of nitric oxide (given as sodium nitroprusside) in acute ischaemic stroke. *Cerebrovasc. Dis.* **8,** 158–165.

Chan, P. H. (1996). Role of oxidants in ischemic brain damage. *Stroke* **24,** 1124–1129.

Chan, P. H., Kamii, H., Yang, G., Gafni, J., Epstein, C. J., Carlson, E., and Reola, L. (1993). Brain infarction is not reduced in SOD-1 transgenic mice after a permanent focal cerebral ischemia. *NeuroReport* **5,** 293–296.

Charriaut-Marlangue, C., Margaill, I., Represa, M. A., Popovici, T., Plotkine, M., and Ben-Ari, Y. (1996). Apoptosis and necrosis after reversible focal ischemia: An in situ DNA fragmentation analysis. *J. Cereb. Blood Flow Metab.* **16,** 186–195.

Cuzzocrea, S., Zingarelli, B., and Caputi, A. P. (1998). Role of constitutive nitric oxide synthase and peroxynitrite production in a rat model of splanchnic artery occlusion shock. *Life Sci.* **63,** 789–799.

Dalkara, T., and Moskowitz, M. A. (1994). Complex role of nitric oxide in cerebral ischemia. *Brain Pathol.* **4,** 49–57.

Dalkara, T., and Moskowitz, M. A. (1997). Neurotoxic and neuroprotective roles of nitric oxide in cerebral ischaemia. *Int. Rev. Neurobiol.* **40,** 319–336.

Dalkara, T., Morikawa, H., Moskowitz, M. A., and Panahian, N. (1994). Blood flow-dependent functional recovery in a rat model of cerebral ischemia. *Am. J. Physiol.* **267,** H837–H838.

Dawson, T. M., and Dawson, V. L. (1995). Nitric oxide: Actions and pathological roles. *Neuroscientist* **1,** 7–18.

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., and Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical culture. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 6368–6371.

Dawson, V. L., Kizushi, V. M., Huang, P. L., Snyder, S. H., and Dawson, T. M. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neurosci.* **16,** 2479–2487.

Eliasson, M. J. L., Blackshaw, S., Schell, M. J., and Snyder, S. H. (1997a). Neuronal nitric oxide synthase alternatively spliced forms: Prominent functional localizations in the brain. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3396–3401.

Eliasson, M. J. L., Sampei, K., Mandir, A. S., Hurn, P. D., Traystman, R. J., Bao, J., Pieper, A., Wang, Z.-Q., Dawson, T. M., Snyder, S. H., and Dawson, V. L. (1997b). Poly(ADP-ribose) polymerase gene disruption renderes mice resistant to cerebral ischemia. *Nat. Med.* **3,** 1089–1095.

Eliasson, M. J. L., Huang, Z., Ferrante, R. J., Sasamata, M., Molliver, M. E., Snyder, S. H., and Moskowitz, M. A. (1999). Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J. Neurosci.* **19,** 5910–5918.

Endres, M., Wang, Z.-Q., Namura, S., Waeber, C., and Moskowitz, M. A. (1997). Ischemic brain injury is mediated by the activation of poly(ADP-ribose) polymerase. *J. Cereb. Blood Flow Metab.* **17,** 1143–1151.

Endres, M., Laufs, U., Huang, Z., Nakamura, T., Huang, P., Moskowitz, M. A., and Liao, J. (1998a). Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8880–8885.

Endres, M., Scott, G., Namura, S., Salzman, A. L., Huang, P. L., Moskowitz, M. A., and Szabo, C. (1998b). Role of peroxynitrite and neuronal nitric oxide synthase in the activation of poly(ADP-ribose) synthetase in a murine model of cerebral ischemia–reperfusion. *Neurosci. Lett.* **248,** 41–44.

Escott, K. J., Beech, J. S., Haga, K. K., Williams, S. C., Meldrum, B. S., and Bath, P. M. (1998). Cerebroprotective effect of the nitric oxide synthase inhibitors, 1-(2-trifluoromethylphenyl)imidazole and 7-nitroindazole, after transient focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.* **18,** 281–287.

Estevez, A. G., Radi, R., Barbeito, L., Shin, J. T., Thompson, J. A., and Beckman, J. S. (1995). Peroxynitrite-induced cytotoxicity in PC12 cells: Evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. *J. Neurochem.* **65,** 1543–1550.

Fässler, R., Martin, K., Forsberg, E., Litzenburger, T., and Iglesias, A. (1995). Knockout mice: How to make them and why. The immunological approach. *Int. Arch. Allergy Immunol.* **106,** 323–334.

Fischer, H. C., and Kuljis, R. O. (1994). Multiple types of nitrogen monoxide synthase/NADPH diaphorase containing neurons in the human cerebral neocortex. *Brain Res.* **654,** 105–117.

Fisher, M., Meadows, M. E., Do, T., Weise, J., Trubetskoy, V., Charette, M., and Finklestein, S. P. (1995). Delayed treatment with intravenous basic fibroblast growth factor reduce infarct size following permenant focal cerebral ischemia in rats. *J. Cereb. Blood Flow Metab.* **15,** 953–959.

Forman, L. J., Liu, P., Nagele, R. G., Yin, K., and Wong, P. Y. (1998). Augmentation of nitric oxide, superoxide, and peroxynitrite production during cerebral ischemia and reperfusion in the rat. *Neurochem. Res.* **23,** 141–148.

Fukuyama, N., Takizawa, S., Ishida, H., Hoshiai, K., Shinohara, Y., and Nakazawa, H. (1998). Peroxynitrite formation in focal cerebral ischemia–reperfusion in rats occurs predominantly in the peri-infarct region. *J. Cereb. Blood Flow Metab.* **18,** 123–129.

Galea, E., Golanov, E. V., Feinstein, D. L., Kobylarz, K. A., Glickstein, S. B., and Reis, D. J. (1998). Cerebellar stimulation reduces inducible nitric oxide synthase expression and protects brain from ischemia. *Am. J. Physiol.* **274,** H2035–H2045.

Gidday, J. M., Park, T. S., Shah, A. R., and Gonzales, E. R. (1998). Modulation of basal and postischemic leukocyte–endothelial adherence by nitric oxide. *Stroke* **29,** 1423–1429.

Gonzalez-Zulueta, M., Ensz, L. M., Muhkin, G., *et al.* (1998). Manganase superoxide dismutase protects nNOS neurons from NMDA and nitric oxide mediated neurotoxicity. *J. Neurosci.* **18,** 2040–2055.

Grandati, M., Verrecchia, C., Revaud, M. L., Allix, M., Boulu, R. G., and Plotkine, M. (1997). Calcium-independent NO-synthase activity and nitrites/nitrates production in transient focal cerebral ischaemia in mice. *Br. J. Pharmacol.* **122,** 625–630.

Gross, S. S., and Wolin, M. S. (1995). Nitric oxide: Pathophysiological mechanisms. *Annu. Rev. Physiol.* **57,** 737–769.

Hara, H., Huang, P. L., Panahian, N., Fishman, M. C., and Moskowitz, M. A. (1996). Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after reversible MCA occlusion. *J. Cereb. Blood Flow Metab.* **16,** 605–611.

Hara, H., Ayata, C., Huang, P. L., Waeber, C., Ayata, G., Fujii, M., and Moskowitz, M. A. (1997). [^{3}H]L-N^G-nitroarginine binding after transient focal ischemia and NMDA-induced excitotoxicity in type I and type III nitric oxide synthase null mice. *J. Cereb. Blood Flow Metab.* **17,** 515–526.

Higuchi, Y., Hattori, H., Kume, T., Tsuji, M., Akaike, A., and Furusho, K. (1998). Increase in nitric oxide in the hypoxic–ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine. *Eur. J. Pharmacol.* **342,** 47–49.

Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993). Targeted disruption of neuronal nitric oxide synthase gene. *Cell* **75,** 1273–1286.

Huang, P. L., Huang, Z., Mashimo, M., Bloch, K. D., Moskowitz, M. A., Bevan, J. S., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for nitric oxide synthase. *Nature* **377,** 239–242.

Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265,** 1883–1885.

Huang, Z., Huang, P. L., Ma, J., Meng, W., Ayata, C., Fishman, M. C., and Moskowitz, M. A. (1996). Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J. Cereb. Blood Flow Metab.* **16,** 981–987.

Huang, Z., *et al.* (1997). bFGF ameliorates focal ischemic injury by blood flow-independent mechanisms in eNOS mutant mice. *Am. J. Physiol.* **272,** H1401–H1405.

Huk, I., Brovkovych, V., Nanobash Vili, J., Weigel, G., Neumayer, C., Partyka, L., Patton, S., and Malinski, T. (1998). Bioflavonoid quercetin scavenges superoxide and increases nitric oxide concentration in ischaemia–reperfusion injury: An experimental study. *Br. J. Surg.* **85,** 1080–1085.

Iadecola, C., Xu, X., Zhang, F., el-Fakahany, E. E., and Ross, M. E. (1995). Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* **15,** 52–59.

Irikura, K., Huang, P. L., Ma, J., Lee, W. S., Dalkara, T., Fishman, M. C., Dawson, T. M., Snyder, S. H., and Moskowitz, M. A. (1995). Cerebrovascular alterations in mice lacking neuronal nitric oxide synthase gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 6823–6827.

Jiang, N., Finklestein, S. P., Do, T., Caday, C. G., Charette, M., Chopp, M. (1996). Delayed administration of basic fibroblast growth factor (bFGF) reduces infarct volume in a model of focal cerebral ischemia/reperfusion in the rat. *J. Neurol. Sci.* **139,** 173–179.

Johnson, D., Freischlag, J. A., Lesniak, R., Kelly, H., Mudaliar, J. H., Cambria, R. A., Seabrook, M. D., and Towne, J. B. (1998). Endothelial damage due to ischemia and reperfusion is prevented with SIN-1. *Cardiovasc. Surg.* **6,** 527–532.

Kader, A., Frazzini, V. I., Solomon, R. A., and Trifiletti, R. R. (1993). Nitric oxide production during focal cerebral ischemia. *Stroke* **24,** 1709–1716.

Kamii, H., Mikawa, S., Murakami, K., Kinouchi, H., Yoshimoto, T., Reola, L., Carlson, E., Epstein, C. J., Chan, P. H. (1996). Effects of nitric oxide synthase inhibition on brain infarction in SOD-1-transgenic mice following transient focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* **16,** 1153–1157.

Keller, J. N., Kindy, M. S., Holtsberg, F. W., St. Clair, D. K., Yen, H. C., Germeyer, A., Steiner, S. M., Bruce-Keller, A. J., Hutchins, J. B., and Mattson, M. P. (1998). Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: Suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J. Neurosci.* **18,** 687–697.

Kirsch, J. R., Bhardwaj, A., Martin, L. J., Hanley, D. F., and Traystman, R. J. (1997). Neither L-arginine nor L-NAME affects neurological outcome after global ischemia in cats. *Stroke* **28,** 2259–2264.

Knowles, R. G., Palacios, M., Palmer, R. M. J., and Moncada, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 5159–5162.

Koh, J., and Choi, D. W. (1988). Vulnerability of cultured cortical neurons to damage by excitotoxins: Differential susceptibility of neurons containing NADPH-diaphorase. *J. Neurosci.* **8,** 2153–2163.

Koketsu, N., Berlowe, D. J., Moskowitz, M. A., Kowall, N. W., Caday, C. G., and Finklestein, S. P. (1994). Pretreatment with intraventricular basic fibroblast growth factor (bFGF) decreases infarct size following focal cerebral ischemia in rats. *Ann. Neurol.* **35,** 451–457.

Li, Y., Chopp, M., Jiang, N., Yao, F., and Zaloga, C. (1995). Temporal profile of in situ DNA fragmentation after middle cerebral artery occlusion in the rat. *J. Cereb. Blood Flow Metab.* **15,** 389–397.

Lin, K. T., Xue, J. Y., Nomen, M., Spur, B., and Wong, P. Y. K. (1995). Peroxynitrite-induced apoptosis in HL-60 cells. *J. Biol. Chem.* **270,** 16487–16490.

Lipton, S. A., Choi, Y., Pan, Z., Lei, S. Z., Chen, H. V., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Liu, R. H., and Hotchkiss, J. H. (1995). Potential genotoxicity of chronically elevated nitric oxide: A review. *Mutat. Res.* **339,** 73–89.

Lo, E. H., Hara, H., Rogowska, J., Trocha, M., Pierce, A. R., Huang, P. L., Fishman, M. C., Wolf, G. L., and Moskowitz, M. A. (1996). Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene. *Stroke* **27,** 1381–1385.

Ma, J., Ayata, C., Huang, P., Fishman, M. C., and Moskowitz, M. A. (1996). Regional cerebral blood flow response to vibrissal stimulation in mice lacking type I NOS gene expression. *Am. J. Physiol.* **270,** H1085–H1090.

MacManus, J. P., and Linnik, M. D. (1997). Gene expression induced by cerebral ischemia: An apoptotic perspective. *J. Cereb. Blood Flow Metab.* **17,** 815–832.

Malinski, T., Bailey, F., Zhang, Z. G., and Chopp, M. (1993). Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.* **13,** 355–358.

Meng, W., Ma, J., Ayata, C., Hara, H., Huang, P. L., Fishman, M. C., and Moskowitz, M. A. (1996). ACh dilates pial arterioles in endothelial and neuronal NOS knockout mice by NO-dependent mechanisms. *Am. J. Physiol.* **271,** H1145–H1150.

Morikawa, E., Moskowitz, M. A., Huang, Z., Yoshida, T., Irikura, K., and Dalkara, T. (1994). L-Arginine infusion promotes nitric oxide-dependent vasodilatation, increases regional cerebral blood flow, and reduces infarction volume in the rat. *Stroke* **25,** 429–435.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., Yuan, J., and Moskowitz, M. A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J. Neurosci.* **18,** 3659–3668.

Nicotera, P., Bonfoco, E., and Brune, B. (1995). Mechanisms for nitric oxide-induced cell death—involvement of apoptosis. *Adv. Neuroimmunol.* **5,** 411–420.

Niu, X. F., Smith, C. W., and Kubes, P. (1994). Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ. Res.* **74,** 1133–1140.

Nossuli, T. O., Hayward, R., Jensen, D., Scalia, R., and Lefer, A. M. (1998). Mechanisms of cardioprotection by peroxynitrite in myocardial ischemia and reperfusion injury. *Am. J. Physiol.* **275,** H509–H519.

Ohmori, H., Dhar, D. K., Nakashima, Y., Hashimoto, M., Masumura, S., and Nagasue, N. (1998). Beneficial effects of FK409, a novel nitric oxide donor, on reperfusion injury of rat liver. *Transplantation* **66,** 579–585.

Ohta, K., Graf, R., Rosner, G., and Heiss, W. D. (1997). Profiles of cortical tissue depolarization in cat focal cerebral ischemia in relation to calcium ion homeostasis and nitric oxide production. *J. Cereb. Blood Flow Metab.* **17,** 1170–1181.

Olesen, S. P., Moller, A., Mordvintcev, P. I., Busse, R., and Mulsch, A. (1997). Regional measurements of NO formed in vivo during brain ischemia. *Acta Neurol. Scand.* **95,** 219–224.

Palluy, O., and Rigaud, M. (1996). Nitric oxide induces cultured cortical neuron apoptosis. *Neurosci. Lett.* **208,** 1–4.

Panahian, N., Yoshida, T., Huang, P. L., Hedley-Whyte, E. T., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1996). Attenuated hippocampal damage after global cerebral ischemia in mice mutant in neuronal nitric oxide synthase. *Neuroscience* **72,** 343–354.

Rao, A. M., Dogan, A., Hatcher, J. F., Dempsey, R. J. (1998). Fluorometric assay of nitrite and nitrate in brain tissue after traumatic brain injury and cerebral ischemia. *Brain Res.* **793,** 265–270.

Ratan, R. R., Murphy, T. H., and Baraban, J. M. (1994). Oxidative stress induces apoptosis in embryonic cortical neurons. *J. Neurochem.* **62,** 376–379.

Rubin, E. M., and Barsh, G. S. (1996). Biological insights through genomics: Mouse to man. *J. Clin. Invest.* **97,** 275–280.

Sacks, F. M., Pfeffer, M. A., Moye, L. A., Rouleau, J. L., Rutherford, J. D., Cole, T. G., Brown, L., Warnica, J. W., Arnold, J. M., Wun, C. C., Davis, B. R., and Braunwald, E. (1996). The effect of pravastatin on coronary events after myocardial infarction on patients with average cholesterol levels. Cholesterol and Recurrent Events Trial Investigators. *N. Engl. J. Med.* **335,** 1001–1009.

Takada, K., Yamashita, K., Sakurai-Yamashita, Y., Shigematsu, K., Hamada, Y., Hioki, K., and Taniyama, K. (1998). Participation of nitric oxide in the mucosal injury of rat intestine induced by ischemia–reperfusion. *J. Pharmacol. Exp. Ther.* **287,** 403–407.

Thomas, K. R. (1995). The knockout mouse: Six years old and growing stronger. *Am. J. Respir. Cell. Mol. Biol.* **12,** 461–463.

Togashi, H., Mori, K., Ueno, K., Matsumoto, M., Suda, N., Saito, H., and Yoshioka, M. (1998). Consecutive evaluation of nitric oxide production after transient cerebral ischemia in the rat hippocampus using in vivo brain microdialysis. *Neurosci. Lett.* **240,** 53–57.

Uemura, Y., Kowall, N. W., and Beal, M. F. (1990). Selective sparing of NADPH–diaphorase–somatostatin–neuropeptide Y neurons in ischemic gerbil striatum. *Ann. Neurol.* **27,** 620–625.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. (1996). Nitiric oxide generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6770–6774.

Yang, G., Chan, P., Chen, J., Carlson, E., Chen, S. F., Weinstein, P., Epstein, C. J., and Kamii, H. (1994). Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. *Stroke* **25,** 165–170.

Yoshida, T., Limmroth, V., Irikura, K., and Moskowitz, M. (1994). The NOS inhibitor 7-nitroindazole decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J. Cereb. Blood Flow Metab.* **14,** 924–929.

Yoshimura, M., Yasue, H., Nakayama, M., Shimasaki, Y., Sumida, H., Sugiyama, S., Kugiyama, K., Ogawa, H., Ogawa, Y., Saito, Y., Miyamoto, Y., and Nakao, K. (1998). A missense Glu298Asp variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese. *Hum. Genet.* **103,** 65–69.

Zhang, F., White, J. G., and Iadecola, C. (1994). Nitric oxide donors increase blood flow and reduce brain damage in focal cerebral ischemia: Evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. *J. Cereb. Blood Flow Metab.* **14,** 217–226.

Zhang, J., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1994). Nitric oxide activation of poly(ADP-ribose) synthase in neurotoxicity. *Science* **263,** 686–689.

Zhang, Z. G., Reif, D., Macdonald, J., Tang, W. X., Kamp, D. K., Gentile, R. J., Shakespeare, W. C., Murray, R. J., and Chopp, M. (1996). ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. *J. Cereb. Blood Flow Metab.* **16,** 599–604.

CHAPTER 43

Neurotoxic Actions and Mechanisms of Nitric Oxide

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NITRIC OXIDE (NO) HAS REVOLUTIONIZED OUR PERCEPTION OF NEUROTRANSMISSION AND NEURONAL SIGNALING. NO IS EMERGING AS A KEY REGULATOR OF NUMEROUS PHYSIOLOGICAL RESPONSES. HOWEVER, EXCESSIVE GENERATION OF NO CAN MEDIATE NEURONAL DAMAGE IN A VARIETY OF NEUROLOGIC DISEASES. UNDERSTANDING THE PATHWAYS THROUGH WHICH NO CAUSES NEURONAL CELL DEATH IS KEY IN THE DEVELOPMENT OF MORE EFFECTIVE THERAPIES. IN THIS CHAPTER WE REVIEW THE CURRENT KNOWLEDGE ON THE MECHANISMS OF NO-MEDIATED NEUROTOXICITY. A PROMINENT PATHWAY THROUGH WHICH NO DAMAGES NEURONS IS THE REACTION WITH THE SUPEROXIDE ANION TO GENERATE PEROXYNITRITE. PEROXYNITRITE IS A POTENT CELLULAR TOXIN AND IS CAPABLE OF ALTERING PROTEINS, LIPIDS, AND DNA. EXCESSIVE ACTIVATION OF THE DNA REPAIR ENZYME POLY(ADP-RIBOSE) POLYMERASE (PARP), WHICH MAY LEAD TO CELL DEATH THROUGH DEPLETION OF THE CELLULAR ENERGY STORES, HAS EMERGED AS AN IMPORTANT MEDIATOR OF NO-INDUCED NEUROTOXICITY. NO MAY INTERACT DIRECTLY WITH A LARGE NUMBER OF PROTEINS AND CAUSE SUBSEQUENT ALTERATION OF THEIR FUNCTION. NO-INDUCED CELL DEATH IS CHARACTERIZED BY TYPICAL FEATURES OF BOTH NECROSIS AND/OR APOPTOSIS. NO PLAYS A ROLE AS A NEURONAL CELL DEATH MEDIATOR IN A VARIETY OF DISORDERS OF THE NERVOUS SYSTEM. IN THIS CHAPTER WE DISCUSS THE INVOLVEMENT OF NO IN EXCITOXICITY, STROKE, ISCHEMIC PRECONDITIONING, AND PARKINSON'S DISEASE.

Introduction

Since its initial discovery, nitric oxide (NO) has become one of the most highly studied and important bioactive molecules. Along with the many essential roles of nitric oxide *in vivo,* such as neurotransmission, vasodilation, and immune defense, it can also be involved in reactions that may result in cell damage and death. NO is a relatively unstable molecule that is potentially toxic owing, in part, to the high reactivity of its unpaired electron. It has long been studied as an environmental pollutant because it contributes to the formation of smog, and acid rain and is also involved in the destruction of the ozone layer (Feldman *et al.,* 1993), so it was surprising when NO was found to play an important role in mammalian physiology. NO is biologically relevant because of its production by numerous cell types and because of its role in many physiological processes. In fact, virtually every type of mammalian cell is under the influence of NO (Schmidt and Walter, 1994). NO is synthesized by one of three distinct NO synthase (NOS) isoforms. Depending on the site of production, the amount of NO produced, and the targets within the local environment, NO can exert many diverse functions. In the nervous system, NO has a dual role as a physiological messenger and a mediator of lethal processes in a variety of neurodegenerative disorders and toxic insults of the nervous system.

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The Unique Characteristics of Nitric Oxide

The enzymatic combination of a single atom of nitrogen with a single oxygen atom forms the smallest synthetic product of mammalian cells. It also forms a molecule with an unpaired electron in a frantic search for another molecule to accept or share this odd electron (Nathan, 1992). Target molecules include oxygen, other free radicals, thiol groups, and metals. In an environment rich in these targets, NO has a short half-life, in the range of a few seconds or less. Whereas NO can be inactivated through its interaction with oxygen to form nitrite and nitrate, the interaction of NO with other free radicals is a mechanism by which NO exerts many of its effects. The combination of NO with superoxide (O_2^-) forms peroxynitrite ($ONOO^-$) with the capacity to injure target cells. When NO interacts with prosthetic iron groups or thiol groups on proteins, it can form complexes that activate or inactivate target enzymes. It is through this mechanism that NO activates one of its main target enzymes, soluble guanylate cyclase, which increases cellular cGMP concentrations. The heme-dependent activation of this enzyme by NO results in vasorelaxation in the vasculature and neurotransmission in the central nervous system. Under conditions of high NO formation, a variety of enzymes can also be inhibited by NO–enzyme interaction (Feldman *et al.,* 1993; T. M. Dawson *et al.,* 1994; Garthwaite and Garthwaite, 1994). For example, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is inactivated by nitrosylation of a thiol group in its active site (Molina y Vedia *et al.,* 1992), whereas aconitase in the Krebs cycle and nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase in the mitochondrial electron transport chain are inhibited when peroxynitrite attacks the iron atom in the iron–sulfur cluster essential to the functions of these enzymes (Stuehr and Nathan, 1989). The inactivation of these and other enzymes is believed to be the mechanism by which cytokine-induced NO can inhibit the growth of target cells such as microorganisms, tumor cells, or lymphocytes.

The action of NO is mostly local; however, NO has the capacity to move rapidly to sites of target molecules. Unlike many messenger molecules and secretory molecules that use membrane receptors or specific membrane transporters, NO is so lipophilic that it readily diffuses across membranes. Thus, NO is a unique messenger that rapidly moves cell to cell, with a short range and duration of action, but exhibiting high biological activity.

Nitric Oxide Synthase Isoforms

In biological systems the amino acid L-arginine is the sole substrate for NO synthesis. L-Arginine contains two guanidine nitrogens that accept five electrons in an oxidation–reduction pathway which results in the stochiometric formation of L-citrulline and NO (Nathan, 1992). This reaction is carried out by one of three isoforms of nitric oxide synthase (NOS), which are named after the tissue from which they were first cloned and numbered in the order in which they were cloned (T. M. Dawson *et al.,* 1998). Neuronal NOS (nNOS or type I) and endothelial NOS (eNOS or type III) are constitutively expressed and are calcium dependent; inducible NOS (iNOS or type II) is expressed after immunological challenge and neuronal injury and is calcium independent under most circumstances (T. M. Dawson *et al.,* 1998).

In vitro biochemical studies have indicated that NOS activity is regulated by the phosphorylation state of the enzyme. Phosphorylation of NOS by protein kinase C, cAMP-dependent protein kinase, or Ca^{2+}/calmodulin-dependent protein kinase inhibits its catalytic activity (Dinerman *et al.,* 1994a), whereas dephosphorylation enhances its activity (T. M. Dawson *et al.,* 1993).

The synthesis of NO can be inhibited by L-arginine analogs such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NA), or N^G-nitro-L-arginine methyl ester (L-NAME) that compete for binding at the NOS catalytic site (Kerwin *et al.,* 1994). Calmodulin antagonists and gangliosides are also potent inhibitors of NOS catalytic activity (T. M. Dawson *et al.,* 1995). These agents inhibit all NOS isoforms. More selective NOS inhibitors have been developed. For instance, 7-nitroindazole (7-NI) was the first relatively selective nNOS inhibitor developed (Babbedge *et al.,* 1993). Although *in vitro* 7-NI inhibits all three isoforms with equal affinity, *in vivo* it has marked selectivity for inhibition of nNOS over eNOS and, thus, has minimal vascular effects. Aminoguanidine is relatively selective for iNOS, and it has been extensively used *in vivo* (Arkovitz *et al.,* 1996; Higuchi *et al.,* 1998; Zhang and Iadecola, 1998). Despite the wide use of aminoguanidine, caution must be used when interpreting experiments because aminoguanidine is a potent inhibitor of diamine oxidase, polyamine oxidase, and ribonucleotide reductase, and it is also a general oxidase, decarboxylase, and catalase inhibitor, as well as an inhibitor of advanced glycosylation end products. The isothioureas in general are potent and selective inhibitors of iNOS, and these agents have been widely used to study the role of iNOS-produced NO in the pathophysiology of inflammation (Jang *et al.,* 1996) and circulatory shock (Thiemermann *et al.,* 1995; Arkovitz *et al.,* 1996). Highly selective nNOS inhibitors have been developed and include ARL-17477 and A-84643 (Z. Zhang *et al.,* 1996); also, analogs of 2-iminohomopiperidinium have been reported to be potent and selective inhibitors of iNOS catalytic activity (Hansen *et al.,* 1998).

All three NOS isoforms have been identified in numerous tissues. Except for a few isolated regions, such as the cerebellum, where nNOS is expressed in most of the granule cells, nNOS expression in the brain is restricted to certain subpopulations of neurons throughout the brain. For instance, nNOS neurons represent about 1 to 2% of the total neuronal population in the cortex and corpus striatum (Bredt *et al.,* 1990). In these regions, nNOS neurons colocalize with somatostatin and neuropeptide Y (T. M. Dawson *et al.,* 1991). In the hippocampus, pyramidal cells of the CA1 re-

gion do not express nNOS or express barely detectable levels, whereas eNOS (Dinerman *et al.,* 1994b) is highly expressed. Both isoforms have been detected in the same neurons in some regions of the cerebellum and olfactory bulb. Several splice variants of nNOS have been identified. Significant structural diversity of nNOS occurs through alternative splicing. Alternative splicing of exon 2 was first noted during the characterization of mice lacking the gene for nNOS in which exon 2 was targeted. Characterization of residual NOS catalytic activity in nNOS null mice revealed that the residual activity was due to alternative nNOS isoforms designated nNOS-β and nNOS-γ (Brenman *et al.,* 1996a). Contained within exon 2 of nNOS is a PDZ domain that may mediate binding of nNOS to synaptic junctions through interactions with postsynaptic density proteins (PSD) (Brenman *et al.,* 1996b). In the nervous system, nNOS is closely associated with *N*-methyl-D-aspartate (NMDA) receptors, probably through PDZ interaction domains of each protein. The lack of the amino terminus of nNOS would prevent the association of nNOS in postsynaptic density, leading to the potential inability to be stimulated by increases in intracellular calcium mediated by NMDA receptor activation. Consistent with this notion is the loss of NMDA-stimulated NO formation in nNOS null mice (V. Dawson *et al.,* 1996). Sattler *et al.* (1999) indicate that disruption of nNOS interactions with PSD-95 uncouple NMDA receptor activity from NO toxicity and thus imparts specificity to excitotoxic calcium signaling.

Mechanisms of Nitric Oxide-Mediated Cytotoxicity

The exact pathways by which excessive NO production causes neuronal death are not known. Disruption of any of the physiological processes in which NO is involved could potentially have deleterious consequences for neuronal survival. Although the exact pathways through which NO causes neuronal death remain controversial, several general mechanisms have been proposed (Garthwaite and Boulton, 1995; Yun *et al.,* 1996; Bolanos *et al.,* 1997; T. M. Dawson *et al.,* 1998). It is felt that the generation of peroxynitrite by the reaction of NO with superoxide accounts for most of the toxicity elicited by NO, although other direct targets of NO have been proposed.

Targets of Nitric Oxide

Although NO is an important and unique messenger molecule, it also plays pathological roles in excitotoxicity, stroke, migraine, epilepsy, and neurodegenerative diseases. Models of potential diffusion of NO indicate that it can diffuse as far as 300 μm from its site of origin, which could include as many as 2 million synapses (Wood and Garthwaite, 1994).

Because of an unpaired electron, NO is a free radical, and its effects are largely mediated through other molecules that accept or share this odd electron (T. M. Dawson *et al.,* 1998).

Table I Reactions of Nitric Oxide[a]

Reaction with molecular oxygen			
$2\,NO\cdot + O_2$	$\rightleftharpoons$	$2NO_2\cdot$	
$2\,NO_2\cdot$	$\rightleftharpoons$	N_2O_4	
$N_2O_4 + H_2O$	$\rightarrow$	$HNO_2 + HNO_3$	
$NO\cdot + NO_2$	$\rightleftharpoons$	N_2O_3	
$N_2O_3 + H_2O$	$\rightleftharpoons$	$2HNO_2$	
Reaction with superoxide ($O_2^{\cdot-}$)			
$NO\cdot + O_2^{\cdot-}$	$\rightleftharpoons$	$OONO^- \rightleftharpoons OONOH \rightleftharpoons NO_2\cdot + \cdot OH$	
Reaction with transition metals			
$NO\cdot + XFe^x$	$\rightleftharpoons$	$[X{-}Fe^x{-}NO\cdot]$	$\rightleftharpoons\ XFe^{x-1}{-}NO^+$
$NO\cdot + YFe^x$	$\rightleftharpoons$	$[Y{-}Fe^x{-}NO\cdot]$	$\rightleftharpoons\ YFe^{x+1}{-}NO^-$
Reactions with oxyferrohemoglobin			
$Hb[Fe^{2+}]O_2 + NO\cdot$	$\rightleftharpoons$	$Hb[Fe^{3+}] + NO_3^-$	
Reaction with amines and thiols			
$R_2NH + NO^+$	$\rightleftharpoons$	$R_2NNO + H^+$	
$RSH + NO^+$	$\rightleftharpoons$	$RSNO + H^+$	

[a] Modified from T. M. Dawson *et al.* (1998).

Target biological molecules include oxygen, other free radicals, thiol groups, and metals (Table I).

There are two well-characterized reactions of NO *in vivo.* The first reaction is with oxyhemoglobin to form methemoglobin and nitrate, which is considered the principal mechanism for NO clearance *in vivo* (Liebler *et al.,* 1998). NO generated by NO-donating compounds *in vitro* can accumulate at micromolar concentrations and lead to formation of $NO_2\cdot$ and N_2O_3 via reactions with oxygen. Generation of 10 μ*M*/min of NO *in vivo* with a half-life of 1 s and with detoxification by oxyhemoglobin can produce steady-state NO levels of 0.2 μ*M.* This leads to approximately one NO molecule in 10,000 reacting with oxygen to form $NO_2\cdot$, while the rest is converted to nitrate by oxyhemoglobin.

The second key reaction of NO *in vivo* is its interaction with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), which occurs with a second-order rate constant of 6.7×10^9/*M*/s. A key consideration in the fate of reactive oxygen species is the presence of biologically relevant levels of antioxidant defense. For example, superoxide dismutases (SODs) in the brain are present at a cellular concentration of 5–10 μ*M,* which maintains superoxide at a steady-state level of about 10 p*M.* The reaction of NO and $O_2^{\cdot-}$ is about seven or eight times faster than scavenging by SODs under physiological conditions, even though there is approximately a million times more SOD than $O_2^{\cdot-}$ at the physiological steady state. *In vivo,* concentrations of NO generally exceed those of $O_2^{\cdot-}$ because of $O_2^{\cdot-}$ consumption by SOD. Peroxynitrite is a relatively stable molecule and can diffuse a few micrometers in half of its biological half-life; thus, it can cross membranes. It can survive millions of collisions with thiols and other biological reductants before reacting. This allows peroxynitrite to survive to react with targets toward which it is highly reactive, such as the iron–sulfur cluster of proteins and zinc fingers. Selective oxidants, such as peroxynitrite, can ultimately be more toxic than more highly reactive oxidants, such as hydroxyl radical (·OH).

A predominant mechanism by which NO kills neurons is through the diffusion-limited reaction of NO with $O_2^{\cdot-}$ to generate $ONOO^-$ (Beckman *et al.,* 1990), which is directly cytotoxic (Beckman and Crow, 1993). Peroxynitrite has the activity of the free radicals ·OH and $NO_2^{\cdot}$, although it may not decompose directly into these species. It nitrates and hydroxylates aromatic rings on amino acid residues, and it is also a potent oxidant that reacts readily with sulfhydryls, zinc–thiolate moieties, lipids, proteins, and DNA (Molina y Vedia *et al.,* 1992; Crow *et al.,* 1995). Most likely, peroxynitrite mediates the interactions of NO with the iron–sulfur cluster of proteins, inhibits DNA synthesis by ribonucleotide reductase, liberates iron by binding to the iron in ferritin, and influences iron metabolism at the posttranscriptional level by interacting with aconitase. In the presence of $ONOO^-$, aconitase activity is disrupted and its RNA binding site exposed, leading to the enzyme function as the iron-responsive binding protein which binds to the iron-responsive element (Weiss *et al.,* 1993, 1994; V. L. Dawson and T. M. Dawson, 1996).

Protein Modification

Reactions of NO with amino acid residues include nitrosation (formation of nitrosamines, nitrosothiols, and deaminations) and oxidation via peroxynitrite. The balance between these effects is dictated by competition between reaction pathways that lead to the formation of more reactive nitrogen oxides (e.g., reaction with oxygen to form N_2O_3), formation of peroxynitrite, trapping of free radicals, and interaction with metals and metalloproteins.

A key interaction is the binding of NO to the heme of guanylate cyclase (Table II), which elicits many of the well-known physiological actions of NO. NO combines with nonheme iron in a variety of enzymes, including NADPH–ubiquinone oxidoreductase, NADH–succinate oxidoreductase, and *cis*-aconitase, which are all iron–sulfur enzymes, resulting in enzyme inactivation (Zhang and Snyder, 1995). NO binds to the iron in ferritin, liberating iron that can result in lipid peroxidation. Other targets of NO include cytochrome *c* oxidase in the mitochondrial respiratory chain, ribonucleotide reductase (the rate-limiting enzyme in DNA synthesis), and glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway. NO can also modify protein function by S-nitrosylation of target molecules such as $p21^{ras}$, protein kinase C, and glyceraldehyde-3-phosphate dehydrogenase (Dimmeler *et al.,* 1992; Stamler *et al.,* 1992; Lander *et al.,* 1995).

Table II Targets of Nitric Oxide[a]

Proteins containing heme groups
Cyclooxygenase 1 and 2
Cytochrome P-450
Guanylate cyclase
Hemoglobin and myoglobin
Nitric oxide synthase
Proteins containing iron–sulfur clusters
cis-Aconitase
Ferritin and transferrin
Mitochondrial complex I
Mitochondrial complex II
Mitochondrial aconitase
Ribonucleotide reductase
Proteins containing thiol groups (nitrosylation)
Adenylate cyclase
G proteins/$p21^{ras}$
GAP-43
GAPDH
Glutathione
Protein kinase C
NMDA receptor
NADPH oxidase
Neurofilaments
Tissue plasminogen activator
Synaptosomal-associated protein of 25 kDa
ADP-ribosylation
Glyceraldehyde-3-phosphate dehydrogenase
DNA
DNA deamination
DNA strand breaks

[a] Modified from T. M. Dawson and V. L. Dawson (1999).

DNA Damage

DNA damage can occur by the actions of N_2O_3 or peroxynitrite. N_2O_3 can damage DNA directly by deamination of cytosine to uracil, and indirectly by inactivation of DNA repair enzymes such as O^6-alkylguanine transferase. NO exposure can also lead to the formation of single-strand breaks in DNA both *in vitro* and *in vivo* (Burney *et al.,* 1998). In contrast to NO, which is involved primarily in the deamination chemistry of DNA, most of the damage inflicted on DNA by peroxynitrite is oxidative (Burney *et al.,* 1998). DNA treatment with peroxynitrite generally leads to much more damage than treatment with an equivalent dose of NO. Not only are the levels of damage higher, but also the spectrum of alterations tends to be much more complex (Burney *et al.,* 1998).

DNA damage by NO·/$ONOO^-$ leads to subsequent activation of the enzyme poly(ADP-ribose) polymerase (PARP) (J. Zhang *et al.,* 1994; V. L. Dawson and T. M. Dawson, 1996). PARP is a nuclear enzyme involved in DNA repair (Lautier *et al.,* 1993). It is activated by breaks in DNA, and it catalyzes the transfer of ADP-ribose to nuclear proteins such as histone and PARP itself. The addition of ADP-ribose groups to nuclear proteins by PARP facilitates separation of the DNA strands and improves access for the DNA repair enzymes. PARP can add hundreds of ADP-ribose units within seconds to minutes of being activated (Lautier *et al.,* 1993; de Murcia and Mennissier de Murcia, 1994). For every mole of ADP-ribose transferred by PARP, one mole of NAD and four free energy equivalents of ATP are consumed. Therefore, activation of PARP can rapidly deplete cellular energy stores. If this depletion is severe and sustained, it can

lead to impaired cellular metabolism and ultimately to cell death. In fibroblasts from mice where the PARP gene has been knocked out, apoptotic death is accelerated due to impaired DNA repair mechanisms (de Murcia *et al.,* 1994). However, in some cell lines genetic knockout of PARP has no evident effect on apoptotic cell death (Wang *et al.,* 1997). Compelling evidence for the involvement of PARP in NO-mediated toxicity was provided by studies on mice with a targeted disruption of PARP (Heller *et al.,* 1995). Islet cells lacking PARP do not show DNA damage-induced energy depletion and are more resistant to NO toxicity. However, NO-mediated toxicity was not completely abolished in the islet cells from mutant mice, which suggests the existence of alternative pathways for NO-mediated toxicity not involving PARP-induced NAD depletion. In neurons and myocytes, lack of PARP protects them dramatically against ischemic damage (Eliasson *et al.,* 1997; Endres *et al.,* 1997), and neuronal cultures from PARP knockout mice are remarkably resistant to excitotoxicity and NO toxicity (Eliasson *et al.,* 1997) (Fig. 1). The link between NO toxicity and PARP activation is a very important extension to the understanding of NO-induced neuronal damage, but an important unanswered question is how PARP activation relates to other downstream mechanisms implicated in neurotoxicity, including mitochondrial dysfunction (White and Reynolds, 1996).

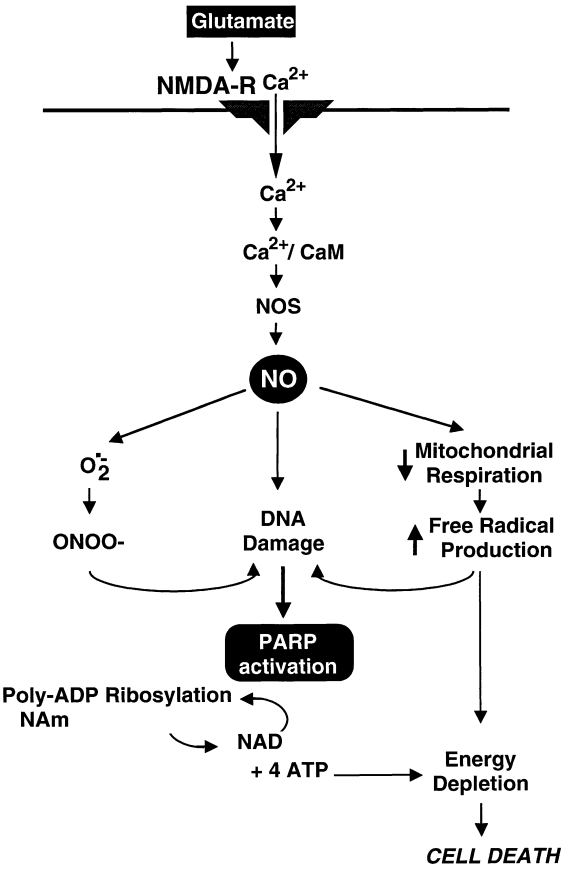


Figure 1 Mechanisms of PARP-mediated neuronal cell death in excitotoxicity. NMDA receptor (NMDA-R) activation causes an increase in intracellular calcium levels, which activates NOS. Excessive generation of NO leads to production of free radicals, mitochondrial dysfunction, and DNA damage. The damaged DNA activates PARP, which transfers ADP-ribose groups to nuclear proteins, consuming one mole of NAD. NAD is resynthesized from nicotinamide (NAm), a reaction that utilizes four high-energy equivalents of ATP. PARP adds ADP-ribose groups to numerous nuclear proteins, consuming a very large amount of energy. It is hypothesized that excitotoxicity-generated free radicals such as NO activate a futile cycle of DNA damage followed by PARP activation, which depletes cells of their energy stores, ultimately leading to cell death.

Nitric Oxide and Mitochondria in Neurodegeneration

Increasing evidence suggests that dysfunction of cellular energy production is an important mechanism in NO-mediated neurotoxicity (Bolanos *et al.,* 1997). Mitochondrial complexes II–III and IV and succinate dehydrogenase are damaged in neurons and glia after NO exposure (Bolanos *et al.,* 1997). Also, NO is able to bind to mitochondrial complex I and II and inhibit oxidative phosphorylation. In addition, NO competes with oxygen for cytochrome oxidase, leading to reversible inhibition of mitochondrial respiration (Brown, 1995). Brorson *et al.* (1999) suggest that NO directly impairs mitochondrial function and energy metabolism in cultured hippocampal neurons. NO is capable of depolarizing the mitochondrial membrane potential, which is accompanied by a progressive concentration-dependent depletion of cellular ATP. Interestingly, the severe ATP-depleting effects of NO are not fully explained by the direct effects on mitochondrial activity, as inhibitors of mitochondrial oxidative phosphorylation such as rotenone or 3-nitroproprionic do not mimic the effects of NO. Thus, the ATP-depleting effects of NO are not only related to its disruption of mitochondrial respiration, but also may be related to its effects on glycolysis or activation of PARP.

Mitochondrial failure results in increased free radical formation and membrane depolarization due to dysfunction of Na^+, K^+-ATPase, and this in turn promotes calcium influx through NMDA receptors, leading to maintained elevation of intracellular calcium concentration and NOS activation, which could result in a self-propagating destructive cycle. Defects in mitochondrial energy metabolism have long been considered to underlie the pathology of neurodegenerative disorders (Beal *et al.,* 1993). For instance, decreased complex I activity has been reported in the substantia nigra of postmortem samples obtained from patients with Parkinson's disease, and impaired complex IV activity has been noted in Alzheimer's disease (Bolanos *et al.,* 1997).

Role for Nitric Oxide in Apoptosis

Although the term apoptosis was originally coined to describe defined morphological alterations, it is now generally used to describe the evolutionally conserved pathway of biochemical and molecular events leading to cell demise (Hale *et al.,* 1996; Leist and Nicotera, 1997). The term pro-

grammed cell death is now used as a synonym for apoptosis in appreciation of the genetic programs that regulate cell death (Vaux and Strasses, 1996).

NO-dependent apoptotic cell death was initially shown in peritoneal macrophages following immunological activation (Albina *et al.,* 1993; Sarih *et al.,* 1993). Evidence for apoptosis was provided by microscopic examination of chromatin condensation and by a specific pattern of internucleosomal DNA fragmentation. The involvement of NO was confirmed by the cell death-preventive effects of L-arginine-restricted medium and of the NOS inhibitor N^G-monomethyl-L-arginine, and more directly by exposing cells to NO gas, leading to apoptosis. Following these initial reports numerous studies have confirmed the ability of NO to initiate apoptosis in a variety of cell types and cell lines (Brune *et al.,* 1998), including neurons (Lipton *et al.,* 1993). Apoptotic cell death as a result of excessive NO production correlates with upregulation of the p53 tumor suppressor gene, activation of caspases, chromatin condensation, and DNA fragmentation (Brune *et al.,* 1998). The p53 tumor suppressor has come to be known as the master guardian of the genome and as a member of the DNA damage response pathways (Oren, 1997), and it induces growth arrest or apoptosis in DNA-damaged cells. Another mechanism by which NO may activate apoptosis is the activation of caspases, a family of cysteine proteases that specifically cleave a growing number of cellular substrates after Asp residues (Cohen, 1997). Caspase activation is inherent to the final executive phase of apoptosis. Some reports have shown that during NO-mediated apoptosis there is cleavage of PARP, which is an established caspase-3 substrate (Messmer *et al.,* 1998), and caspase activation by NO donor agents has been confirmed in human leukemia cells (Yabuki *et al.,* 1997), mesangial cells (Sandau *et al.,* 1997), and neuronal excitotoxicity (Leist *et al.,* 1997). It has been suggested that ceramide generation might be a mediator of NO signaling during apoptosis through caspase-3 activation (Takeda *et al.,* 1999).

In contrast to the proapoptotic role of NO, exposure to NO donor agents and activation of NOS have been reported to inhibit apoptosis in T lymphocytes and human umbilical vein endothelial cells (Mannick *et al.,* 1997) through cGMP-dependent actions, or by direct inhibition of caspase-3 through S-nitrosylation of cysteine-163, a key residue for caspase activity (Dimmeler *et al.,* 1997). The discrepancy in the NO effect on apoptosis may be due to differences in intensity and duration of NO exposure and the cellular environment. Indeed, low concentrations of NO donor agents (<100 μM) show a protective effect against apoptosis, whereas induction of apoptosis is observed with >250 μM NO donor agents (Takeda *et al.,* 1999). NO at low levels has the potential to modulate caspase activity, which is a further indication of the complexity of the cellular response to death signals. The current view on the role of NO in apoptosis is that low concentrations of NO are protective against apoptosis, whereas high NO concentrations induce apoptosis (Fig. 2).

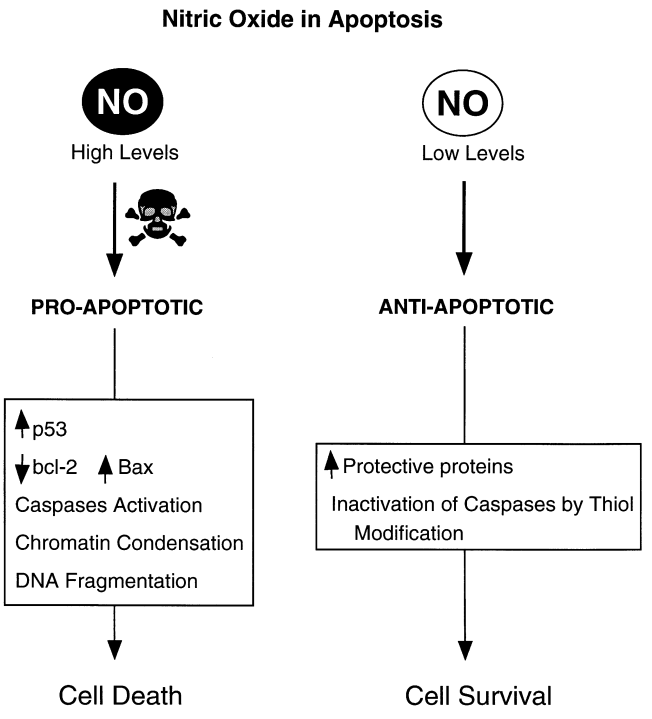


Figure 2 The dual role of NO in apoptotic cell death. The toxicity of NO is influenced by the existing biological milieu. Relative rates of NO formation, its oxidation and reduction, and the combination with oxygen, superoxide, and other molecules will determine the signaling pathway(s) initiated by NO. Large amounts of NO promote cell death, which is characterized by typical biochemical and morphological features that resemble apoptosis. Apoptosis associated with high NO levels is accompanied by an early accumulation of the tumor suppressor p53, decreased B-cell leukemia/lymphoma-2 (bcl-2) expression, upregulation of Bax, as well as activation of caspase-3, chromatin condensation, and DNA fragmentation. In contrast to its toxic effects at high levels, NO at low levels signals cell protection and has an antiapoptotic effect. Mechanisms that mediate the protective effect of NO include inhibition of caspase activity as well as activation of signaling pathways that mediate cell survival through the upregulation of protective proteins.

Neuronal Nitric Oxide Synthase Neurons Are Resistant to Toxic Insults

In 1961, Thomas and Pearse described a histochemical stain, NADPH diaphorase, that was detected in a unique subpopulation of neurons that comprises approximately 1–2% of the total neuronal population. Over the years, investigators have observed that these NADPH diaphorase-positive neurons are uniquely resistant to a variety of toxic insults. Thus, nNOS neurons are relatively spared from cell death in NMDA neurotoxicity, Huntington's disease, Alzheimer's disease, and vascular stroke (Ferrante *et al.,* 1985; Koh *et al.,* 1986; Choi, 1988; Uemura *et al.,* 1990; Hyman *et al.,* 1992). With the cloning and expression of nNOS, it was appreciated that this NADPH diaphorase staining was in fact produced by nNOS (T. M. Dawson *et al.,* 1991; Hope *et al.,* 1991). The mechanisms that lead nNOS neurons to preferentially survive in such profound neuronal loss in many neurologic disorders

are only now starting to be understood. Toxicity studies on primary cortical cultures from nNOS knockout mice have shown that such resistance is not due to nNOS itself (V. Dawson *et al.,* 1996). Although it is always feasible that alternative splice variants of nNOS are expressed at low levels and may provide some protection to nNOS neurons, this is unlikely. We hypothesize that nNOS neurons must possess unique protective mechanisms that render them resistant to the potentially toxic concentrations of NO they can produce.

In order to identify candidate proteins with a potential neuroprotective role, we performed serial analysis of gene expression (SAGE) in wild-type PC12 cells and in PC12 cells that had become resistant to NO toxicity (Gonzalez-Zulueta *et al.,* 1998). The first gene identified with the highest differential level of expression between the two cell populations was the manganese superoxide dismutase (MnSOD) gene. Knockdown of MnSOD levels by antisense oligonucleotides restored vulnerability to NO in the PC12 NO-resistant cells, whereas adenoviral overexpression of MnSOD conferred resistance to NO toxicity in wild-type PC12 cells (Gonzalez-Zulueta *et al.,* 1998). Inagaki and colleagues (Inagaki *et al.,* 1991) showed that MnSOD is localized in somatostatin-containing neurons in the striatum, suggesting that nNOS neurons are enriched in MnSOD as somatostatin and nNOS are coexpressed in these neurons (V. L. Dawson *et al.,* 1991). We have shown that in primary cortical cultures MnSOD is enriched in nNOS neurons (Gonzalez-Zuluet *et al.,* 1998). Antisense knockdown and genetic knockout of MnSOD renders nNOS neurons susceptible to NMDA and NO toxicity, and overexpression of MnSOD via adenoviral vectors provides dramatic protection against NMDA and NO toxicity to cortical cultures (Gonzalez-Zulueta *et al.,* 1998). Our results indicated that MnSOD constitutes a major defense against excitotoxic and oxidative damage and appears to be essential for the resistance of nNOS neurons to NMDA and NO-induced neurotoxicity (Gonzalez-Zulueta *et al.,* 1998) (Fig. 3). MnSOD is one of the first cellular responses to oxidative stress and scavenges the highly reactive free radical superoxide anion. MnSOD in nNOS neurons may decrease the formation of peroxynitrite, rendering these neurons relatively resistant to the toxic actions of NO.

nNOS was shown to interact with phosphofructokinase-M (PFK-M) through the nNOS PDZ domain (Firestein and Bredt, 1999). In the brain, nNOS and PFK-M are both en-

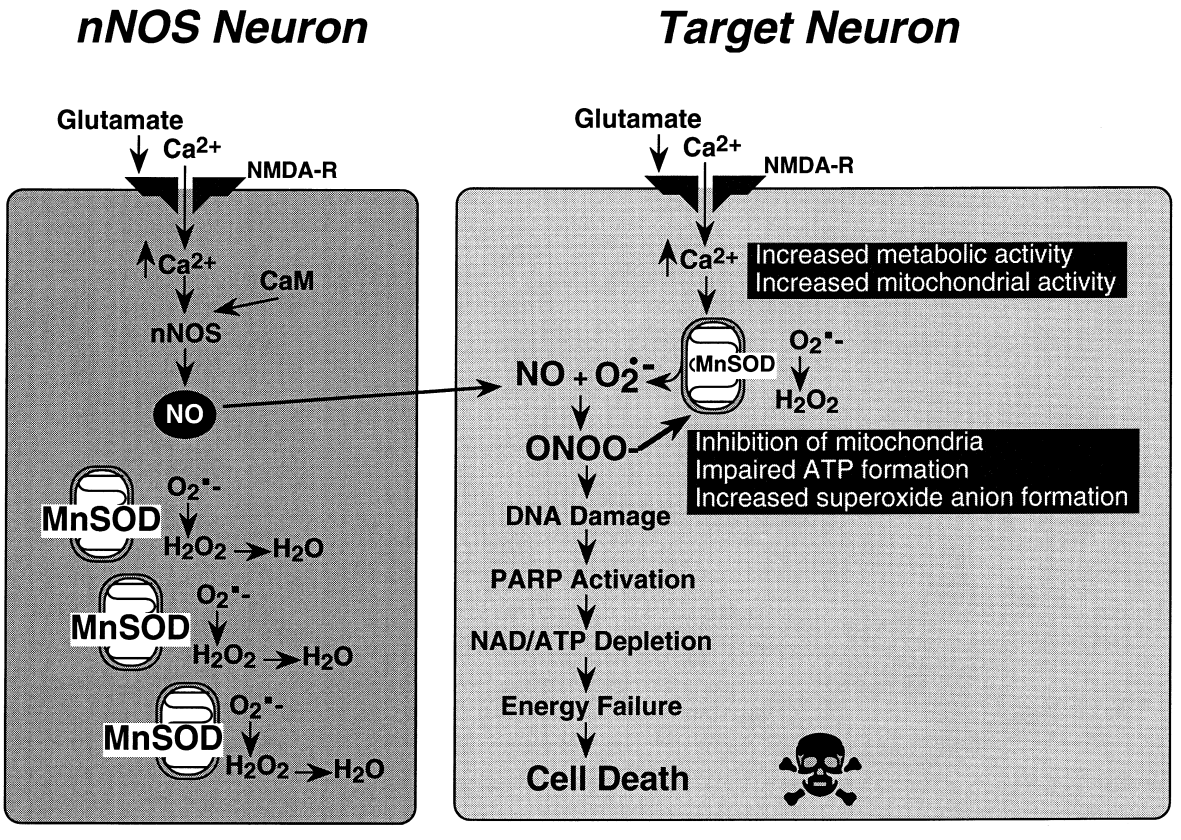


Figure 3 MnSOD protects nNOS neurons against NMDA and NO toxicity. MnSOD is highly expressed in nNOS neurons in comparison to adjacent neurons. NO generated in nNOS neurons does not have the opportunity to react with superoxide anion ($O_2^{\cdot-}$), as it is efficiently scavenged by MnSOD. NO readily diffuses to adjacent neurons where the low levels of MnSOD are not enough to scavenge the high concentrations of $O_2^{\cdot-}$ formed in the mitochondria following NMDA receptor stimulation by glutamate. In target neurons NO and $O_2^{\cdot-}$ react and generate high concentrations of peroxynitrite ($ONOO^-$). Peroxynitrite is a highly toxic oxidant that damages DNA and ultimately causes neuronal death.

riched in synaptosomes and, specifically, in the synaptic vesicle fraction, where they can interact. At the cellular level, PFK-M is enriched in neurons that express nNOS, and since fructose-1,6-bisphosphate, the product of PFK activity, is neuroprotective, interaction of nNOS and PFK may contribute to neuroprotection of nNOS-positive cells (Firestein and Bredt, 1999). Other putative protective mechanisms are also likely to exist in nNOS neurons.

Nitric Oxide in Neurologic Diseases

Role for Nitric Oxide in NMDA Receptor-Induced Excitotoxicity

The emergence of the hypothesis that excitotoxic injury is a major cause of neuronal death in the central nervous system in neurologic disorders is one of the most important developments in neuroscience (Meldrum and Garthwaite, 1990). A paradox that has come with the development of a better understanding of excitatory synaptic transmission is that excitatory neurotransmitters can also act as potent neurotoxins. Excessive release of excitatory amino acids is associated with convulsions and neurotoxicity (Moncada, 1993). Excitatory amino acids induce neurotoxicity via a three-stage process involving induction, amplification, and expression (Choi, 1992). Induction is due to overstimulation of glutamate receptors, which leads to increases in intracellular calcium, sodium, and chloride. Amplification occurs as a result of a further increase in cytoplasmic calcium concentration, which mediates the activation of enzymes such as protein kinase C, calmodulin-dependent enzymes such as nNOS, calpains, and phospholipases. Glutamate released from injured neurons can cause further glutamatergic receptor stimulation and the propagation of toxicity. Intracellular calcium overload can also activate catabolic enzymes such as proteases and endonucleases, leading to neuron degeneration. Excess glutamate acting via *N*-methyl-D-aspartate (NMDA) receptors mediates cell death in focal cerebral ischemia (Choi, 1988), trauma, and epilepsy, and it has been implicated in neurodegenerative diseases such as Huntington's disease and Alzheimer's disease (Meldrum and Garthwaite, 1990).

Since 1990, a major significant development in the study of excitotoxicity has been the demonstration that NO mediates a component of NMDA receptor-mediated neurotoxicity (V. L. Dawson *et al.,* 1991) (Fig. 4), and this advance paralleled the recognition and understanding of the important role played by NO in the central nervous system (Garthwaite and Boulton, 1995). The link between stimulation of NMDA receptors by glutamate and activation of nNOS was first demonstrated by Garthwaite and colleagues (Garthwaite *et al.,* 1988), and the first evidence for NO involvement in excitotoxicity was reported by V. L. Dawson and colleagues (1991). Our work in primary neuronal cultures showed that (1) NMDA-elicited neuronal death is prevented by NOS inhibitors or depletion of L-arginine from the culture medium and (2) the NO donor sodium nitroprusside induces dose-dependent neuronal death that parallels the formation of cGMP. The addition of hemoglobin, which complexes with NO, prevents NMDA- and sodium nitroprusside-elicited neurotoxicity. These findings suggested that NO is involved in glutamate-mediated neuronal death, and they have been confirmed by several *in vitro* studies with NO inhibitors (Izumi *et al.,* 1992; V. L. Dawson *et al.,* 1993; Reif, 1993), and NO donor agents (Chen *et al.,* 1991; Lustig *et al.,* 1992; V. L. Dawson *et al.,* 1993). The inhibition of nNOS also protects against NMDA-induced lesions *in vivo* (Buisson *et al.,* 1992; Moncada *et al.,* 1992). Coexposure of primary cortical cultures to agents that decrease NOS catalytic activity, such as NMDA and arginine analogs, flavoprotein inhibitors, calmodulin antagonists, and calcineurin inhibitors, results in neuroprotection against NMDA toxicity. Compounds that release NO are neurotoxic and follow the same time course as NMDA neurotoxicity (V. L. Dawson *et al.,* 1991; Garthwaite and Boulton, 1995). Targeted disruption of the nNOS gene results in neuronal cultures that are resistant to NMDA toxicity, which indicates that nNOS neurons are the primary source of neurotoxic NO. Furthermore, cultures from nNOS null mice are markedly resistant to combined oxygen–glucose deprivation (V. Dawson *et al.,* 1996). These studies strongly suggest that NO plays a role in the mechanisms underlying NMDA-induced neuronal death.

The role of NO in NMDA-mediated neurotoxicity has been at times a controversial subject, owing to the publication of studies failing to demonstrate a role for NO in NMDA-induced toxicity in cortical cultures (Demerle-Pallardy *et al.,* 1991; Hewett *et al.,* 1993) or *in vivo* after a stroke (Buchan *et al.,* 1994). There are several experimental factors that appear to contribute to the variable detection of the NO component in NMDA excitotoxicity *in vitro,* including variable expression of NOS in neuronal cultures and the timing and intensity of the insult. Samdani *et al.* (1996) showed that the expression of nNOS is dependent on the culture paradigm, and that plating of neurons on glial feeder layers, which is a common practice, is associated with an impoverishment of nNOS-containing neurons and associated nNOS protein and catalytic activity. It is likely that the resistance of neurons in the cultures on feeder layers is due to the failure of the cultures to reach a mature phenotype. NMDA receptor-mediated NO-dependent neurotoxicity is developmentally regulated and may reach full expression in cultures only after a few weeks *in vitro.* Neurons plated on feeder layers show an inmature phenotype with respect to nNOS expression in that total nNOS activity and number of nNOS-positive neurons is low (Samdani *et al.,* 1996). Also, serum-free medium prevents expression of nNOS at high enough levels to mediate toxicity. Furthermore, since nNOS is not expressed in cultured neurons grown on a polyornithine matrix until approximately 14 days *in vitro,* experiments performed before then will not yield any NO-mediated toxicity. Strijbos *et al.* (1996) showed that the timing and exposure of cultured neurons to NMDA is crucial to the detection of a NO-dependent component of toxicity. It was shown that the NO component

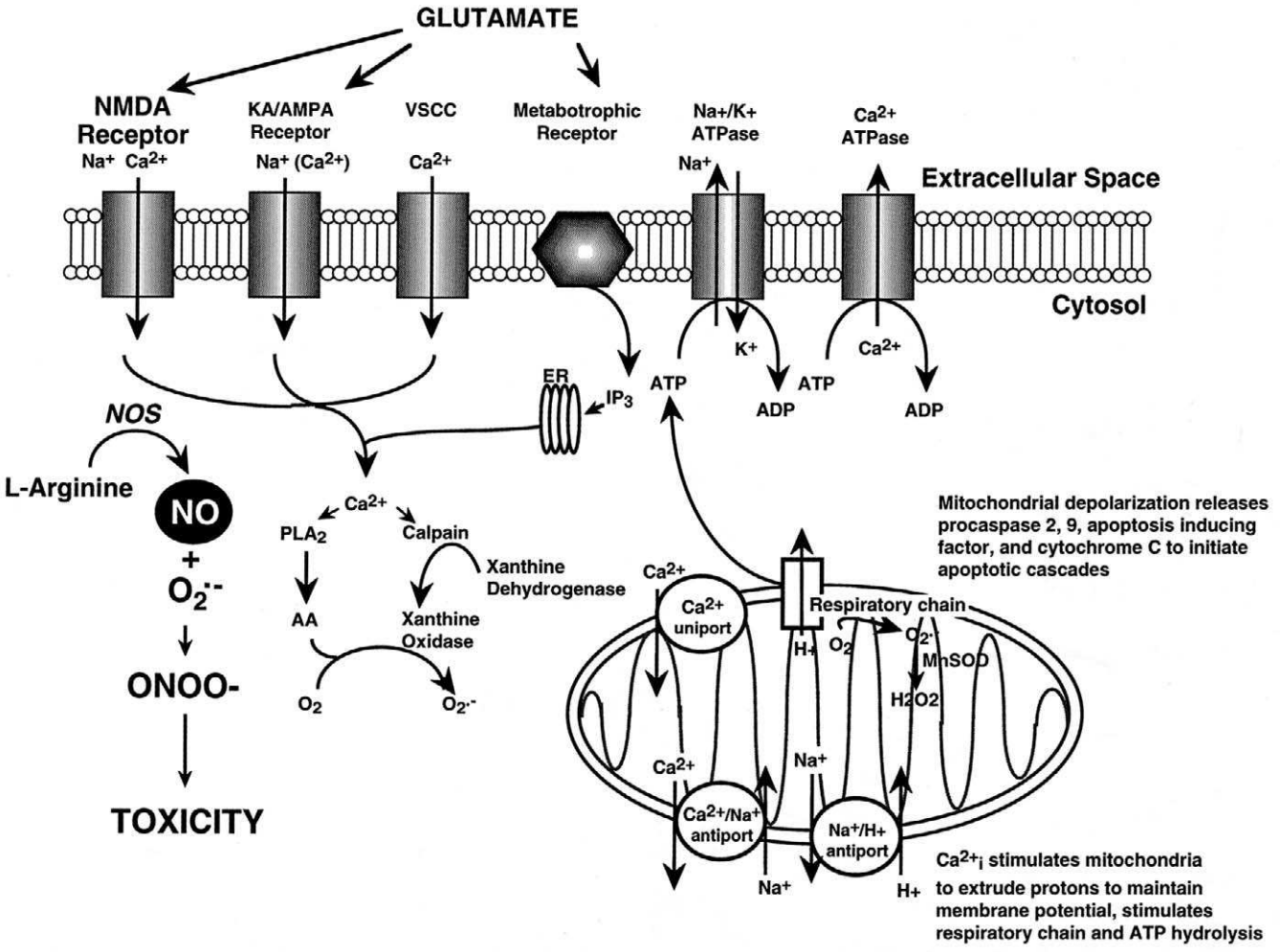


Figure 4 Mechanisms of glutamate-induced oxidative stress and excitotoxicity. Activation of the NMDA receptor increases intracellular calcium (Ca^{2+}) levels. The depolarization activates voltage-sensitive calcium channels (VSCC), further increasing intracellular Ca^{2+}. Activation of kainate/AMPA receptors allows in some Ca^{2+} but mostly sodium (Na^+). Na^+ depolarizes the cell, relieving the magnesium block of the NMDA receptor and increasing the activation of this receptor by glutamate. Activation of the metabotropic receptor releases inositol trisphosphate (IP_3) which raises intracellular Ca^{2+} levels from intracellular stores. Elevated intracellular Ca^{2+} levels activate nitric oxide synthase (NOS) to form NO, activate phospholipase A_2 (PLA_2) to initiate the arachidonic acid cascade which generates free radicals, and activate calpain, which converts xanthine to xanthine oxidase resulting in the generation of superoxide anion. NO produced at high levels reacts with $O_2^{\cdot-}$ to generate high concentrations of peroxynitrite, which is a potent cellular toxin. Mitochondrial depolarization releases procaspase 2 and 9, apoptosis inducing factor, and cytochrome *c* to initiate apoptotic death cascades. Following depolarization, Na^+,K^+-ATPase is activated to maintain membrane polarity. ATP is generated by the mitochondria through oxidative phosphorylation, which can also generate $O_2^{\cdot-}$ through the loss of electrons from the electron transport chain to oxygen.

of NMDA toxicity diminished as duration of exposure to NMDA was extended longer than 5 min, suggesting the existence of NO-independent mechanisms of toxicity in cultures as well (Strijbos *et al.,* 1996).

The Role for Nitric Oxide in Ischemic Brain Injury

Glutamate has risen as a key mediator of ischemic brain injury (Choi and Rothman, 1990). Cerebral ischemia increases the extracellular concentrations of glutamate and aspartate, which stimulate the glutamatergic receptors and may lead to neuronal cell death if overstimulation occurs. Numerous studies indicate that the NMDA glutamate receptor mediates in large part neuronal damage following focal ischemia. The observation that NOS inhibitors attenuate NMDA toxicity in neuronal cultures (V. L. Dawson *et al.,* 1991) and decrease brain damage produced by occlusion of the middle cerebral artery in mice (Nowicki *et al.,* 1991) provided strong initial evidence that NO plays a role in the pathways of cerebral ischemic injury. Elimination of the nNOS gene through transgenic technology results in primary neuronal cultures with significantly increased resistance to combined oxygen–glucose deprivation when compared to cultures from wild-type mice (V. Dawson *et al.,* 1996). Furthermore, exposure to the nonspecific NOS inhibitor L-NAME protects wild-type cultures from oxygen–glucose deprivation.

Tissue NO levels can be measured directly using a porphyrinic microsensor. Malinski and colleagues (1993) determined that after occlusion of the middle cerebral artery

(MCA), NO concentration in the ischemic area increases from nanomolar to micromolar levels within 20 min and then begins to decline owing to substrate depletion for NO synthesis. The initial peak of NO after the ischemic insult can be prevented by glutamate receptor antagonists. The increase in NO production correlates with an upregulation of nNOS gene transcription and catalytic activity, as well as with an increase in the number of nNOS neurons in the ischemic region (Z. G. Zhang *et al.,* 1994). After 60 min, nNOS activity returns to normal, and after 24 hours nNOS neurons are lost and nNOS activity decreases (Iadecola *et al.,* 1995a). Inhibition of nNOS with concentrations of NOS inhibitors that do not alter eNOS activity results in a reduction of infarct volume after MCA occlusion in rats and cats (Buisson *et al.,* 1992; Nishikawa *et al.,* 1994). In addition, the more selective nNOS inhibitor 7-nitroindazole, which effectively decreases nNOS activity but does not affect eNOS activity, is neuroprotective in models of cerebral ischemia (Yoshida *et al.,* 1994).

nNOS null mice have helped in the clarification of the role of nNOS in ischemic brain injury. These mice show reduced infarct size when compared with wild-type controls following permanent focal MCA occlusion (Huang *et al.,* 1994), as well as in a reperfusion model of transient MCA occlusion (Hara *et al.,* 1996). Furthermore, nNOS null mice show reduced hippocampal damage in a model of global cerebral ischemia (Panahian *et al.,* 1996). Interestingly, when nNOS null mice are treated with the nonselective NOS inhibitor nitro-L-arginine methyl ester, there are adverse effects on cerebral blood flow, and stroke volume is increased (Huang *et al.,* 1994).

In ischemic brain tissue, eNOS protein levels and catalytic activity also increase shortly after induction of ischemia (Z. G. Zhang *et al.,* 1993). If eNOS activity is diminished by high doses of NOS inhibitors, deleterious alterations of cerebral blood flow occur, and infarction volume is exacerbated. Consistent with this notion is the observation that MCA occlusion of eNOS null mice leads to larger infarcts in wild-type mice, and when eNOS null mice are treated with nonselective NOS inhibitors during MCA occlusion, infarct volume is reduced. Therefore, the currently available evidence suggests that the production of NO during cerebral ischemia has both deleterious and protective effects (Fig. 5), with neuronal NO playing an important role in mediating neuronal cell death and endothelial derived NO being neuroprotective by maintaining proper cerebral blood flow (Dalkara *et al.,* 1994).

iNOS protein and catalytic activity are also increased in the postischemic brain, but in contrast to nNOS and eNOS, iNOS expression does not occur until 6 to 12 hours after MCA occlusion (Iadecola *et al.,* 1995). In transient ischemia, iNOS is expressed in vascular cells, whereas in permanent ischemia, iNOS is primarily detected in neutrophils that infiltrate the ischemic area (Iadecola *et al.,* 1995). It is thought that the postischemic induction of iNOS expression leads to sustained generation of large amounts of NO, which subsequently causes delayed neuronal injury as indicated by the reduction in infarct volume in iNOS null mice compared to wild-type mice following MCA occlusion (Iadecola *et al.,* 1997).

The Role of Nitric Oxide in Ischemic Preconditioning

Ischemic preconditioning is a well-known phenomenon in which sublethal ischemic insults induce robust protection against subsequent lethal ischemia in a variety of organ systems including brain, heart, liver, intestine, kidney, and lung (Kitagawa *et al.,* 1990; Ishida *et al.,* 1997). In the brain, ischemic preconditioning is mediated largely through the activation of NMDA glutamate receptors through increases in intracellular calcium (Kato *et al.,* 1992; Kasischke *et al.,* 1996). Because ischemic preconditioning induces such dramatic protection, understanding the early cellular events that account for the development of ischemic tolerance could lead to the development of novel therapeutic strategies for the treatment of patients at risk for a variety of neurologic disorders. Although ischemic preconditioning was first described in the 1980s (Murry *et al.,* 1986), the mechanisms underlying the powerful, protective effects of ischemic preconditioning remain uncertain. It appears as though activation of adenosine A1 receptors and K_{ATP} channels during the preconditioning ischemia appears to be important for the induction of tolerance (Heurteaux *et al.,* 1995). Mounting evidence indicates that NO may play a key role in the development of tolerance (Gidday *et al.,* 1999; Ping *et al.,* 1999). A potential clue linking the induction of ischemic tolerance to NO stems from our work showing that NO activates $p21^{Ras}$ (Ras) (Yun *et al.,* 1998). Stimulation of NMDA receptors in cultured cortical neurons activates the Ras/extracellular signal activated kinase (Erk) pathway via calcium-dependent activation of nNOS and NO generation. This activation occurs through a non-cGMP-dependent mechanism and probably occurs through a redox-sensitive activation of Ras (Yun *et al.,* 1998). Our studies suggest that ischemic preconditioning induces Ras activation in an NMDA receptor- and NO-dependent, but cGMP-independent manner, and furthermore, we show that the Ras/Erk pathway is both necessary and sufficient for ischemic tolerance in neurons (Fig. 6).

Nitric Oxide in Parkinson's Disease

Parkinson's disease is a movement disorder characterized by the selective loss of dopamine neurons, particularly in the substantia nigra, that project to the striatum (Jenner *et al.,* 1992). It can be modeled in mice by injection of the toxin 1-methyl-4-phenyl-1,2,3,6–tetrahydropyridine (MPTP), which is metabolized to 1-methyl-4-phenylpyridium (MPP^+) (Langston *et al.,* 1984; Heikkila *et al.,* 1989). MPP^+ selectively targets dopaminergic neurons through the high-affinity dopamine reuptake transporter. MPP^+ is a mitochondrial toxin, inhibiting mitochondrial complex I and causing a deficit in ATP production and an increase in superoxide anion formation in neurons (Kitayana *et al.,* 1992). The superoxide anion appears to play an important role in MPTP dopaminergic

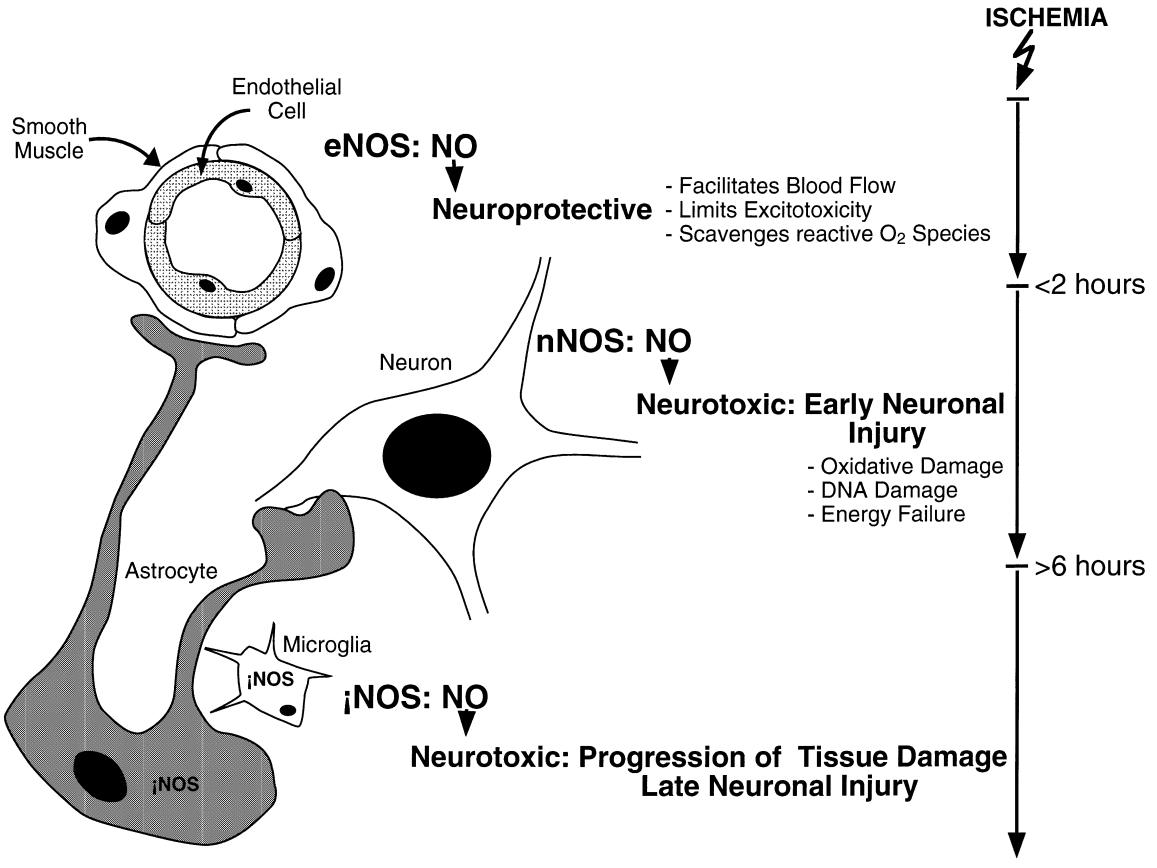


Figure 5 Role of NO in ischemia-induced brain injury. NO is protective or destructive depending on the stage of evolution of the ischemic process and on the cellular source of NO. In the early stages following cerebral ischemia (<2 hours), the vascular actions of endothelial NOS (eNOS)-derived NO are beneficial by, mostly, promoting collateral circulation and microvascular flow. At the same time, glutamate-induced Ca^{2+} overload in ischemic neurons leads to activation of neuronal NOS (nNOS), resulting in production of NO. Large amounts of NO contribute to early neuronal death in the ischemic penumbra, a region in which glutamate excitotoxicity is most marked. At later times (>6 hours), inducible NOS (iNOS) expression is upregulated in astrocytes and in microglia, and large amounts of NO generated by this isoform contribute to late neuronal death and the progression of tissue damage.

toxicity, as mice overexpressing copper/zinc superoxide dismutase (SOD1) are significantly more resistant to MPTP-induced dopaminergic toxicity than their wild-type littermates (Przedborski *et al.,* 1992). Although the superoxide anion appears to play a prominent role in MPTP neurotoxicity, it has been suggested that the superoxide anion is poorly reactive and that it exerts many or most of its toxic effects through the generation of other reactive species (Halliwell *et al.,* 1992). Endogenous NO may react with superoxide to form peroxynitrite, which further inhibits mitochondrial function and damages DNA. DNA damage may lead to excessive activation of PARP in a cell with an already compromised ATP production capability. This pathway appears to play a prominent role in MPTP neurotoxicity, as selective inhibition of nNOS or deletion of nNOS attenuates, in a dose-dependent fashion, MPTP-induced striatal dopaminergic loss in mice (Przedborski *et al.,* 1996; Schulz *et al.,* 1995), and the selective inhibition of nNOS also protects against MPTP-induced dopaminergic neuronal cell death (Przedborski *et al.,* 1996). These protective effects were subsequently demonstrated in a monkey model of MPTP-induced parkinsonism (Hantraye *et al.,* 1996).

Both neuronally derived NO and iNOS-derived NO from microglia may contribute to the loss of dopaminergic terminals and dopaminergic cell bodies, respectively (Liberatore *et al.,* 1999). Because nNOS is not localized within the vicinity of the substantia nigra dopamine-containing neurons, but is densely enriched within the striatum which is densely innervated by dopamine, it is likely that nNOS-derived NO contributes to terminal damage of dopaminergic neurons. Indeed, NO interaction with the superoxide anion to form peroxynitrite leads to the nitration of phenolic rings, particularly those of tyrosine contained within tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. MPTP administration in mice leads to a time-dependent inactivation of TH by tyrosine nitration that appears to be mainly mediated through the actions of neuronally derived NO (Ara *et al.,* 1998). In contrast to the absence of nNOS within the substantia nigra, data suggest that iNOS is significantly upregulated within the substantia nigra following

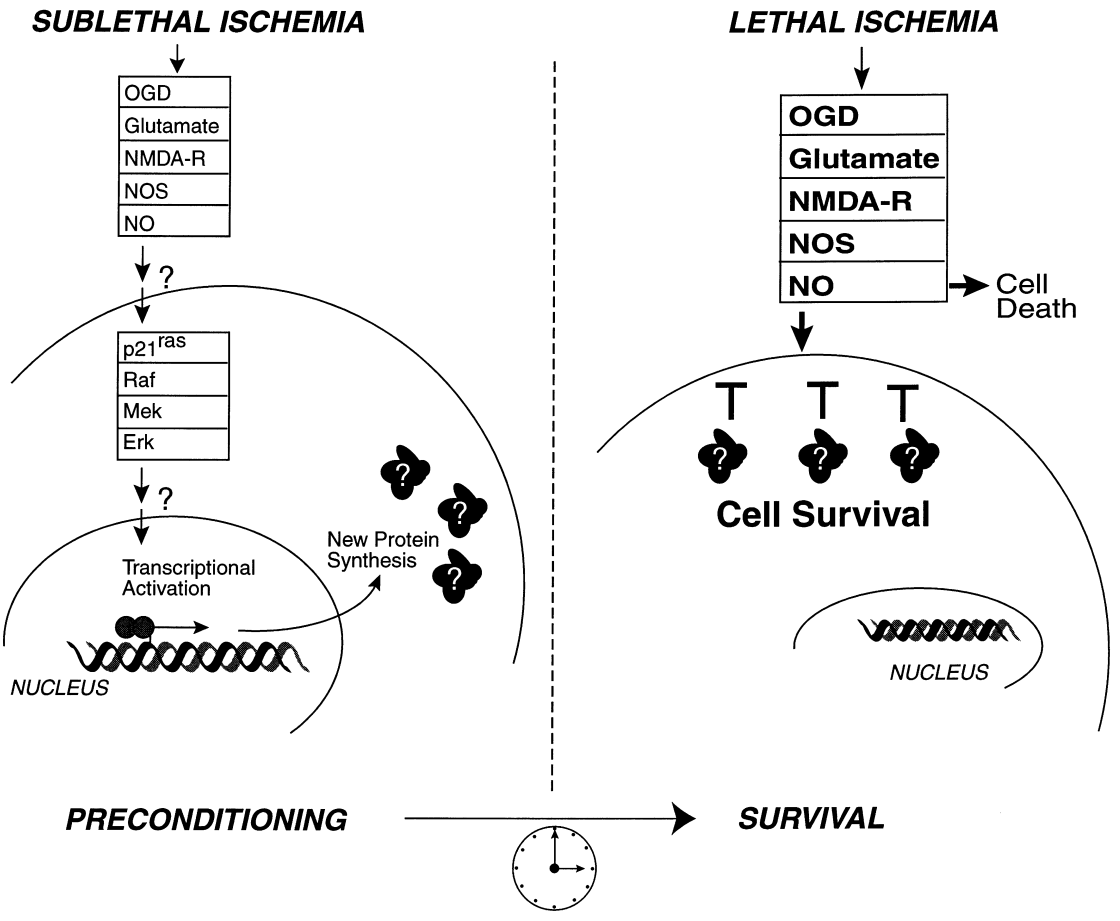


Figure 6 Model for NO/Ras/Erk signaling in ischemic preconditioning. Oxygen–glucose deprivation (OGD) or ischemia induces a multitude of cellular events that can result in preconditioning if the ischemic insult is brief, or in cell death if the insult is sustained. Cellular events triggered by a brief ischemic episode result in the reduction of the resting membrane potential of glia and neurons, leading to release of glutamate. Acting via NMDA receptors, glutamate induces a rapid elevation of intracellular calcium levels that activates a variety of signaling cascades, including activation of neuronal nitric oxide synthase (nNOS) and production of NO. NO leads to activation of $p21^{ras}$, which through the Raf–Mek–Erk signaling cascade can result in induction of gene transcription and new protein synthesis. Some of the newly made proteins may be neuroprotective and sufficient for cellular resistance to subsequent sustained ischemic insults that would normally be lethal for a naive neuron.

MPTP administration to mice. These changes precede or parallel MPTP-induced dopaminergic neuronal cell death, and mice lacking the gene for iNOS are significantly resistant to MPTP compared to wild-type littermates. Thus, iNOS appears to play a significant role in the death of dopaminergic neurons, whereas nNOS-derived NO appears to play a prominent role in the terminal damage and subsequent target-deprived dopaminergic cell death (Fig. 7).

Coupled with the emerging role of nNOS- and iNOS-derived NO in MPTP neurotoxicity is the identification of the downstream targets of NO- and MPTP-induced dopaminergic cell death. NO, superoxide anion, and peroxynitrite have vast potential targets, but they share at least one common downstream target in that they damage DNA (Burney *et al.,* 1998). DNA damage is a prime activator of PARP, which uses NAD as a substrate to transfer ADP-ribose groups to a variety of nuclear proteins. Activation of PARP appears to be critical in MPTP-induced dopaminergic cell death, as mice lacking the gene for PARP are dramatically spared from MPTP neurotoxicity. Furthermore, MPTP potently activates PARP exclusively in vulnerable dopamine-containing neurons of the substantia nigra, and MPTP elicits a novel pattern of poly-ADP-ribosylation of nuclear proteins that completely depends on neuronally derived NO. Thus, NO, DNA damage, and PARP activation appear to play a critical role in MPTP-induced parkinsonism (Mandir *et al.,* 1999).

Conclusion

The concepts of neuronal signaling have clearly been revolutionized by NO. The prominence of the interaction of NO with superoxide anion to form peroxynitrite as an important mediator of neuronal damage is also emerging. Elucidation of the downstream mediators of NO, superoxide, and peroxynitrite should contribute toward a better understanding of the neuronal messenger properties of NO versus its neuro-

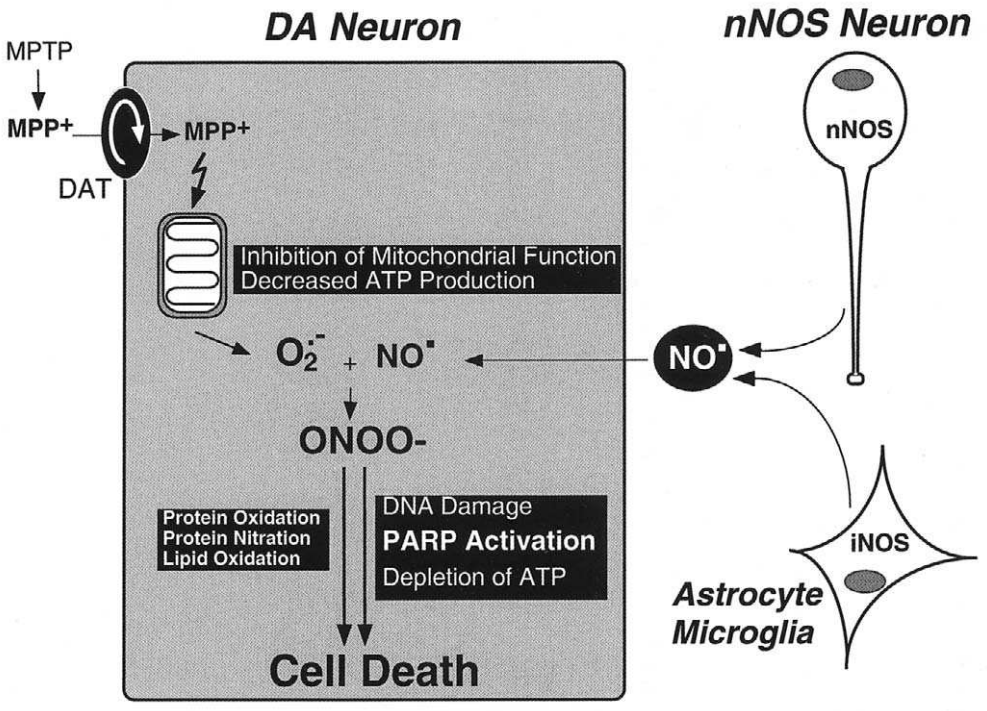


Figure 7 Model for MPTP-induced neurotoxicity. MPTP is converted to MPP^+, which is selectively uptaken by dopamine (DA) neurons through the high-affinity DA transporter (DAT). MPP^+ is a mitochondrial toxin that inhibits mitochondrial function, leading to decreased energy production and increased generation of superoxide ($O_2^{\cdot-}$). NO generated in neurons and astrocytes by neuronal NOS and inducible NOS, respectively, diffuses into DA neurons. NO reacts with $O_2^{\cdot-}$ to produce high concentrations of $ONOO^-$, a potent oxidant which injures cells by oxidizing proteins and lipids, nitrating proteins, and damaging DNA. DNA damage leads to PARP activation, energy depletion, and DA neuronal cell death.

toxic activities. Ultimately, it is hoped that knowledge of these processes will lead to the development of selective therapeutic agents that can be used to treat various neurologic disorders.

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References

Albina, J., Cui, S., Mateo, R., and Reichner, J. (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150,** 5080–5085.

Ara, J., Przedborski, S., Naini, A., Jackson-Lewis, V., Trifiletti, R., Horwitz, J., and Ischiropoulos, H. (1998). Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7659–7663.

Arkovitz, M., Wispe, J., Garcia, V., and Szabo, C. (1996). Selective inhibition of the inducible isoform of nitric oxide synthase prevents pulmonary transvascular flux during acute endotoxemia. *J. Pediatr. Surg.* **31,** 1009–1015.

Babbedge, R., Bland-Ward, P., Hart, S., and Moore, P. (1993). Inhibition of rat cerebellar nitric oxide synthase by 7-nitro indazole and related substituted indazoles. *Br. J. Pharmacol.* **110,** 225–228.

Beal, M., Hyman, B., and Koroshetz, W. (1993). Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative disorders? *Trends Neurosci.* **16,** 25–131.

Beckman, J. S., and Crow, J. P. (1993). Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem. Soc. Trans.* **21,** 330–334.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Bolanos, J., Almeida, A., Stewart, V., Peuchen, S., Land, J., Clark, J., and Heales, S. (1997). Nitric oxide-mediated mitochondrial damage in the brain: Mechanisms and implications for neurodegenerative diseases. *J. Neurochem.* **68,** 2227–2240.

Bredt, D. S., Hwang, P. M., and Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* **347,** 768–770.

Brenman, J., Chao, D., Gee, S., McGee, A., Craven, S., Santillano, D., Wu, Z., Huang, F., Xia, H., Peters, M., Froehne, R. S., and Bredt, D. (1996a). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alphal-syntrophin mediated by PDZ domains. *Cell* **84,** 757–767.

Brenman, J., Christopherson, K., Craven, S., McGee, A., and Bredt, D. (1996b). Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. *J. Neurosci.* **16,** 7407–7415.

Brorson, J., Schumacker, P., and Zhang, H. (1999). Nitric oxide acutely inhibits neuronal energy production. *J. Neurosci.* **19,** 147–158.

Brown, G. C. (1995). Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett.* **369,** 136–139.

Brune, B., von Knethen, A., and Sandau, K. (1998). Nitric oxide and its role in apoptosis. *Eur. J. Pharmacol.* **351,** 261–272.

Buchan, A. M., Gertler, S. Z., Huang, Z. G., Li, H., Chaundry, K. E., and Xue, D. (1994). Failure to prevent selective CA1 neuronal death and reduce cortical infaction following cerebral ischemia with inhibition of nitric oxide synthase. *Neuroscience* **61,** 1–11.

Buisson, A., Plotkine, M., and Boulu, R. G. (1992). The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischaemia. *Br. J. Pharmacol.* **106,** 766–767.

Burney, S., Caulfield, J., Niles, J., Wishnok, J., and Tannenbaum, S. (1998). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat. Res.* **424,** 37–49.

Chen, J., Chang, B., Williams, M., and Murad, F. (1991). Sodium nitroprusside degenerates cultured rat striatal neurons. *Neuroreport* **2,** 121–3.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1,** 623–634.

Choi, D. W. (1992). Excitotoxic cell death. *J. Neurobiol.* **23,** 1261–1276.

Choi, D. W., and Rothman, S. M. (1990). The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death. *Annu. Rev. Neurosci.* **13,** 171–182.

Cohen, G. (1997). Caspases: The executioners of apoptosis. *Biochem. J.* **326,** 1–16.

Crow, J. P., Beckman, J. S., and McCord, J. M. (1995). Sensitivity of the essential zinc–thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry* **34,** 3544–3552.

Dalkara, T., Yoshida, T., Irikura, K., and Moskowitz, M. A. (1994). Dual role of nitric oxide in focal cerebral ischemia. *Neuropharmacology* **33,** 1447–1452.

Dawson, T. M., and Dawson, V. L. (1999). Nitric oxide in the nervous system. *In* ''Elsevier's Encyclopedia of Neuroscience'' (G. Adelman and B. H. Smith, eds.), 2nd enlarged and revised edition, pp. 1473–1476. Elsevier, Amsterdam.

Dawson, T. M., Bredt, D. S., Fotuhi, M., Hwang, P. M., and Snyder, S. H. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and periphral tissues. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7797–7801.

Dawson, T. M., Steiner, J. P., Dawson, V. L., Dinerman, J. L., Uhl, G. R., and Snyder, S. H. (1993). Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9808–9812.

Dawson, T. M., Zhang, J., Dawson, V. L., and Snyder, S. H. (1994). Nitric oxide: Cellular regulation and neuronal injury. *Prog. Brain Res.* **103,** 365–369.

Dawson, T. M., Hung, K., Dawson, V. L., Steiner, J. P., and Snyder, S. H. (1995). Neuroprotective effects of gangliosides may involve inhibition of nitric oxide synthase. *Ann. Neurol.* **37,** 115–118.

Dawson, T. M., Gonzalez-Zulueta, M., Kusel, J., and Dawson, V. L. (1998). Nitric oxide: Diverse actions in the central and peripheral nervous systems. *Neuroscientist* **4,** 96–112.

Dawson, V. L., and Dawson, T. M. (1996). Free radicals and neuronal cell death. *Cell Death Differ.* **3,** 71–78.

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., and Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 6368–6371.

Dawson, V. L., Dawson, T. M., Bartley, D. A., Uhl, G., and Snyder, S. (1993). Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13,** 2651–2661.

Dawson, V., Kizushi, V., Huang, P., Snyder, S., and Dawson, T. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase deficient mice. *J. Neurosci.* **16,** 2479–2487.

de Murcia, G., and Mennissier de Murcia, J. (1994). Poly(ADP-ribose) polymerase: A molecular nick-sensor. *Trends Biochem. Sci.* **19,** 172–176.

de Murcia, G., Schreiber, V., Molinete, M., Saulier, B., Poch, O., Masson, M., Niedergang, C., and Menissier de Murcia, J. (1994). Structure and function of poly(ADP-ribose) polymerase. *Mol. Cell. Biochem.* **138,** 15–25.

Demerle-Pallardy, C., Lonchampt, M. O., Chabrier, P. E., and Braquet, P. (1991). Absence of implication of L-arginine/nitric oxide pathway on neuronal cell injury induced by L-glutamate or hypoxia. *Biochem. Biophys. Res. Commun.* **181,** 456–464.

Dimmeler, S., Lottspeich, F., and Brune, B. (1992). Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* **267,** 16771–16774.

Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1 b-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.* **185,** 601–608.

Dinerman, J. L., Steiner, J. P., Dawson, T. M., Dawson, V. L., and Snyder, S. H. (1994a). Protein phosphorylation inhibits neuronal nitric oxide synthase. *Neuropharmacology* **33,** 1245–1252.

Dinerman, J. L., Dawson, T. M., Schell, M. J., Snowman, A., and Snyder, S. H. (1994b). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: Implications for synaptic plasticity. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4214–4218.

Eliasson, M., Sampei, K., Mandir, A., Hurn, P., Traystman, R., Bao, J., Pieper, A., Wang, Z.-Q., Dawson, T., Snyder, S., and Dawson, V. (1997). Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med.* **3,** 1–8.

Endres, M., Wang, Z.-Q., Namura, S., Waeber, C., and Moskowitz, M. (1997). Ischemic brain injury is mediated by the activation of poly(ADP-ribose) polymerase. *J. Cereb. Blood Flow Metab.* **17,** 1143–1151.

Feldman, P., Griffith, D., and Stuehr, D. (1993). The surprising life of nitric oxide. *Chem. Eng. News* **20,** 26–38.

Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Bird, E. D., and Martin, J. B. (1985). Selective sparing of a class of striatal neurons in Huntington's disease. *Science* **230,** 561.

Firestein, B., and Bredt, D. (1999). Interaction of neuronal nitric-oxide synthase and phosphofructokinase-M. *J. Biol. Chem.* **274,** 10545–10550.

Garthwaite, G., and Garthwaite, J. (1994). Nitric oxide does not mediate acute glutamate neurotoxicity, nor is it neuroprotective, in rat brain slices. *Neuropharmacology* **33,** 1431–1438.

Garthwaite, J., and Boulton, C. L. (1995). Nitric oxide signaling in the central nervous system. *Annu. Rev. Physiol.* **57,** 683–706.

Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336,** 385–388.

Gidday, J., Shah, A., Maceren, R., Wang, Q., Pelligrino, D., Holtzman, D., and Park, T. (1999). Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *J. Cereb. Blood Flow Metab.* **19,** 331–340.

Gonzalez-Zulueta, M., Mukhin, G., Ensz, L., Zwacka, R., Engelhardt, J., Oberley, L., Dawson, V., and Dawson, T. (1998). Manganese superoxide dismutase protects nNOS neurons from NMDA and nitric oxide mediated neurotoxicity. *J. Neurosci.* **18,** 2040–2055.

Hale, A., Smith, C., Sutherland, L., Stoneman, V., Longthorne, V., Culhane, A., and Williams, G. (1996). Apoptosis: Molecular regulation of cell death. *Eur. J. Biochem.* **236,** 1–16.

Halliwell, B., Gutteridge, J., and Cross, C. (1992). Free radicals, antioxidants, and human disease: Where are we now? *J. Lab. Clin. Med.* **119,** 598–620.

Hansen, D. J., Peterson, K., Trivedi, M., Kramer, S., Webber, R., Tjoeng, F., Moore, W., Jerome, G., Kornmeier, C., Manning, P., Conno, J., Misko, T., Currie, M., and Pitzele, B. (1998). 2-Iminohomopiperidinium salts as selective inhibitors of inducible nitric oxide synthase (iNOS). *Med. Chem.* **41,** 1361–1366.

Hantraye, P., Brouillet, E., Ferrante, R., Palfi, S., Dolan, R., Matthews, R., and Beal, M. (1996). Inhibition of neuronal nitric oxide synthase prevents MPTP-induced parkinsonism in baboons. *Nat. Med.* **2,** 1017–1021.

Hara, H., Huang, P. L., Panahian, N., Fishman, M. C., and Moskowitz, M. A. (1996). Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. *J. Cereb. Blood Flow Metab.* **16,** 605–611.

Heikkila, R., Sieber, B., Manzino, L., and Sonsalla, P. (1989). Some features of the nigrostriatal dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) in the mouse. *Mol. Chem. Neuropathol.* **10,** 171–183.

Heller, B., Wang, Z. Q., Wagner, E. F., Radons, J., Burkle, A., Fehsel, K., Burkart, V., and Kolb, H. (1995). Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J. Biol. Chem.* **270,** 11176–11180.

Heurteaux, C., Lauritzen, I., Widmann, C., and Lazdunski, M. (1995). Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K^+ channels in cerebral ischemic preconditioning. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4666–4670.

Hewett, S. J., Corbett, J. A., McDaniel, M. L., and Choi, D. W. (1993). Inhibition of nitric oxide formation does not protect murine cortical cell cultures from *N*-methyl-D-aspatate neurotoxicity. *Brain Res.* **625,** 337–341.

Higuchi, Y., Hattori, H., Kume, T., Tsuji, M., Akaike, A., and Furusho, K. (1998). Increase in nitric oxide in the hypoxic–ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine. *Eur. J. Pharmacol.* **342,** 47–49.

Hope, B. T., Michael, G. J., Knigge, K. M., and Vincent, S. R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2811–2814.

Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265,** 1883–1885.

Hyman, B. T., Marzloff, K., Wenniger, J. J., Dawson, T. M., Bredt, D. S., and Snyder, S. H. (1992). Relative sparing of nitric oxide-containing neurons in the hippocampal formation in Alzheimer disease. *Ann. Neurol.* **32,** 818.

Iadecola, C., Xu, X., Zhang, F., el-Fakahany, E. E., and Ross, M. E. (1995a). Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* **15,** 52–59.

Iadecola, C., Zhang, F., Xu, S., Casey, R., and Ross, M. (1995b). Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J. Cereb. Blood Flow Metab.* **15,** 378–384.

Iadecola, C., Zhang, F., Casey, R., Nagayama, M., and Ross, M. (1997). Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J. Neurosci.* **17,** 9157–9164.

Inagaki, S., Susuki, K., Taniguchi, N., and Takagi, H. (1991). Localization of Mn-superoxide dismutase (Mn-SOD) in cholinergic and somatostatin-containing neurons in the rat neostriatum. *Brain Res.* **549,** 174–177.

Ishida, T., Yarimizu, K., Gute, D. C., and Korthuis, R. J. (1997). Mechanisms of ischemic preconditioning. *Shock* **8,** 86–94.

Izumi, Y., Benz, A. M., Clifford, D. B., and Zorumski, C. F. (1992). Nitric oxide inhibitors attenuate *N*-methyl-D-aspartate excitotoxicity in rat hippocampal slices. *Neurosci. Lett.* **135,** 227–230.

Jang, D., Szabo, C., and Murrell, G. (1996). S-substituted isothioureas are potent inhibitors of nitric oxide biosynthesis in cartilage. *Eur. J. Pharmacol.* **312,** 341–347.

Jenner, P., Schapira, A., and Marsden, C. (1992). New insights into the cause of Parkinson's disease. *Neurology* **42,** 2241–2250.

Kasischke, K., Ludolph, A., and Riepe, M. (1996). NMDA antagonists reverse increased hypoxic tolerance by preceding chemical hypoxia. *Neurosci. Lett.* **214,** 175–178.

Kato, H., Liu, Y., Araki, T., and Kogure, K. (1992). MK801, but not anisomycin, inhibits the induction of tolerance to ischemia in the gerbil hippocampus. *Neurosci. Lett.* **139,** 118–121.

Kerwin, J. F., Heller, J., and Heller, M. (1994). The arginine–nitric oxide pathway: A target for new drugs. *Med. Res. Rev.* **14,** 23–74.

Kitagawa, K., Matsumoto, M., Kuwabara, K., Tagaya, M., Ohtsuki, T., Hata, R., Ueda, H., Handa, N., Kimura, K., and Kamada, T. (1990). ''Ischemic tolerance'' phenomenon found in the brain. *Brain Res.* **528,** 21–24.

Kitayana, S., Shimada, S., and Uhl, G. (1992). Parkinsonism-inducing neurotoxin MPP^+: Uptake and toxicity in non-neuronal COS cells expressing dopamine transporter cDNA. *Ann. Neurol.* **32,** 109–111.

Koh, J. Y., Peters, S., and Choi, D. W. (1986). Neurons containing NADPH-diaphorase are selectively resistant to quinolinate toxicity. *Science* **234,** 73–76.

Lander, H., Ogiste, J., Teng, K., and Novogrodsky, A. (1995). p21ras as a common signaling target of reactive free radicals and cellular redox stress. *J. Biol. Chem.* **270,** 21195–21198.

Langston, J., Langston, E., and Irwin, I. (1984). MPTP-induced Parkinsonism in human and non-human primates—clinical and experimental aspects. *Acta Neurol. Scand. Suppl.* **100,** 49–54.

Lautier, D., Lagueux, J., Thibodeau, J., Menard, L., and Poirier, G. G. (1993). Molecular and biochemical features of poly(ADP-ribose) metabolism. *Mol. Cell. Biochem.* **122,** 171–193.

Leist, M., and Nicotera, P. (1997). The shape of cell death. *Biochem. Biophys. Res. Commun.* **236,** 1–9.

Leist, M., Volbracht, C., Kuhnle, S., Fava, E., and Nicotera, P. (1997). Caspase-mediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. *Mol. Med.* **3,** 750–764.

Liberatore, G. T., Jackson-Lewis, V., Vukosavic, S., Mandir, A. S., Vila, M., McAuliffe, W. G., Dawson, V. L., Dawson, T. M., and Przedborski, S. (1999). Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Nat. Med.* **5,** 1403–1409.

Liebler, D., Aust, A., Wilson, G., and Copeland, E. (1998). Reactive oxidants from nitric oxide, oxidants and cellular signalling, and repair of oxidative DNA damage: A chemical pathology study section workshop. *Mol. Carcinogen.* **22,** 209–220.

Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Lustig, H. S., von Brauchitsch, K. L., Chan, J., and Greenberg, D. A. (1992). Cyclic GMP modulators and excitotoxic injury in cerebral cortical cultures. *Brain Res.* **577,** 343–346.

Malinski, T., Bailey, F., Zhang, Z. G., and Chopp, M. (1993). Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.* **13,** 355–358.

Mandir, A., Przedborski, S., Jackson-Lewis, V., Wang, Z., Simbulan-Rosenthal, C., Smulson, M., Hoffman, B., Guastella, D., Dawson, V., and Dawson, T. (1999). Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 5774–5779.

Mannick, J., Miao, X., and Stamler, J. (1997). Nitric oxide inhibits Fas-induced apoptosis. *J. Biol. Chem.* **272,** 24125–24128.

Messmer, U., Reimer, D., Reed, J., and Brune, B. (1998). Protease activation during nitric oxide-induced apoptosis: Comparison between poly(ADP-ribose) polymerase and U1-70kDa cleavage. *Eur. J. Pharmacol.* **349,** 333–343.

Meldrum, B., and Garthwaite, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11,** 379–87.

Molina y Vedia, L., McDonald, B., and Reep, B. (1992). Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.* **267,** 24929–24932.

Moncada, C., Lekieffre, D., Arvin, B., and Meldrum, B. (1992). Effect of NO synthase inhibition on NMDA- and ischaemia-induced hippocampal lesions. *Neuroreport* **3,** 530–532.

Moncada, S. (1993). The L-arginine: Nitric oxide pathway, cellular transduction and immunological roles. *Adv. Second Messenger Phosphoprotein Res.* **28,** 97–99.

Murry, C., Jenning, R., and Reimer, K. (1986). Preconditioning with ischemia: A delay in lethal injury in ischemic myocardium. *Circulation* **74,** 1124–1136.

Nathan, C. F. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6,** 3051–3064.

Nishikawa, T., Kirsch, J. R., Koehler, R. C., Miyabe, M., and Traystman, R. J. (1994). Nitric oxide synthase inhibition reduces caudate injury following transient focal ischemia in cats. *Stroke* **25,** 877–885.

Nowicki, J. P., Duval, D., Poignet, H., and Scatton, B. (1991). Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. *Eur. J. Pharmacol.* **204,** 339–340.

Oren, M. (1997). Lonely no more: P53 finds its kin in a tumor suppressor haven. *Cell* **90,** 829–832.

Panahian, N., Yoshida, T., Huang, P. L., Hedley-Whyte, E. T., Dalkara, T., and Fishman, M. C. (1996). Attenuated hippocampal damage after global cerebral ischemia in mice mutant in neuronal nitric oxide synthase. *Neuroscience* **72,** 343–354.

Ping, P., Takano, H., Zhang, J., Tang, X., Qiu, Y., Li, R., Banerjee, S., Dawn, B., Balafonova, Z., and Bolli, R. (1999). Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: A signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ. Res.* **84,** 587–607.

Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A., Simonetti, S., Fahn, S., Carlson, E., Epstein, C., and Cadet, J. (1992). Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J. Neurosci.* **12,** 1658–1567.

Przedborski, S., Jackson-Lewis, V., Yokohama, R., Shibata, T., Dawson, V. L., and Dawson, T. M. (1996). Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4565–4571.

Reif, D. W. (1993). Delayed production of nitric oxide contributes to NMDA-mediated neuronal damage. *Neuroreport* **4,** 566–568.

Samdani, A. F., Newcamp, C., Resink, A., Facchinetti, F., Hoffman, B. F., Dawson, V. L., and Dawson, T. M. (1996). Differential susceptibility to neurotoxicity mediated by neurotrophins and nitric oxide synthase. *J. Neurosci.* **17,** 4633–4541.

Sandau, K., Pfeilschifter, J., and Brune, B. (1997). Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis. *Kidney Int.* **52,** 378–386.

Sarih, M., Souvannavong, V., and Adam, A. (1993). Nitric oxide induces macrophage death by apoptosis. *Biochem. Biophys. Res. Commun.* **191,** 503–508.

Sattler, R., Xiong, Z., Lu, W., Hafner, M., MacDonald, J., and Tymianski, M. (1999). Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* **284,** 1845–1848.

Schmidt, H. H. H. W., and Walter, U. (1994). NO at work. *Cell* **78,** 919–925.

Schulz, J. B., Matthews, R. T., Muqit, M. M., Browne, S. E., and Beal, M. F. (1995). Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *J. Neurochem.* **64,** 936–939.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Strijbos, P., Leach, M., and Garthwaite, J. (1996). Vicious cycle involving Na^+ channels, glutamate release and NMDA receptors mediates delayed neurodegeneration through nitric oxide formation. *J. Neurosci.* **16,** 5004–5013.

Stuehr, D., and Nathan, C. (1989). Nitric oxide: A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169,** 1543–1545.

Takeda, Y., Tashima, M., and Okazaki, T. (1999). Ceramide generation in nitric oxide-induced apoptosis. *J. Biol. Chem.* **274,** 10654–10660.

Thiemermann, C., Ruetten, H., Wu, C., and Vane, J. (1995). The multiple organ dysfunction syndrome caused by endotoxin in the rat: Attenuation of liver dysfunction by inhibitors of nitric oxide synthase. *Br. J. Pharmacol.* **116,** 2845–2851.

Thomas, E., and Pearse, A. (1961). The fine localization of dehydrogenases in the nervous system. *Histochemistry* **2,** 266–282.

Uemura, Y., Kowall, N. W., and Beal, M. F. (1990). Selective sparing of NADPH-diaphorase-somatostatin-neuropeptide Y neurons in ischemic gerbil striatum. *Ann. Neurol.* **27,** 620–625.

Vaux, D., and Strasses, A. (1996). The molecular biology of apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2239–2244.

Wang, Z.-Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K., and Wagner, E. (1997). PARP is important for genomic stability but dispensable in apoptosis. *Gene Dev.* **11,** 2347–2358.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., and Hentze, M. W. (1993). Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *EMBO J.* **12,** 3651–3657.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Grunewald, K., Wachter, H., and Hentze, M. W. (1994). Stimulation of IRE-BP activity of IRF by tetrahydrobiopterin and cytokine dependent induction of nitric oxide synthase. *Adv. Exp. Med. Biol.* **356,** 133–139.

White, R. J., and Reynolds, I. J. (1996). Mitochondrial depolarization in glutamate-stimulated neurons: An early signal specific to excitotoxin exposure. *J. Neurosc.* **16,** 5688–5697.

Wood, J., and Garthwaite, J. (1994). Models of the diffusional spread of nitric oxide: Implications for neural nitric oxide signalling and its pharmacological properties. *Neuropharmacology* **33,** 1235–1244.

Yabuki, M., Kariya, S., Inai, Y., and Usumi, K. (1997). Molecular mechanism of apoptosis in HL-60 cells induced by a nitric oxide-releasing compound. *Free Radical Res.* **27,** 325–335.

Yoshida, T., Limmroth, V., Irikura, K., and Moskowitz, M. A. (1994). The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J. Cereb. Blood Flow Metab.* **14,** 924–929.

Yun, H.-Y., Dawson, V. L., and Dawson, T. M. (1996). Neurobiology of nitric oxide. *Crit. Rev. Neurobiol.* **10,** 291–316.

Yun, H.-Y., Gonzalez-Zulueta, M., Dawson, V., and Dawson, T. (1998). Nitric oxide mediates *N*-methyl-D-aspartate receptor-induced activation of p21ras. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5773–5778.

Zhang, F., and Iadecola, C. (1998). Temporal characteristics of the protective effect of aminoguanidine on cerebral ischemic damage. *Brain Res.* **802,** 104–110.

Zhang, J., and Snyder, S. H. (1995). Nitric oxide in the nervous system. *Annu. Rev. Pharmacol. Toxicol.* **35,** 213–233.

Zhang, J., Dawson, V., Dawson, T., and Snyder, S. (1994). Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science* **263,** 687–689.

Zhang, Z. G., Chopp, M., Zaloga, C., Pollock, J. S., and Forstermann, U. (1993). Cerebral endothelial nitric oxide synthase expression after focal cerebral ischemia in rats. *Stroke* **24,** 2016–2021.

Zhang, Z. G., Chopp, M., Gautam, S., Zaloga, C., Zhang, R. L., Schmidt, H. H., Pollock, J. S., and Forstermann, U. (1994). Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. *Brain Res.* **654,** 85–95.

Zhang, Z., Reif, D., Macdonald, J., Tang, W., Kamp, D., Gentile, R., Shakespeare, W., Murray, R., and Chopp, M. (1996). ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. *J. Cereb. Blood Flow Metab.* **16,** 599–604.

CHAPTER 44

Role of Macrophage-Derived Nitric Oxide in Target Cell Injury

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MACROPHAGES CAN RECOGNIZE AND ELIMINATE TUMOR CELLS USING, FOR THIS PURPOSE, A VARIETY OF CYTOTOXIC EFFECTORS. ABUNDANT EVIDENCE SUPPORTS THE CONCEPT THAT NITRIC OXIDE (NO) IS ONE OF THESE CYTOTOXIC EFFECTORS, CAPABLE OF INDUCING APOPTOTIC DEATH IN A VARIETY OF NEOPLASTIC CELLS. JUST AS INTERESTING, HOWEVER, ARE MORE RECENT RESULTS THAT INDICATE NO CAN ACTUALLY SUPPRESS APOPTOSIS INITIATED BY EXPOSURE TO NO AND PROTECT CELLS FROM OTHER APOPTOSIS-INDUCING AGENTS. THIS CHAPTER, THEREFORE, WILL INITIALLY DISCUSS THE CHARACTERISTICS OF MACROPHAGE TUMOR CYTOTOXICITY AND THE POTENTIAL MECHANISMS BY WHICH NO CAN INDUCE APOPTOSIS IN TUMOR CELLS. IN ADDITION, OBSERVATIONS OF SPONTANEOUS AND ACQUIRED RESISTANCE TO NO WILL BE ANALYZED. LAST, THE RELEVANCE OF RESULTS OBTAINED USING ANIMAL CELLS TO THE BIOLOGY OF THE HUMAN MACROPHAGE WILL BE CONSIDERED.

Introduction

The nonspecific stimulation of macrophages by intracellular bacteria determines the development of host resistance to the proliferation of neoplastic cells that is not mediated by specific antibodies or by classic, cell-mediated immune responses (Jun *et al.,* 1996; Hibbs *et al.,* 1990). In this regard, the experimental induction of chronic protozoan infections in AKR mice was shown to delay the onset of spontaneous leukemia in the animals (Aliprantis *et al.,* 1996). Subsequent work identified the activated macrophage as the mediator of this acquired and nonspecific antitumor activity (Brüne *et al.,* 1996; MacMicking *et al.,* 1997; Hirokawa *et al.,* 1994). Peritoneal macrophages obtained from mice chronically infected with unrelated pathogens such as protozoa or intracellular bacteria were shown to be cytotoxic for syngeneic, allogeneic, and xenogeneic tumor cells *in vitro.* Moreover, macrophages mobilized to sites of infection were found to undergo the functional and morphological modifications characteristic of the activated state and to acquire the capacity to recognize and destroy neoplastic cells (Clark *et al.,* 1997). The presence of an ongoing local cell-mediated immune response was shown to be necessary to induce macrophage activation, endowing the cell with tumoricidal capacity (Aliprantis *et al.,* 1996). The paradigm of a two-stepped sequence of macrophage priming and activation for tumoricidal activity evolved from investigations which revealed that activation *in vitro* required the presence of interferon-γ (IFN-γ), a T-lymphocyte-derived cytokine, and bacterial products such as lipopolysaccharide (LPS) or muramyl dipeptide as second signals (Adams and Hamilton, 1984; Cohn, 1978).

Tumor cell lysis and cytostasis have been shown to be accomplished through an arsenal of effector mechanisms elaborated by the activated macrophage. These cytotoxic effectors include various proteases, reactive oxygen intermediates, tumor necrosis factor α (TNF-γ), and reactive nitrogen species (Adams *et al.,* 1982; Adams and Nathan, 1983). Central to this chapter will be evidence regarding the participation of NO or its more reactive derivatives in macrophage-dependent tumor cell cytotoxicity and, more

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narrowly, that which characterizes NO-mediated tumor cell death as apoptotic in mechanism (Cavalli *et al.,* 1994; Tsang *et al.,* 1993).

Role of Macrophage-Derived NO as an Antitumor Effector

Activated murine macrophages induced cytostasis in tumor cell lines only when the culture medium contained L-arginine (Hibbs *et al.,* 1987a, b). This antitumor effect correlated with the metabolism of L-arginine to NO_2^- and L-citrulline, as inhibition of this pathway by the L-arginine analog N^G-monomethyl-L-arginine (*N*-MMA) suppressed cytostasis. Moreover, cytostasis was accompanied by the inhibition of aconitase, an enzyme in the citric acid cycle. NO was subsequently shown to be the precursor for NO_2^- and NO_3^- produced by macrophages from L-arginine, and authentic NO gas duplicated the cytostatic injury (Drapier *et al.,* 1988; Hibbs *et al.,* 1988; Stuehr and Nathan, 1989). Previous reports had shown that tumor cells cocultured with activated macrophages had reduced mitochondrial respiration and that macrophages could not lyse L1210 leukemia cells when cultured in the presence of excess glucose (Hibbs *et al.,* 1988, 1987b). Although NO inhibited mitochondrial oxidative phosphorylation, a compensatory increased reliance on glycolysis to generate ATP by tumor cells injured by NO seemed to explain these findings. Indeed, the oxidoreductases of electron transport chain complexes I and II were specifically inhibited by the activated macrophages, whereas other more distal enzymes of the electron transport chain were thought to be unaffected (Hamilton *et al.,* 1996). A common mechanistic explanation for these diverse findings emerged when iron loss was identified as a component of the metabolic damage of tumor cells caused by activated macrophages (Drapier and Hibbs, 1988; Hibbs *et al.,* 1984). All enzymes initially shown to be inhibited in tumor cells exposed to macrophages contain nonheme iron–sulfur clusters. The degradation of these clusters through iron sequestration by NO into iron–nitrosyl complexes results in loss of enzyme activity. Exposure of tumor cells to authentic NO gas was able to mimic the L-arginine-dependent antitumor effects of activated macrophages, including loss of intracellular iron as well as inhibition of mitochondrial respiration, aconitase, and DNA synthesis (Hibbs *et al.,* 1988; Stuehr and Nathan, 1989). The provision of excess iron to these cells restored enzyme activity and prevented tumor cell death. Since none of the enzymes in the glycolytic pathway contain iron–sulfur centers, glycolysis remains functional as an ATP-generating system in the presence of NO (Albina and Mastrofrancesco, 1993).

The observed suppression of DNA synthesis in tumor cells injured by cytotoxic macrophages could be explained by the NO-dependent inhibition of ribonucleotide reductase, a rate-limiting enzyme in DNA synthesis (Soo Kwon *et al.,* 1991; Lepoivre *et al.,* 1992; Kwon *et al.,* 1991). Further contributions by Hibbs and co-workers and other investigators led to the conclusion that NO is a mediator of the cytotoxic activity of activated macrophages and to the proposal that NO kills or limits growth in tumor cells by inducing metabolic failure and inhibiting DNA replication through the perturbation of key enzymes via iron sequestration. The profound inhibition of protein synthesis that is also found in cells exposed to NO was more recently explained by the NO-dependent phosphorylation of the initiation factor eiF-2α and the inhibition of the 80S ribosomal complex formation (Kim *et al.,* 1998).

Since the aforementioned seminal publications, a better understanding of the complexity of NO synthesis and chemistry has mandated the reconsideration of the potential mechanisms of NO-dependent cellular injury. NO production is now recognized as resulting from the five-electron oxidation of one of the chemically equivalent guanidino nitrogens of L-arginine through the action of nitric oxide synthase (NOS) (reviewed in Morris and Billiar, 1994). Two basic isoforms of NOS have been described to date based on their constitutive (cNOS) or inducible (iNOS) expression. The inducible form of NOS can be found in several cell types including macrophages, hepatocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells following stimulation with a variety of factors including microbes, microbial products, and inflammatory cytokines. Expression of iNOS is often increased synergistically by combining several of these agents.

Mentioned earlier was the intricacy of NO chemistry. Indeed, NO per se has been shown to behave rather poorly as a free radical. It is its reactivity with molecular oxygen, transition metals, and superoxide that results in the formation of compounds with potentially profound cytotoxic effects. These derivatives of NO include highly reactive intermediates such as its oxidation product nitrosonium ion (NO^+), its reduction product nitroxyl radical (NO^-), the product of its reaction with superoxide anion, peroxynitrite ($ONOO^-$), as well as other compounds generated from these precursors (Cavalli *et al.,* 1994; Tsang *et al.,* 1993; Muijsers *et al.,* 1997). In reacting with dioxygen, NO forms *N*-nitrosamines, and the nitrosation of thiols results in the generation of *S*-nitrosothiols. The nitrosation of amines and the deamination of DNA bases could, as will be discussed later, contribute to the toxicity of NO (Nguyen *et al.,* 1992; Sonoki *et al.,* 1997).

The inhibition of iron–sulfur cluster-containing enzymes by NO has already been mentioned. Disruption of nonheme iron enzymes by NO, including aconitase, NADH:ubiquinone oxidoreductase, succinate:ubiquinone oxidoreductase, and cytochrome oxidase, explains some of the most prominent metabolic consequences of cellular exposure to NO (Hibbs *et al.,* 1990). Interestingly, NO can also lead to cellular zinc depletion (Berendji *et al.,* 1997) with metal ion loss from zinc-containing proteins, as shown for metallothionein, and destruction of Cys-type zinc fingers, an effect that will potentially alter transcription factor binding to DNA (Kröncke *et al.,* 1994).

The reactivity of NO with superoxide anion and the resulting formation of peroxynitrite have received considerable attention (Muijsers *et al.,* 1997). Beckman and others have contended that most biological effects of NO thought to mediate cytotoxicity, including mitochondrial inhibition, suppression of ribonucleotide reductase, deamination of DNA, and direct induction of apoptosis, can be attributed to $ONOO^-$ (Beckman and Koppenol, 1996; Lin *et al.,* 1995). Moreover, $ONOO^-$ nitrosylates protein-bound tyrosine residues. Such modification can have profound functional significance. It has been shown, for example, that nitrosylation inhibits tyrosine phosphorylation, an effect with potentially important consequences in signal transduction (Schäffer *et al.,* 1997). Nitration of cytoskeletal proteins, in turn, may target them for proteolysis or fundamentally alter their structure and function.

Because of the limited stability and diffusion range of $ONOO^-$, it is unlikely that this compound is the direct effector molecule released by macrophages (Saran and Bors, 1994). It is conceivable that NO released from the activated macrophages reacts with superoxide within the target cell to generate $ONOO^-$. Because superoxide anion is mainly produced at the electron transport chain in cells lacking NADPH oxidase activity, and considering that NO has been shown to increase the release of superoxide by mitochondria (Poderoso *et al.,* 1996, 1998), the site of intracellular $ONOO^-$ formation could be at or in close proximity to mitochondria, subcellular organelles thought to have a central role in the development of apoptosis.

NO-mediated cytotoxicity may also occur as a result of a chemical interaction with hydrogen peroxide (H_2O_2). In the presence of catalytic levels of a trace metal (perhaps intracellular iron liberated from iron-containing prosthetic groups by NO), a potent cytotoxic oxidizing species can be generated from a chemical reaction involving NO and H_2O_2 (Farias-Eisner *et al.,* 1996). Two inferences may be drawn by the existence of such a pathway regarding mechanisms of cellular sensitivity or resistance to NO toxicity. First, because NO inhibits catalase, the relative sensitivity of a target cell to this NO/H_2O_2/metal cytotoxic mechanism will depend on the activity of the glutathione peroxidase–glutathione reductase system to degrade H_2O_2. Second, this mechanism predicts that increased H_2O_2 formed by the action of superoxide dismutase may accentuate, rather than protect from, oxidative damage.

More recently, another potential mechanism by which NO may cause tumor cell cytostasis was reported (Buga *et al.,* 1998). NO caused a potent (1–10 μM) inhibition of growth of Caco-2 human colon carcinoma cells by mechanisms attributed to S-nitrosylation and inhibition of ornithine decarboxylase. Moreover, N^G-hydroxy-L-arginine, the precursor to NO in the NO synthase-catalyzed conversion of L-arginine to NO plus citrulline, also inhibited tumor cell growth, but by inhibiting the catalytic activity of arginase. Both of these cytostatic effects were independent of any action of cyclic GMP (Buga *et al.,* 1998). The observations that N^G-hydroxy-L-arginine and NO inhibit sequential steps in the arginine–polyamine pathway provide yet another possible mechanism by which elevated NO synthase activity could lead to tumor cell cytostasis.

It follows from the preceding paragraphs that, although the cytotoxicity of NO or its derivatives and the identification of their cellular targets are growing, the adjudication of specific mediators for the toxic effects of NO remains in flux.

Recent results have added another level of complexity to the understanding of the specific role of NO in determining macrophage-dependent cytotoxicity. In this regard, macrophages isolated from experimental wounds in rats are capable of producing NO just as activated peritoneal macrophages (Mateo *et al.,* 1996). In contrast to the peritoneal macrophages, however, the wound-derived cells are not cytotoxic toward NO-sensitive P815 cells. Moreover, macrophages from knockout mice for the NFIL-6 (C/EBPβ) transcription factor produce NO in quantities similar to those from wild-type mice but, like wound-derived cells, are incapable of killing P815 targets (Tanaka *et al.,* 1995). Whether, as it appears, NO is necessary but not sufficient to explain NO-dependent cytotoxicity is under current investigation.

Autotoxicity of NO: Induction of Macrophage Apoptosis by Endogenous or Exogenous NO

Although an important role for macrophage-derived NO as an antineoplastic effector molecule has been established at least *in vitro,* it is also true that the generation of NO exerts significant negative effects on the macrophage that produces it. Work from our laboratory indicated that phagocytosis and the production of reactive oxygen intermediates are profoundly suppressed in rat resident or *Corynebacterium parvum*-elicited peritoneal macrophages cultured in conditions allowing NO production (Albina *et al.,* 1989, 1990). Moreover, NO-producing macrophages exhibit reduced electron transport chain activity, enhanced reliance on glycolysis for energy generation, a reduced ATP content, and decreased protein synthesis. These metabolic alterations are similar to the effects of NO on tumor cells.

In an additional observation, macrophages activated with IFN-γ and LPS rapidly lose their L-arginine-dependent cytotoxic capacity when cultured in media containing L-arginine, and this loss of tumoricidal activity correlates with macrophage death (Albina *et al.,* 1989; Takema *et al.,* 1991). Investigation of the mechanism of macrophage death associated with the production of NO revealed, through morphologic and molecular means, that death in these cells exhibits the characteristics of apoptosis (Albina *et al.,* 1993). Apoptosis, or programmed cell death, is an active process of cell self-destruction with distinct morphologic and molecular characteristics. Morphologically, cells undergoing apoptosis demonstrate prominent cytoplasmic and nuclear condensation, with progressive formation of cell surface blebs and shedding of membrane-bound apoptotic bodies. At the molecular level, DNA is fragmented by a specific Ca^{2+},

Mg^{2+}-dependent endonuclease into oligonucleotides 180 to 200 base pairs in length. Necrosis, in contrast, is a passive and unregulated result of cell death caused by catastrophic toxic or traumatic events. Necrotic cells exhibit organelle swelling and early dissolution of the plasma membrane. DNA in necrotic cells is degraded in a random fashion and appears in gel electrophoresis analysis as a smear without the distinct banding or laddering characteristically found in apoptotic cells.

Macrophages expressing iNOS or exposed to NO gas show cytoplasmic and nuclear condensation, as well as the internucleosomal DNA fragmentation diagnostic of apoptosis (Albina *et al.,* 1993). The role of NO in determining apoptotic death was confirmed by the protective effects of culture in L-arginine-deficient media or the inclusion of inhibitors of NOS in the cultures (Albina *et al.,* 1993). Thus, the production of NO by activated macrophages leads to their functional suppression and, ultimately, to their apoptotic death.

The diagnosis of cell death through apoptosis was originally based on morphologic changes including chromatin condensation, cell blebbing, apoptotic body formation, cell detachment, and the lack of a prominent inflammatory response (Möröy and Zörnig, 1996; Gerschenson and Rotello, 1992). Following the classic studies of Wyllie (Harbrecht *et al.,* 1997) indicating the disruption of DNA during apoptosis by specific nucleases, more recent work has relied on the detection of more or less specific patterns of DNA fragmentation through electrophoretic, isotopic, or enzymatic means for the molecular diagnosis of apoptosis. It has been shown, however, that DNA fragmentation is a dispensable feature of apoptosis and that morphologic evidence for apoptotic death can be found in circumstances where DNA damage is not detectable (Rubin *et al.,* 1989; Bortner *et al.,* 1995; Cohen *et al.,* 1992). Indeed, the relevance of nuclear changes in apoptosis has been ingeniously challenged by the demonstration of clear-cut apoptotic morphology in the absence of a cell nucleus (Jacobson *et al.,* 1994). Moreover, although the original paradigm of programmed cell death indicated a requirement for *de novo* protein synthesis, that is, the engagement of a protein-based death machinery in cells undergoing apoptosis, this also has been shown to be a nonessential component of the apoptotic process, and, indeed, protein synthesis inhibition can initiate apoptosis in certain cells.

The preceding paragraph highlights some of the difficulties encountered when reviewing existing information on the role of specific mediators, in this case NO, in determining apoptosis. Whereas morphologic and molecular evidence should ideally be brought together to confirm the diagnosis of apoptosis, this is not always the case. In the body of literature pertaining to the induction of apoptosis by NO, seldom is morphologic characterization employed for diagnosis. Most published data rely on some or other determination of DNA damage in the cells. Whether this is sufficient to diagnose apoptotic death to the exclusion of necrotic death is questionable. In our own work, we have found that cell exposure to certain doses of H_2O_2 or NO results in simultaneous DNA fragmentation and cell membrane failure, the first a putative measure of apoptosis, the second, of necrosis. In short, documentation of DNA damage, even if meeting the criteria thought to be pathognomonic of apoptosis, does not appear to rule out necrotic cell death. With this caveat in mind, then, we review existing evidence on the causation and mechanisms of NO-dependent tumor cell apoptosis.

Mechanisms for NO-Dependent Apoptosis

Given the gaseous, cell-permeant nature of NO and in view of the multiple potential molecular targets for NO in living cells, it is not surprising that the intimate mechanism for NO-dependent apoptosis is not yet known. By the same token, mechanisms by which cells can escape its toxic effects are difficult to envision. Results from our laboratory demonstrated that activated macrophages induce apoptotic death in some tumor cells (i.e., P815, RAW 264.7) using NO as the cytotoxic effector, that intimate cell contact is not necessary for induction of apoptosis, and that authentic NO gas or NO derived from chemical donor agents could substitute for macrophages in inducing apoptosis (Cui *et al.,* 1994). In contrast, L929 tumor cells were refractory to NO-mediated effects regardless of whether NO was derived from activated macrophages, authentic gas, or chemical donors, even when delivered at high concentrations and for prolonged periods. These cells were killed, incidentally, by either macrophage-derived or recombinant TNF-α through an apoptotic pathway. Reports by others have highlighted the relative NO resistance of other cell types, including hepatocytes (Nussler and Billiar, 1993; Schwarz *et al.,* 1995) and rat mesangial cells (Brüne *et al.,* 1996). By exploiting the insensitivity of certain tumor cells to NO, certain speculative considerations can be made regarding the pathways to apoptotic death.

Impact of NO on Oxidative Metabolism

As mentioned before, early descriptions of the effects of NO on tumor cells and on macrophages indicated the suppression of oxidative metabolism through the inhibition of aconitase and the electron transport chain as causal alterations in the induction of cell death. A reduced oxidative capacity mandates the enhanced generation of metabolic energy through glycolysis. Indeed, the macrophage, a prototypically glycolytic cell, responds to iNOS expression with increased glucose uptake and metabolism through glycolysis and the hexose monophosphate shunt, as if to compensate for a diminished energy production through oxidative metabolism (Albina and Mastrofrancesco, 1993b). In apparent contradiction to these observations, it was demonstrated (Häussinger, 1983; Vedia *et al.,* 1992) that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be inhibited by nitric oxide. However, and probably be-

cause of the large GAPDH protein content found in most cells, the flux of glucose carbon through GAPDH is unimpeded in peritoneal macrophages even when this enzyme is significantly suppressed by NO (Mateo *et al.,* 1995). It is not until GAPDH is almost fully inhibited that lactate production is diminished. It appears that complete suppression of GAPDH constitutes a terminal event, since cell viability is compromised at that time (Albina and Reichner, 1995).

Although the construct of NO-dependent metabolic suppression as a mechanism for apoptosis is attractive and supported by the observed increased reliance on glycolysis of NO-injured cells, other results tend to reduce its apparent causative importance. In this regard, NO-mediated inhibition of key iron–sulfur cluster enzymes is thought to be determined by iron sequestration and loss. Experiments comparing NO-sensitive RAW 264.7 cells to NO-resistant L929 cells demonstrated identical rates of iron loss on exposure to the NO donor agent *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Albina and Reichner, 1998). Additionally, culture of either cell type with SNAP was followed by similar and moderate suppression of mitochondrial respiration. However, whereas RAW 264.7 cells readily underwent apoptosis, L929 cells maintained viability even at the highest concentrations of SNAP. These findings suggest that the classic paradigm holding NO cytotoxicity as mediated through metabolic inhibition may not be fully explanatory.

Role of Antioxidant Species

The free radical nature of NO and its derivatives allows the hypothesis that NO induces apoptosis through mechanisms common to those used by reactive oxygen intermediates. When speculating on potential mechanisms of NO sensitivity or resistance of different cell types, one should consider that the cellular content of antioxidant systems may be involved in determining the sensitivity or resistance to NO-dependent apoptosis (Walker *et al.,* 1995). Cells characterized by a high antioxidant content resist NO and lose such resistance when depleted of glutathione (GSH), yet the role of this antioxidant in cell death or survival remains unclear. Since NO or its by-products can S-nitrosylate GSH, and because *S*-nitroso-GSH (GSNO) can, in turn, release NO, the possibilities exist for GSH to serve as a NO trap and/or as a NO transporter and subsequent donor (Wink *et al.,* 1994). Indeed, because GSH can react with peroxynitrite and with intermediates of the reaction of NO with molecular oxygen, it has been proposed that formation of GSNO can serve to detoxify reactive nitrogen species (Wink *et al.,* 1994). In vascular smooth muscle cells exogenous or endogenous NO induced apoptosis in the context of markedly reduced intracellular GSH content (Zhao *et al.,* 1997). Addition of a cell-permeant form of GSH restored its intracellular concentration and prevented apoptosis. The protective effects of the GSH precursor *N*-acetylcysteine (NAC) have been reported, and the depletion of GSH resulted in higher toxicity of NO in V79 fibroblasts (Chesrown *et al.,* 1994). In contrast, cells derived from the chronic exposure of RAW 264.7 to IFN-γ and endotoxin, which are resistant to endogenously generated NO, were found to contain less GSH than the parental, NO-sensitive RAW 264.7 cells (Boggs *et al.,* 1998). If GSH was, in turn, depleted from the parental cells, these became resistant to the effects of a NO donor agent. The authors proposed that a high intracellular GSH content would serve to reverse the inactivating S-nitrosylation of caspases by NO and allow for the apoptotic process to proceed (see later). Interestingly, a decreased intracellular GSH concentration has been shown to correlate with enhanced toxicity of a variety of agents and to be associated with a change in the type of cell death from apoptosis to necrosis (Shimizu *et al.,* 1994). Moreover, whereas GSH, NAC, and ascorbic acid prevented the apoptotic effects of NO, they augmented those of a nitrosonium ion donor (Khan *et al.,* 1997).

Our experimental data do not support a role for NO-dependent oxidative injury as a mechanism for tumor cell apoptosis. NO-dependent apoptosis of sensitive cell lines (P815, RAW 264.7) was not prevented by antioxidants or radical scavengers including DMSO (1–100 m*M*), ethanol (1–100 m*M*), mannitol (1–100 m*M*), thiourea (1–10 m*M*), or uric acid (1–5 m*M*), nor was it exaggerated by the addition of menadione (0–200 μ*M*), an intracellular superoxide generator. A protective effect against NO-dependent apoptosis was found, however, when NAC was added to cultures (J. E. Albina and J. S. Reichner, 1998, unpublished observations). NAC can act as a direct extracellular antioxidant, or it can be incorporated into cells and support the production of GSH (Stuehr and Nathan, 1989). It has been shown, in this connection, that the affinity of NO for sulfur groups in GSH is $>10^6$-fold higher than that for nucleic acid bases. As mentioned earlier, then, GSH could act as an intracellular ''sink'' for NO and neutralize some of its toxic effects. However, others have shown that the antiapoptotic effects of NAC are not explained by changes in intracellular GSH content (Jones *et al.,* 1995). In particular connection to its effects on NO-dependent cytotoxicity, NAC (and other thiols) can theoretically alter the kinetics of NO release from the NO donor agent used in our experiment, SNAP (Huot *et al.,* 1995). As thiols have been proposed to accelerate the rate of NO release from donors, it may be possible that NAC (and/or other thiols) act by provoking the explosive release of NO from SNAP, thus shortening its presence in the cultures. It is not clear whether it is the maximal NO concentration reached in culture, the duration of NO exposure, or the product of these two factors that is essential in determining NO-mediated apoptosis. Evidence has been provided indicating that it is the total exposure to NO, as defined by integrated concentration–time curves, that correlates best with the induction of apoptosis (Messmer and Brüne, 1996a).

NO and the Induction of Direct DNA Damage

It is also possible that NO may induce apoptosis through direct DNA damage, as NO can deaminate purine and pyrim-

idine bases in DNA and result in increased strand breaks and mutagenesis (Nguyen *et al.,* 1992; deRojas-Walker *et al.,* 1995). Moreover, exposure of isolated nuclei to NO donor agents is followed by DNA strand breaks (Schwarz *et al.,* 1995). Therefore, the process of apoptosis could be initiated directly as a result of DNA damage and may occur in addition to, or independently from, the metabolic effects of NO. In this regard, a mechanism of energetic failure and cell death invokes the activation of poly(ADP-ribose) polymerase (PARP) by DNA damage (Heller *et al.,* 1995; Zhang *et al.,* 1994). PARP is a nuclear enzyme that binds to and is activated by DNA strand breaks (Berger, 1985). Overactivation of PARP would lead to the rapid consumption of its substrate, NAD^+, and through NAD^+ depletion to the inhibition of glycolysis and a catastrophic decrease in the ATP content of the cells (Schraufstatter *et al.,* 1986). Evidence for PARP activation and for its correlation with cell death following H_2O_2 injury has been presented (Schraufstatter *et al.,* 1986).

In regard to a role for PARP in NO-mediated cytotoxicity, a mutant cell line with inactive PARP was shown to undergo DNA damage on exposure to low concentrations (<0.6 m*M*) of the NO donor agent sodium nitroprusside (SNP), just like its wild-type counterpart, but not to undergo lysis (Heller *et al.,* 1995). Higher concentrations of SNP elicited cell lysis in wild-type and mutant cells alike, indicating other, PARP-independent mechanisms of NO toxicity. Consistent with a PARP-independent pathway are the findings that the PARP inhibitors nicotinamide and 3-aminobenzamide failed to protect RAW 264.7 or P815 cells from NO release from SNAP, and that PARP can be cleaved and inactivated in the process of NO-mediated RAW 264.7 apoptosis (Yamazaki and Birnboim, 1995) (see later). This observation appears particularly germane because PARP inhibition in thymocytes increases the number of DNA strand breaks and actually induces apoptosis. Indeed, in our own work, high concentrations of the PARP inhibitors mentioned earlier dramatically increased spontaneous DNA fragmentation in RAW 264.7 cells. Thus, the evidence for the direct involvement of PARP activation or inhibition in mediating NO-dependent apoptosis remains controversial.

NO and Direct Mitochondrial Injury

In connection with the role of mitochondria in NO-mediated apoptosis, NO induces permeability transition in isolated mitochondria, resulting in the release of factors capable of triggering apoptotic changes in isolated cell nuclei (Hortelano *et al.,* 1997). Moreover, exposure of thymocytes to NO determined the disruption of the mitochondrial membrane potential, increased generation of reactive oxygen species and exposure of phosphatidylserine on the outer plasma membrane, and increased apoptotic nuclear changes. Most importantly, chemical prevention of the mitochondrial permeability transition prevented NO-mediated apoptosis of the cells (Hortelano *et al.,* 1997).

NO Resistance: NO as a Positive and Negative Regulator of Apoptosis

Work from our laboratory mentioned previously identified some cell types as particularly resistant to the apoptotic effects of NO. In other experimental models it has been shown that NO resistance can be acquired by a variety of cell types. In this connection, Kim *et al.* (1995) reported that pre-exposure of rat hepatocytes to low concentrations of the NO donor agent SNAP offered protection to a second exposure to higher concentrations of this compound, that protein synthesis was required for expression of acquired resistance, and that the protective effect could be mediated through the upregulation of ferritin levels or the induction of heme oxygenase. Favoring the oxidant hypothesis of NO toxicity, they also reported that SNAP pretreatment resulted in cross-protection against H_2O_2.

Metallothionein overexpression induced by zinc chloride also afforded some protection against NO in NIH 3T3 cells (Schwarz *et al.,* 1995). Experiments in our laboratory also used zinc salts to prevent SNAP-induced apoptosis. Indeed, zinc salts were, in our hands, the most potent tested inhibitors of SNAP-induced apoptosis. The inclusion of zinc in our experiments, and in those by others, was predicated on its reported inhibitory effects on the endonucleases thought to mediate DNA fragmentation (Messmer *et al.,* 1995; Lin *et al.,* 1997). Additional mechanisms by which NO can protect cells from apoptotic death have been reported, including the heat-shock induction of protein 70 (HSP70) (Müller *et al.,* 1997), heme oxygenase 1 (Ceneviva *et al.,* 1998), and cyclooxygenase 2 (von Knethen and Brüne, 1997).

Acquired resistance to NO was also documented in studies where RAW 264.7 cells were exposed to low-dose *S*-nitrosoglutathione (25–200 μ*M*) or pretreated with IFN-γ and LPS in the presence of a NOS inhibitor prior to culture with an otherwise lethal concentration of *S*-nitrosoglutathione (1 m*M*) (Brüne *et al.,* 1996). Pretreatment with the NO donor agent or with the cytokine–LPS–NOS inhibitor combination prevented apoptotic death and was associated with a substantial decrease in p53 accumulation (Brüne *et al.,* 1996).

In a similar manner, Genaro *et al.* (1995) reported that murine B cells exposed to NO or to NO donor agents in low concentrations avoided spontaneous apoptosis, that treatment prevented the natural decay in cellular bcl-2 levels, and, most interestingly, that the protective effects of NO could be duplicated by cell-permeant analogs of cGMP. The latter observation is particularly interesting because it connects apoptosis with one of the earliest recognized effects of NO, namely, the activation of guanylate cyclase. It has been contested in the literature, however, by work demonstrating a lack of effect of cGMP analogs in preventing NO-mediated apoptosis in RAW 264.7 cells (Messmer *et al.,* 1995).

Just mentioned was the potential involvement of pro- and antiapoptotic molecules in determining cell resistance or sensitivity to NO. In this regard, the signal transduction and gene expression events leading to apoptosis remain incom-

pletely defined (Stewart, 1994). The concept that apoptosis results from the activation of a death gene(s) stemmed from observations that cycloheximide or actinomycin D could prevent apoptosis in some circumstances. As mentioned earlier, this model was challenged by findings of apoptosis following treatment with inhibitors of protein or RNA synthesis. A release mechanism through which these inhibitors allow the expression of a gene or genes that mediate apoptosis was proposed to explain cases of inhibitor-induced apoptosis. There are, however, instances where apoptosis is not influenced one way or another by inhibitors of protein or RNA synthesis.

This confusing picture recurs where specific signal transduction pathways and transcription factor physiology have been studied in relation to the development of apoptosis. The apparent similarities between NO- and H_2O_2-mediated responses may offer some guidance to begin to clarify this issue. It was originally proposed that H_2O_2-mediated cellular events, including apoptosis, were initiated by DNA damage. More recent evidence, however, has indicated that H_2O_2 is capable of altering cell membranes through lipid peroxidation and is capable of initiating signaling at the membrane through tyrosine kinases, ion channels, and G proteins, and that downstream activation of redox-sensitive factors, including activator protein 1 (AP-1) and nuclear factor κB (NF-κB), follows the aforementioned membrane signaling (Schreck *et al.,* 1991). The similarities with NO-mediated responses can be readily seen when it is considered that NO has been shown to induce membrane peroxidation and to modulate ion channels, G proteins, kinases, and AP-1/NF-κB (Radi *et al.,* 1991; Tabuchi *et al.,* 1994; Lander *et al.,* 1993). More specifically, NO, both exogenous and cell generated, has been shown to inactivate protein kinase C (PKC), directly activate G proteins, and induce nuclear translocation of NF-κB (Gopalakrishna *et al.,* 1993; Lander *et al.,* 1993). Interestingly, PKC activation suppresses the development of apoptosis in several models, including its induction by NO (Messmer *et al.,* 1995), presumably through binding and activation of AP-1 by TRE (Lucas *et al.,* 1994; Walker *et al.,* 1993). AP-1, in turn, has been characterized as an antiapoptotic and antioxidant responsive factor that maps to a negative response site in the promoter region of the c-*myc* gene, the expression of which correlates with the initiation of apoptosis in T-cell hybridomas (Walker *et al.,* 1993). It can be proposed, then, that by inactivating PKC NO may suppress the structuring of a functional AP-1 transcription factor and thus block the expression of ''protective'' genes. Alternatively, NF-κB has been demonstrated to be an oxidative-stress responsive element, which translocates extensively to the nucleus following cellular exposure to oxidants and presumably upregulates the transcription of ''death genes'' (Schreck *et al.,* 1991).

Insight into the genetic regulation of apoptosis has also been gained from the identification of pro- and antiapoptotic gene products (Stewart, 1994; Hawkins and Vaux, 1994; Canman and Kastan, 1995). In regard to NO-mediated apoptosis, Messmer *et al.* (1994) first reported a correlation between NO exposure and the expression of p53 in studies using RAW 264.7 and RINm5F cells. The nuclear phosphoprotein p53, which was originally characterized as a tumor suppressor agent, acts both as a sequence specific transcription factor to block cells in the G_1/S transition in response to DNA damage and as a trigger for apoptosis (Canman and Kastan, 1995; MacLeod *et al.,* 1990). Although the mechanism of action of p53 in determining these alternative paths of proliferation block or apoptosis remain unclear, it is thought that this gene product can induce apoptosis through altering the balance of the anti- and proapoptosis gene products bcl-2 and Bax (Canman and Kastan, 1995; MacLeod *et al.,* 1990). Indeed, treatment of cells with the NO donor agents *S*-nitrosoglutathione or spermine–NO resulted in the accumulation of Bax (Messmer *et al.,* 1996a). Although these results suggest a role for p53 in the induction of apoptosis by endogenous or exogenous NO, treatment of the p53-negative cell line U937 with *S*-nitrosoglutathione also resulted in the characteristic laddering of DNA that is a hallmark of apoptosis (Messmer and Brüne, 1995).

In contrast to the proapoptotic effects of p53, the expression of the protooncogene *bcl-2* has been shown to prevent apoptosis resulting from oxidative injury, growth factor withdrawal, γ irradiation, heat shock, and the application of certain chemotherapeutic drugs (Hawkins and Vaux, 1994). Experiments in our laboratory explored whether the overexpression of *bcl-2* conferred cell immunity from NO-mediated apoptosis (Albina *et al.,* 1996). In these experiments, P815 cells transfected with whole-length human *bcl-2* were exposed to NO liberated from SNAP. Transfected cells were resistant to a wide dose of SNAP (0–1 m*M*) present during an overnight culture in terms of DNA fragmentation. They were, however, only partially protected from SNAP-dependent cell lysis. In regard to biologically relevant concentrations of NO, the *bcl-2* transfectants were almost completely protected from NO-associated cytotoxicity when exposed to activated murine peritoneal macrophages (Albina *et al.,* 1996).

The mechanism of action of bcl-2 remains unclear (Korsmeyer *et al.,* 1995). Evidence for and against a role for this mitochondrial membrane protein as an antioxidant has been presented (Hockenbery *et al.,* 1990, 1993; Jacobson and Raff, 1995; Muschel *et al.,* 1995). Bcl-2 has been shown, in addition, to prevent the release of cytochrome *c* from mitochondria and to block the cleavage of PARP in NO-producing cells that is putatively associated with the activation of caspase 3 (Kim *et al.,* 1999). Regardless of its mechanism of action, the observation that bcl-2 affords certain protection from NO-dependent apoptosis has been confirmed by others (Messmer *et al.,* 1996b; Genaro *et al.,* 1995; Xie *et al.,* 1996; Raingeaud *et al.,* 1995).

Although the prominent nuclear alterations found in apoptotic cells attracted attention to the endonucleases that ultimately result in the internucleosomal cleavage of DNA that characterizes apoptotic death, more recent reports have centered on the role of a variety of proteases, including serine proteases, calpains, and caspases, thought to convey apoptotic signals received at the cell membrane by initiating

a complex intracellular cascade of activating and inactivating proteolytic events. Progress has been particularly rewarding in the understanding of the caspases, a family of at least 10 proteases maintaining homology with death-specific gene products originally described in *Caenorhabditis elegans* (see Cohen, 1997, for a review). These proteases are produced as inactive zymogens, become activated through proteolytic cleavage, and are associated with promotion of cell death. Several inducers of apoptosis, including Fas ligand or Fas cross-linking, actinomycin D, and etoposide, have been shown to result in activation of caspases.

With regard to the dual effect of NO as promoter or suppressor of apoptosis, reports indicate the ability of this mediator to stimulate or suppress the activation of caspases. Considering evidence consistent with NO stimulation of caspase activity, human leukemic lymphocytes were shown to undergo apoptosis when exposed to a NO donor; apoptosis correlated with activation of caspase 8 and was prevented by a broad-spectrum caspase inhibitor (Chlichlia *et al.,* 1998). Messmer and Brüne (1996) reported the detection of PARP cleavage in RAW 264.7 cells expressing iNOS or exposed to NO donor agents. The inactivating cleavage of PARP is known to reflect the activation of ICE (interleukin 1-β-converting enzyme)-like caspases, most likely caspases 3 and 7 (Cohen, 1997). Interestingly, cleavage of PARP was suppressed by transfection with *bcl-2*. Providing further connections between NO-dependent apoptosis and caspase activation, the activation of caspase 3 was demonstrated together with the accumulation of p53 and reduction of bcl-2 in rabbit aortic vascular smooth muscle cells treated with the NO donor agent SNAP (Nishio and Watanabe, 1998). Brockhaus and Brüne (1998) showed that exposure of the p53-negative human leukemic U937 cells to NO donor agents activated caspase 3-like activity that resulted in cleavage of PARP. In this study the authors also reported that NAC prevented apoptosis and the cleavage of PARP. Most importantly, and in agreement with the results of others (Wang *et al.,* 1997), they concluded that PARP cleavage is not essential for the progression of apoptosis, as caspase inactivation prevented apoptosis but not PARP cleavage. Treatment of HL-60 cells with peroxynitrite (100 μ*M*) also induced the activation of caspase 3 (Lin *et al.,* 1998).

As mentioned before, NO can act as a negative regulator of apoptosis. Either endogenous or exogenously provided NO has been shown to halt apogenesis induced by a variety of triggers in a number of cell types including hepatocytes, endothelial cells, cardiac myocytes, splenocytes, B cells, and eosinophils (Mannick *et al.,* 1994; Kim *et al.,* 1995; Dimmeler *et al.,* 1997). Infusion of a NO donor agent was also able to inhibit hepatic apoptosis in rats challenged with TNF-α or galactosamine (Kim *et al.,* 1997). This protective effect of NO was shown to coincide with suppression of intracellular caspase 3 proteolytic activity.

NO donor agents were shown to inhibit caspase 3 when its activity had been previously increased by treatment of U937 cells with actinomycin D (Mohr *et al.,* 1997). The inhibitory effects on caspase 3 of the NO donor compounds in lysates from actinomycin D-treated cells could, in the case of GSNO, spermine–NO, and BF_4NO, be reversed with dithiothreitol. The inhibition of caspase 3 activity by 1,3-morpholino-sydnonimine hydrochloride (SIN-1) or peroxynitrite could not be reversed by the reducing agent. These findings suggested the S-nitrosylation of active site cysteine in caspase 3 as the reversible mechanism of enzyme inhibition, and the further oxidation of this site as the irreversible mechanism. Most interestingly, whereas the addition of GSNO to intact cells cultured with actinomycin D reduced caspase 3 activity, its use in untreated cells actually increased caspase 3 activity. At the biochemical level, the inhibition of seven different recombinant caspases by a NO donor agent was shown together with its reversal by dithiothreitol, indicating S-nitrosylation of the catalytic cysteine residue (Li *et al.,* 1997). In addition, caspase 3 activity in lysates of TNF-α/actinomycin D-treated hepatocytes was suppressed by the addition of a NO donor compound to the culture medium. Work from the same laboratory reported by Kim *et al.* (1997) extended these observations by demonstrating in rat and mouse hepatocytes that the induction of NOS or the addition of NO donor agents prevented spontaneous, TNF-α-associated, and anti-Fas antibody-induced apoptosis, and the inhibitory S-nitrosylation of caspase 3 by NO *in vitro* and *in vivo.* These authors, in addition, proposed an additional mechanism for the antiapoptotic effect of NO in these cells that is dependent on the production of cGMP and does not involve S-nitrosylation. In this regard, others showed that low concentrations of NO donor agents could inhibit TNF-α-induced apoptosis in endothelial cells through a cGMP-dependent mechanism, but that higher concentrations of NO donor compounds induced apoptotic death in the cells. Additional results obtained in pulmonary artery endothelial cells subjected either to adenoviral transfer of inducible NOS or to NO donor exposure indicated these cells to be protected from LPS-mediated apoptosis (Ceneviva *et al.,* 1998). Interestingly, no correlation was found between the protective effect of NO and the induction of putatively protective genes, including those for HSP-70, metallothionein, heme oxygenase-1, or bcl-2. In contrast, NO prevented the activation of caspases in a dithiothreitol-sensitive fashion.

The inhibitory effects of endogenous and exogenous NO on caspase 3 were also demonstrated in TNF-α-treated human endothelial cells (Dimmeler *et al.,* 1997). Protection by NO from Fas-mediated apoptosis has been reported in human hemopoietic cell lines (Mannick *et al.,* 1997; Dimmeler *et al.,* 1998). Ogura *et al.* (1997), in turn, reported the inhibition of recombinant caspase 3 in lysates of cells treated with vincristine by a NO donor agent. Addition of GSH in rather modest concentrations to the assay mixture prevented the inhibitory effect of the NO donor, supporting the concept that the inhibition of caspase 3 activity by NO is under redox control and mediated though the S-nitrosylation of active site cysteines.

Although the findings reviewed so far associate caspase 3 inhibition with suppression of apoptosis, it has been shown that culture of Jurkat cells with SNAP is associated with necrotic cell death despite inhibition of caspase 3 activation. The dose-dependent capacity of NO to induce apoptosis or necrosis has been also shown for peroxynitrite in cell cul-

tures; exposure to this product at concentrations lower than 1 m*M* resulted in apoptosis, whereas concentrations higher than 2 m*M* induced cell necrosis (Estévez *et al.,* 1995). Similar findings in thymocytes were reported by others (Virág *et al.,* 1998). Shen *et al.* (1998), in turn, reported that low concentrations of SNAP inhibited TNF-α-induced apoptosis in a cGMP-dependent manner, but also that higher concentrations of the NO donor agent actually induced apoptosis in their human endothelial cell preparations.

Evidence at hand, therefore, indicates that NO can both activate and inhibit caspases, or at least caspase 3. This ability of NO may relate to its capacity to induce or prevent apoptosis. It must, however, be kept in mind that cells lacking PARP, the putative substrate for activated caspase 3, do undergo apoptosis after treatment with TNF-α, anti-Fas, γ irradiation, and dexamethasone (Wang *et al.,* 1997).

Recognition of Apoptotic Cells by Macrophages and Dendritic Cells: Mechanisms and Relevance to Antitumor Immune Responses

The removal of apoptotic cells prior to dissolution of the plasma membrane prevents the release of potentially toxic and/or immunogenic molecules, which may instigate or exacerbate tissue injury. It is easy, in this context, to appreciate the value of cleanly removing senescent neutrophils from a site of acute inflammation, or eosinophils from a local site of atopy. Macrophages have been shown to internalize apoptotic cell debris without elicitation of a phlogistic response, in distinction to phagocytosis of pathogens via Fc or complement receptors. In fact, phagocytosis of apoptotic material by macrophages actively inhibits production of proinflammatory mediators such as interleukin 1β (IL-1β), IL-8, leukotriene C4, and TNF-α while inducing anti-inflammatory products such as transforming growth factor β (TGF-β), prostaglandin E_2 (PGE_2), and platelet-activating factor (Fadok *et al.,* 1998a).

Cells undergoing apoptosis exhibit certain common characteristics, including exposure of anionic phospholipids in the plasma membrane and modification of cell surface carbohydrate moieties (Savill, 1996). A number of receptors have been described on phagocytes that take advantage of these alterations to mediate recognition and removal of apoptotic bodies. These include the vitronectin receptor ($\alpha_v\beta_3$ integrin), CD36, an undefined receptor specific for phosphatidylserine, scavenger receptors, CD14, and a lectin with specificity for *N*-acetylglucosamine or glucosamine (Stern *et al.,* 1996). The receptors for vitronectin and CD36 are thought to form a complex on the cell surface capable of recognizing thrombospondin (TSP), a macrophage secretory protein. TSP, or a 140-kDa COOH-terminal domain proteolytic fragment from this protein, in turn forms a bridge between the receptor complex on the phagocyte and the apoptotic cell. There may be a hierarchy of importance among the seemingly redundant battery of apoptotic receptors with CD36 at the top. Several cell types with no capacity for phagocytosis of apoptotic bodies became efficiently phagocytic when transfected to overexpress CD36 regardless of whether the apoptotic cells were neutrophils, lymphocytes, or fibroblasts (Ren *et al.,* 1995). In other work, blockade of CD36 with specific antibodies inhibited uptake by both $\alpha_v\beta_3$- and phosphatidylserine-dependent pathways (Fadok *et al.,* 1998b). The mechanism by which CD36 interacts with these molecules at the cell surface is not yet understood.

The receptor–ligand interactions just described suggest a redundant system of recognition of apoptotic cells, which may contribute to the overall efficiency of this process. However, the mechanisms that prevail are regulated at the level of the phagocytic cell and, perhaps, at the level of the type of apoptotic target cell. Monocytes and macrophages utilize different mechanisms depending on their state of activation. For example, human peripheral mononuclear cells use primarily the CD36/$\alpha_v\beta_3$/TSP mechanism for recognition of apoptotic neutrophils, but they switch to phosphatidylserine-mediated recognition on activation (Fadok *et al.,* 1998b). In an animal model, resident peritoneal, immune-activated, and wound-derived macrophages all used distinct mechanisms in the phagocytosis of aged, apoptotic neutrophils (Meszaros *et al.,* 1999).

Dendritic cells share a common bone marrow-derived lineage with macrophages, but they express a distinct functional and phenotypic profile. They are particularly well suited for a specialized role in antigen presentation, as they express high levels of cell surface major histocompatibility complex (MHC) antigens and costimulatory molecules and, as a result, are the most potent antigen-presenting cells for the initiation of T-cell-dependent immune responses (Steinman, 1991). Dendritic cells can be found in immature and mature states. When in their immature state, dendritic cells are distributed widely in peripheral tissues but decidedly outside lymphoid tissues. These cells are also capable of phagocytosis of apoptotic cells, a function that may be restricted to dendritic cells in the immature state (Albert *et al.,* 1998). Immature dendritic cells have been shown, in addition, to acquire antigens expressly from ingested apoptotic, but not necrotic, cells. Once engorged, dendritic cells mature as they migrate to regional lymph nodes, where processed antigen is presented in the context of MHC class I molecules to $CD8^+$ cytotoxic T cells. Although the multitude of receptors capable of recognizing apoptotic ligands endows the macrophage with a highly efficient capacity for bulk clearance of apoptotic cells, the dendritic cell may prove to be the effector cell of choice for presentation of antigen to the immune system. As is the case with macrophages, immature dendritic cells demonstrate high levels of cell surface CD36; however, the α_v integrin chain in these cells is expressed in the context of β_5, forming the $\alpha_v\beta_5$ integrin, which is not found on macrophages (Albert *et al.,* 1998). Antibodies blocking either $\alpha_v\beta_5$ or CD36 are sufficient to inhibit phagocytosis of apoptotic cells, raising the possibility of intermolecular cooperation as seen for $\alpha_v\beta_3$ and CD36 on macrophages.

Dendritic cells, in response to allogeneic T cells or immunomodulators such as LPS and IFN-γ, express iNOS and produce NO in levels that rival macrophages (Lu *et al.,*

1996). As in the case of the macrophage, dendritic-cell derived NO inhibits lymphocyte proliferation and induces apoptosis in the cells that produce it (Lu *et al.,* 1996). However, there is no evidence to suggest that a dendritic cell can distinguish a transformed from a normal cell and utilize NO as an effector molecule to manifest its own tumoricidal effect. Absent also is any evidence that dendritic cells cooperate with tumoricidal macrophages by ingesting apoptotic fragments of targeted tumor cells and then migrating to the nearest lymph node to initiate a tumor-specific cytotoxic T-lymphocyte response. Whether the tumor itself has an active role in suppressing such a pathway to immunization is unclear, although some tumors are capable of elaborating factors such as IL-10 that could limit dendritic cell differentiation and function (Schuler and Steinman, 1997). A large body of evidence is being accrued which suggests that dendritic cells primed with tumor antigens *ex vivo* generate a potent antitumor response when returned to the tumor-bearing host (Schuler and Steinman, 1997). Clinical studies are currently underway exploring the usefulness of dendritic cells as a basis for immunotherapy and tumor vaccination protocols.

As described earlier, the caspases are an integral aspect of the cellular decision to abandon the efforts of PARP to repair damaged DNA and proceed with the apoptotic pathway. Caspase activity marks one of the earliest steps in the cidal cascade, such that interference with caspase activity may be a mechanism by which NO mediates cytoprotection. This protection may also be manifested in another way. Caspase activity can be detected prior to morphologic evidence of apoptosis including chromatin condensation, nuclear fragmentation, mitochondrial potential alteration, and translocation of phosphatidylserine to the outer leaflet of the plasma membrane. Interestingly, cells that demonstrate caspase activity but no other morphologic evidence of apoptosis are fodder for phagocytosis. Therefore, the cytoprotective effect of NO through caspase inhibition may include protection from premature engulfment by tumor-associated macrophages (Durrieu *et al.,* 1998).

NO, Tumor Cell Apoptosis, and the Human Macrophage

Although the concept of the macrophage as a cytotoxic effector for tumor cells is well established, the limitations of this model must be kept in mind. First, the nonspecific elimination of tumor cells by macrophages is anatomically restricted to the site where macrophages are activated. Indeed, although the systemic distribution of macrophage-activating agents (i.e., bacillus Calmette-Guerin [BCG]) could result in some degree of systemic macrophage activation, most macrophages endowed with cytotoxic activity congregate at the site where the activating agent is delivered. Second, macrophage activation for cytotoxic activity appears to require the action of T-cell-derived cytokines, bacterial products, or other exogenous agents. How this activation would occur at the tumor site remains unclear. Most importantly, the presence of macrophages within tumors (tumor-associated macrophages or TAMs) has been interpreted as both suppressive and supportive of tumor growth. In connection with presumed antitumor effects of TAMs, the production by these cells of cytotoxic molecules (NO, TNF-α, etc.) may be envisioned as antagonistic to tumor development. The release from TAMs of matrix metalloproteinases, angiogenic factors, or growth factors may, alternatively, promote tumor growth.

Within the specific area of concentration of this chapter, that is, NO cytotoxicity for tumor cells, remarkably few publications have addressed the potential role of NO as a cytotoxic molecule *in vivo.* Two publications deserve special discussion. DiNapoli *et al.* (1996) reported that TAMs obtained from a transplantable mammary carcinoma in mice were unable to produce NO and lyse tumor cells even after treatment with IFN-γ and endotoxin. The same TAMs shown not to be able to express NOS, however, were capable of phagocytosis, showed adherence to plastic, and produced as much IL-6 as those obtained from other anatomical sites in the animal. Thus, whether macrophages actually present at a tumor site can express NOS remains open to investigation.

In an additional *in vivo* study, Gal *et al.* (1997) injected SJL mice with superantigen-bearing RcsX lymphoma cells. Growth of this tumor results in a systemic inflammatory reaction in the hosts. Histochemical interrogation of spleens and lymph nodes from these animals revealed intense iNOS staining in macrophages and apoptotic features and 3-nitrotyrosine staining in neighboring cells. Interestingly, iNOS-positive macrophages did not present apoptotic features, and the apoptotic cells did not show evidence of direct DNA damage as measured by 8-oxoguanidine accumulation. Unfortunately, the authors did not report on the presence of iNOS-positive macrophages or apoptotic cells at the site of tumor development.

It is particularly important to highlight that all observations described thus far on the induction of apoptosis in tumor cells through macrophage-derived NO have used rodent macrophages as the effector cells. Not one report has demonstrated the capacity of human effectors to elicit NO-dependent tumor cell apoptosis.

In this connection, the remarkable weakness of data supporting the production of NO by human monocytes and macrophages has been reviewed (Albina, 1995). Moreover, the report by Schneemann *et al.* (1997) of a nonenzymatic explanation for the accumulation of NO by products in cultures of human monocytes following stimulation with IL-4 refutes one of the more firmly established models for the induction of NOS in human cells (Mautino *et al.,* 1994; Defer *et al.,* 1994).

With regard to human neoplastic disease, cells identified as macrophages were shown to contain immunoreactive inducible NOS in stromal tissue in breast carcinoma (Thomsen *et al.,* 1995). Additionally, calcium-dependent NOS activity was found in those as well as in gynecological and central nervous system tumors (Thomsen *et al.,* 1994; Cobbs *et al.,*

1995). The potential tumor-supporting role of endothelial-type NOS in these malignancies through its vasodilating activity or through the proposed suppression of caspases by low levels of NO remains to be determined.

As far as can be known today, the high-output production of NO through the inducible form of NOS appears to be restricted to macrophages of certain species. If indeed human monocytes or macrophages are capable of releasing low levels of NO, as proposed by some (Albina, 1995), then they may, along the lines discussed in the previous section, be protective rather than toxic toward tumor cells.

Summary

Results reviewed here address the cytotoxicity of macrophage-derived NO and the mechanisms for NO-dependent apoptosis. Although data obtained using wound cells and nuclear factor IL-6-negative macrophages suggest NO may be necessary but not sufficient to explain macrophage cytotoxicity, no doubts remain that at least chemically derived NO induces apoptosis in certain tumor cells. The nature of the NO derivatives ultimately responsible for the induction of apoptosis under physiological conditions, as well as the mechanisms through which apoptotic pathways are induced, awaits clarification. Most interestingly, reports from multiple laboratories indicate that selected cell types can resist NO using innate or acquired defense mechanisms. The relevance of these mechanisms to tumor development remains to be explored. Last, regarding the relevance of NO to human macrophage-dependent cytotoxicity, clarification of the production of NO by these cells will be required before firm conclusions can be reached regarding a role for NO in human neoplastic disease.

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References

Adams, D. O., and Hamilton, T. A. (1984). The cell biology of macrophage activation. *Am. Rev. Immunol.* **2,** 283–318.

Adams, D. O., and Nathan, C. F. (1983). Molecular mechanisms in tumor-cell killing by activated macrophages. *Immunol. Today* **4,** 166–170.

Adams, D. O., Johnson, W. J., and Marino, P. A. (1982). Mechanisms of target recognition and destruction in macrophage-mediated tumor cytotoxicity. *Fed. Proc.* **41,** 2212–2221.

Albert, M. L., Pearce, F. A., Francisco, L. M., *et al.* (1998). Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* **188,** 1359–1368.

Albina, J. E. (1995). On the expression of nitric oxide synthase by human macrophages. Why no NO? *J. Leukocyte Biol.* **58,** 643–649.

Albina, J. E., and Mastrofrancesco, B. (1993). Modulation of glucose metabolism in macrophages by products of nitric oxide synthase. *Am. J. Physiol.* **264,** C1594–C1599.

Albina, J. E., and Reichner, J. S. (1995). Nitric oxide in inflammation and immunity. *New Horizons* **3,** 46–64.

Albina, J. E., and Reichner, J. S. (1998). Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. *Cancer Metastasis Rev.* **17,** 39–53.

Albina, J. E., Mills, C. D., Henry, W. L., Jr., *et al.* (1989). Regulation of macrophage physiology by L-arginine: Role of the oxidative L-arginine deiminase pathway. *J. Immunol.* **143,** 3641–3646.

Albina, J. E., Mills, C. D., and Caldwell, M. D. (1990). Alterations in macrophage physiology associated with the metabolism of L-arginine through the oxidative L-arginine deiminase pathway. *In* ''Nitric Oxide from L-Arginine: A Bioregulatory System'' (S. Moncada and E. A. Higgs, eds.), pp. 243–248. Elsevier, Amsterdam.

Albina, J. E., Cui, S., Mateo, R. B., *et al.* (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150,** 5080–5085.

Albina, J. E., Martin, B.-A., Henry, W. L., Jr., *et al.* (1996). B cell lymphoma-2 transfected P815 cells resist reactive nitrogen intermediate-mediated macrophage-dependent cytotoxicity. *J. Immunol.* **157,** 279–283.

Aliprantis, A. O., Diez-Roux, G., Mulder, L. C. F., *et al.* (1996). Do macrophages kill through apoptosis? *Immunol. Today* **17,** 573–576.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Berendji, D., Kolb-Bachofen, V., Meyer, K. L., *et al.* (1997). Nitric oxide mediates intracytoplasmic and intranuclear zinc release. *FEBS Lett.* **405,** 37–41.

Berger, N. A. (1985). Symposium: Cellular response to DNA damage. The role of poly(ADP-ribose). Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat. Res.* **101,** 4–15.

Boggs, S. E., McCormick, T. S., and Lapetina, E. G. (1998). Glutathione levels determine apoptosis in macrophages. *Biochem. Biophys. Res. Commun.* **247,** 229–233.

Bortner, C. D., Oldenburg, N. B. E., and Cidlowski, J. A. (1995). The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* **5,** 21–26.

Brockhaus, F., and Brüne, B. (1998). U937 apoptotic cell death by nitric oxide: Bcl-2 downregulation and caspase activation. *Exp. Cell Res.* **238,** 33–41.

Brüne, B., Gölkel, C., and von Knethen, A. (1996). Cytokine and low-level nitric oxide prestimulation block p53 accumulation and apoptosis of Raw 264.7 macrophages. *Biochem. Biophys. Res. Commun.* **229,** 396–401.

Buga, G. M., Wei, L. H., Bauer, P. M., Fukuto, J. M., and Ignarro, L. J. (1998). N^G-Hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms. *Am. J. Physiol.* **275,** R1256–R1264.

Canman, C. E., and Kastan, M. B. (1995). Induction of apoptosis by tumor suppressor genes and oncogenes. *Semin. Cancer Biol.* **6,** 17–25.

Cavalli, R. C., Burke, C. J., Kawamoto, S., *et al.* (1994). Mutagenesis of rat liver arginase expressed in *Escherichia coli:* Role of conserved histidines. *Biochemistry* **33,** 10652–10657.

Ceneviva, G. D., Tzeng, E., Hoyt, D. G., *et al.* (1998). Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells. *Am. J. Physiol.* **275,** L717–L728.

Chesrown, S. E., Monnier, J., Visner, G., *et al.* (1994). Regulation of inducible nitric oxide synthase mRNA levels by LPS, INF-γ, TGF-β, and IL-10 in murine macrophage cell lines and rat peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **200,** 126–134.

Chlichlia, K., Peter, M. E., Rocha, M., *et al.* (1998). Caspase activation is required for nitric oxide-mediated CD95(APO-1/Fas)-dependent and independent apoptosis in human neoplastic lymphoid cells. *Blood* **91,** 4311–4320.

Clark, R. A. F., McCoy, G. A., Folkvord, J. M., *et al.* (1997). TGF-β1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: A fibronectin matrix-dependent event. *J. Cell. Physiol.* **170,** 69–80.

Cobbs, C. S., Brenman, J. E., Aldape, K. D., *et al.* (1995). Expression of nitric oxide synthase in human central nervous system tumors. *Cancer Res.* **55,** 727–730.

Cohen, G. M. (1997). Caspases: The executioners of apoptosis. *Biochem. J.* **326,** 1–16.

Cohen, G. M., Sun, X.-M., Snowden, R. T., *et al.* (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.* **286,** 331–334.

Cohn, Z. A. (1978). The activation of mononuclear phagocytes: Fact, fancy, and future. *J. Immunol.* **121,** 813–816.

Cui, S., Reichner, J. S., Mateo, R. B., *et al.* (1994). Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res.* **54,** 2462–2467.

Defer, M.-C., Dugas, B., Paul-Eugène, N., *et al.* (1994). Rôle de l'interleukine-4 dans la régulation de la production de monoxyde d'azote (NO) par les monocytes humains normaux. Interleukin-4 regulates nitric oxide production by normal human monocytes. *C. R. Acad. Sci. Paris* **317,** 1021–1025.

deRojas-Walker, T., Tamir, S., Ji, H., *et al.* (1995). Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chem. Res. Toxicol.* **8,** 473–477.

Dimmeler, S., Haendeler, J., Nehls, M., *et al.* (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1β-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.* **185,** 601–607.

Dimmeler, S., Haendeler, J., Sause, A., *et al.* (1998). Nitric oxide inhibits APO-1/Fas-mediated cell death. *Cell Growth Differ.* **9,** 415–422.

DiNapoli, M. R., Calderon, C. L., and Lopez, D. M. (1996). The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduced expression of the inducible nitric oxide synthase gene. *J. Exp. Med.* **183,** 1323–1329.

Drapier, J.-C., and Hibbs, J. B., Jr. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron–sulfur enzymes in the macrophage effector cells. *J. Immunol.* **140,** 2829–2838.

Durrieu, F., Belloc, F., Lacoste, L., *et al.* (1998). Caspase activation is an early event in anthracycline-induced apoptosis and allows detection of apoptotic cells before they are ingested by phagocytes. *Exp. Cell Res.* **240,** 165–175.

Estévez, A. G., Radi, R., Barbeito, L., *et al.* (1995). Peroxynitrite-induced cytotoxicity in PC12 cells: Evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. *J. Neurochem.* **65,** 1543–1550.

Fadok, V. A., Bratton, D. L., Konowal, A., *et al.* (1998a). Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF. *J. Clin. Invest.* **101,** 890–898.

Fadok, V. A., Warner, M. L., Bratton, D. L., *et al.* (1998b). CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor ($\alpha_v\beta3$). *J. Immunol.* **161,** 6250–6257.

Farias-Eisner, R., Chaudhuri, G., Aeberhard, E., *et al.* (1996). The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. *J. Biol. Chem.* **271,** 6144–6151.

Gal, A., Tamir, S., Kennedy, L. J., *et al.* (1997). Nitrotyrosine formation, apoptosis, and oxidative damage: Relationships to nitric oxide production in SJL mice bearing the RcsX tumor. *Cancer Res.* **57,** 1823–1828.

Genaro, A. M., Hortelano, S., Alvarez, A., *et al.* (1995). Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. *J. Clin. Invest.* **95,** 1884–1890.

Gerschenson, L. E., and Rotello, R. J. (1992). Apoptosis: A different type of cell death. *FASEB J.* **6,** 2450–2455.

Gopalakrishna, R., Chen, Z. H., and Gundimeda, U. (1993). Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *J. Biol. Chem.* **268,** 27180–27185.

Hamilton, T. A., and Ohmori, Y. (1996). Intracellular signaling pathways regulating inflammatory gene expression in mononuclear phagocytes. *In* "Signal Transduction in Leukocytes. G Protein-Related and Other Pathways" (P. M. Lad, J. S. Kaptein, and C.-K. E. Lin, eds.), pp. 325–343. CRC Press, Boca Raton.

Harbrecht, B. G., Di Silvio, M., Chough, V., *et al.* (1997). Glutathione regulates nitric oxide synthase in cultured hepatocytes. *Ann. Surg.* **225,** 76–87.

Häussinger, D. (1983). Hepatocyte heterogeneity in glutamine and ammonia metabolism and the role of an intercellular glutamine cycle during ureogenesis in perfused rat liver. *Eur. J. Biochem.* **133,** 269–275.

Hawkins, C. J., and Vaux, D. L. (1994). Analysis of the role of *bcl-2* in apoptosis. *Immunol. Rev.* **142,** 127–139.

Heller, B., Wang, Z.-Q., Wagner, E. F., *et al.* (1995). Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J. Biol. Chem.* **270,** 11176–11180.

Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1984). Iron depletion: Possible cause of tumor cell cytotoxicity induced by activated macrophages. *Biochem. Biophys. Res. Commun.* **123,** 716–723.

Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1987a). Macrophage cytotoxicity: Role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235,** 473–476.

Hibbs, J. B., Jr., Vavrin, Z., and Taintor, R. R. (1987b). L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* **138,** 550–565.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., *et al.* (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., *et al.* (1990). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: A molecular mechanism regulating cellular proliferation that targets intracellular iron. *In* "Nitric Oxide from L-Arginine: A Bioregulatory System" (S. Moncada and E. A. Higgs, eds.), pp. 189–223. Elsevier, Amsterdam.

Hirokawa, K., O'Shaughnessy, K. M., Ramrakha, P., *et al.* (1994). Inhibition of nitric oxide synthesis in vascular smooth muscle by retinoids. *Br. J. Pharmacol.* **113,** 1448–1454.

Hockenbery, D. M., Nuñez, G., Milliman, C., *et al.* (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348,** 334–336.

Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., *et al.* (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75,** 241–251.

Hortelano, S., Dallaporta, B., Zamzami, N., *et al.* (1997). Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS Lett.* **410,** 373–377.

Huot, J., Lambert, H., Lavoie, J. N., *et al.* (1995). Characterization of 45-kDa/54-kDa HSP27 kinase, a stress-sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27-kDa heat-shock protein HSP27. *Eur. J. Biochem.* **227,** 416–427.

Jacobson, M. D., and Raff, M. C. (1995). Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374,** 814–816.

Jacobson, M. D., Burne, J. F., and Raff, M. C. (1994). Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* **13,** 1899–1910.

Jones, D. P., Maellaro, E., Jiang, S., *et al.* (1995). Effects of *N*-acetyl-L-cysteine on T-cell apoptosis are not mediated by increased cellular glutathione. *Immunol. Lett.* **45,** 205–209.

Jun, C.-D., Han, M.-K., Kim, U.-H., *et al.* (1996). Nitric oxide induces ADP-ribosylation of actin in murine macrophages: Association with the inhibition of pseudopodia formation, phagocytic activity, and adherence on a laminin substratum. *Cell. Immunol.* **174,** 25–34.

Khan, S., Kayahara, M., Joashi, U., *et al.* (1997). Differential induction of apoptosis in Swiss 3T3 cells by nitric oxide and the nitrosonium cation. *J. Cell Sci.* **110,** 2315–2322.

Kim, Y.-M., Bergonia, H., and Lancaster, J. R., Jr. (1995). Nitrogen oxide-induced autoprotection in isolated rat hepatocytes. *FEBS Lett.* **374,** 228–232.

Kim, Y.-M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Kim, Y.-M., Son, K., Hong, S.-J., *et al.* (1998). Inhibition of protein synthesis by nitric oxide correlates with cytostatic activity: Nitric oxide induces phosphorylation of initiation factor eIF-2α. *Mol. Med.* **4,** 179–190.

Kim, Y.-M., Bombeck, C. A., and Billiar, T. R. (1999). Nitric oxide as a bifunctional regulator of apoptosis. *Circ. Res.* **84,** 253–256.

Korsmeyer, S. J., Yin, X.-M., Oltvai, Z. N., *et al.* (1995). Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. *Biochim. Biophys. Acta* **1271,** 63–66.

Kröncke, K.-D., Fchsel, K., Schmidt, T., *et al.* (1994). Nitric oxide destroys zinc–sulfur clusters inducing zinc release from metallothionein and inhibition of the zinc finger-type yeast transcription activator LAC9. *Biochem. Biophys. Res. Commun.* **200,** 1105–1110.

Kwon, N. S., Stuehr, D. J., and Nathan, C. F. (1991). Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.* **174,** 761.

Lander, H. M., Sehajpal, P. K., and Novogrodsky, A. (1993). Nitric oxide signaling: A possible role for G proteins. *J. Immunol.* **151,** 7182–7187.

Lepoivre, M., Flaman, J. M., and Henry, Y. (1992). Early loss of the tyrosyl radical in ribonucleotide reductase of adenocarcinoma cells producing nitric oxide. *J. Biol. Chem.* **267,** 22994–23000.

Li, J., Billiar, T. R., Talanian, R. V., *et al.* (1997). Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem. Biophys. Res. Commun.* **240,** 419–424.

Lin, K.-T., Xue, J.-Y., Nomen, M., *et al.* (1995). Peroxynitrite-induced apoptosis in HL-60 cells. *J. Biol. Chem.* **270,** 16487–16490.

Lin, K.-T., Xue, J.-Y., Sun, F. F., *et al.* (1997). Reactive oxygen species participate in peroxynitrite-induced apoptosis in HL-60 cells. *Biochem. Biophys. Res. Commun.* **230,** 115–119.

Lin, K.-T., Xue, J.-Y., Lin, M. C., *et al.* (1998). Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. *Am. J. Physiol.* **274,** C855–C860.

Lu, L., Bonham, C. A., Chambers, F. G., *et al.* (1996). Induction of nitric oxide synthase in mouse dendritic cells by IFN-γ, endotoxin, and interaction with allogeneic T cells. Nitric oxide production is associated with dendritic cell apoptosis. *J. Immunol.* **157,** 3577–3586.

Lucas, M., Sánchez-Margalet, V., Sanz, A., *et al.* (1994). Protein kinase C activation promotes cell survival in mature lymphocytes prone to apoptosis. *Biochem. Pharmacol.* **47,** 667–672.

MacLeod, C. L., Finley, K., Kakuda, D., *et al.* (1990). Activated T cells express a novel gene on chromosome 8 that is closely related to the murine ecotropic retroviral receptor. *Mol. Cell. Biol.* **10,** 3663–3674.

MacMicking, J., Xie, Q.-W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.

Mannick, J. B., Asano, K., Izumi, K., *et al.* (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* **79,** 1137–1146.

Mannick, J. B., Miao, X. Q., and Stamler, J. S. (1997). Nitric oxide inhibits Fas-induced apoptosis. *J. Biol. Chem.* **272,** 24125–24128.

Mateo, R. B., Reichner, J. S., Mastrofrancesco, B., *et al.* (1995). Impact of nitric oxide on macrophage glucose metabolism and glyceraldehyde-3-phosphate dehydrogenase activity. *Am. J. Physiol.* **268,** C669–C675.

Mateo, R. B., Reichner, J. S., and Albina, J. E. (1996). NO is not sufficient to explain maximal cytotoxicity of tumoricidal macrophages against an NO-sensitive cell line. *J. Leukocyte Biol.* **60,** 245–252.

Mautino, G., Paul-Eugène, N., Chanez, P., *et al.* (1994). Heterogeneous spontaneous and interleukin-4-induced nitric oxide production by human monocytes. *J. Leukocyte Biol.* **56,** 15–20.

Messmer, U. K., and Brüne, B. (1995). Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem. J.* **319,** 299–305.

Messmer, U. K., and Brüne, B. (1996). Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: The role of NO-donor exposure, NAD^+ content, and p53 accumulation. *Arch. Biochem. Biophys.* **327,** 1–10.

Messmer, U. K., Ankarcrona, M., Nicotera, P., *et al.* (1994). p53 expression in nitric oxide-induced apoptosis. *FEBS Lett.* **355,** 23–26.

Messmer, U. K., Lapetina, E. G., and Brüne, B. (1995). Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds. *Mol. Pharmacol.* **47,** 757–765.

Messmer, U. K., Reimer, D. M., Reed, J. C., *et al.* (1996a). Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2. *FEBS Lett.* **384,** 162–166.

Messmer, U. K., Reed, J. C., and Brüne, B. (1996b). Bcl-2 protects macrophages from nitric oxide-induced apoptosis. *J. Biol. Chem.* **271,** 20192–20197.

Meszaros, A. J., Reichner, J. S., and Albina, J. E. (1999). Macrophage phagocytosis of wound neutrophils. *J. Leukocyte Biol.* **65,** 35–42.

Mohr, S., Zech, B., Lapetina, E. G., *et al.* (1997). Inhibition of caspase-3 by s-nitrosation and oxidation caused by nitric oxide. *Biochem. Biophys. Res. Commun.* **238,** 387–391.

Möröy, T., and Zörnig, M. (1996). Regulators of life and death: The Bcl-2 gene family. *Cell. Physiol. Biochem.* **6,** 312–336.

Morris, S. M., Jr., and Billiar, T. R. (1994). New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.* **266,** E829–E839.

Muijsers, R. B. R., Folkerts, G., Henricks, P. A. J., *et al.* (1997). Peroxynitrite: A two-faced metabolite of nitric oxide. *Life Sci.* **60,** 1833–1845.

Müller, C., de Vera, M. E., Watkins, S. C., *et al.* (1997). Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-α-induced apoptosis by inducing heat shock protein 70 expression. *J. Biol. Chem.* **272,** 1402–1411.

Muschel, R. J., Bernhard, E. J., Garza, L., *et al.* (1995). Induction of apoptosis at different oxygen tensions: Evidence that oxygen radicals do not mediate apoptotic signaling. *Cancer Res.* **55,** 995–998.

Nguyen, T., Brunson, D., Crespi, C. L., *et al.* (1992). DNA damage and mutation in human cells exposed to nitric oxide *in vitro. Proc. Natl. Acad. Sci. U.S.A.* **89,** 3030–3034.

Nishio, E., and Watanabe, Y. (1998). NO induced apoptosis accompanying the change of oncoprotein expression and the activation of CPP32 protease. *Life Sci.* **62,** 239–245.

Nussler, A. K., and Billiar, T. R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. *J. Leukocyte Biol.* **54,** 171–178.

Ogura, T., Tatemichi, M., and Esumi, H. (1997). Nitric oxide inhibits CPP32-like activity under redox regulation. *Biochem. Biophys. Res. Commun.* **236,** 365–369.

Poderoso, J. J., Carreras, M. C., Lisdero, C., *et al.* (1996). Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch. Biochem. Biophys.* **328,** 85–92.

Poderoso, J. J., Peralta, J. G., Lisdero, C. L., *et al.* (1998). Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *Am. J. Physiol.* **274,** C112–C119.

Radi, R., Beckman, J. S., Bush, K. M., *et al.* (1991). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288,** 481–487.

Raingeaud, J., Gupta, S., Rogers, J. S., *et al.* (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270,** 7420–7426.

Ren, Y., Silverstein, R. L., Allen, J., *et al.* (1995). CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. *J. Exp. Med.* **181,** 1857–1862.

Rubin, B. Y., Anderson, S. L., Lunn, R. M., *et al.* (1989). Fragmentation of cellular DNA is a nonspecific indicator of responsiveness to tumor necrosis factor. *J. Biol. Response Mod.* **8,** 553–559.

Saran, M., and Bors, W. (1994). Signalling by O_2^- and NO: how far can either radical, or any specific reaction product, transmit a message under *in vivo* conditions? *Chem.–Biol. Interact.* **90,** 35–45.

Savill, J. (1996). Phagocyte recognition of apoptotic cells. *Biochem. Soc. Trans.* **24,** 1065–1069.

Schäffer, M. R., Efron, P. A., Thornton, F. J., *et al.* (1997). Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. *J. Immunol.* **158,** 2375–2381.

Schneemann, M., Schoedon, G., Linsheid, P., *et al.* (1997). Nitrite generation in interleukin-4-treated human macrophage cultures does not involve the nitric oxide synthase pathway. *J. Infect. Dis.* **175,** 130–135.

Schraufstatter, I. U., Hinshaw, D. B., Hyslop, P. A., *et al.* (1986). Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J. Clin. Invest.* **77,** 1312–1320.

Schreck, R., Rieber, P., and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.* **10,** 2247–2258.

Schuler, G., and Steinman, R. M. (1997). Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J. Exp. Med.* **186,** 1183–1187.

Schwarz, M. A., Lazo, J. S., Yalowich, J. C., *et al.* (1995). Metallothionein protects against the cytotoxic and DNA-damaging effects of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4452–4456.

Shen, Y. H., Wang, X. L., and Wilcken, D. E. L. (1998). Nitric oxide induces and inhibits apoptosis through different pathways. *FEBS Lett.* **433,** 125–131.

Shimizu, S. I., Saitoh, Y., Yamamoto, T., *et al.* (1994). Stimulation by hydrogen peroxide of L-arginine metabolism to L-citrulline coupled with nitric oxide synthesis in cultured endothelial cells. *Res. Commun. Chem. Pathol. Pharmacol.* **84,** 315–329.

Sonoki, T., Nagasaki, A., Gotoh, T., *et al.* (1997). Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues *in vivo* by lipopolysaccharide. *J. Biol. Chem.* **272,** 3689–3693.

Soo Kwon, N., Stuehr, D. J., and Nathan, C. F. (1991). Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.* **174,** 761–767.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9,** 271–296.

Stern, M., Savill, J., and Haslett, C. (1996). Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by $\alpha_v\beta_3$/CD36/thrombospondin recognition mechanism and lack of phlogistic response. *Am. J. Pathol.* **149,** 911–921.

Stewart, B. W. (1994). Mechanisms of apoptosis: Integration of genetic, biochemical, and cellular indicators. *J. Natl. Cancer Inst.* **86,** 1286–1296.

Stuehr, D. J., and Nathan, C. F. (1989). Nitric oxide: A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169,** 1543–1555.

Tabuchi, A., Sano, K., Oh, E., *et al.* (1994). Modulation of AP-1 activity by nitric oxide (NO) *in vitro:* NO-mediated modulation of AP-1. *FEBS Lett.* **351,** 123–127.

Takema, M., Inaba, K., Uno, K., *et al.* (1991). Effect of L-arginine on the retention of macrophage tumoricidal activity. *J. Immunol.* **146,** 1928–1933.

Tanaka, T., Akira, S., Yoshida, K., *et al.* (1995). Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell* **80,** 353–361.

Thomsen, L. L., Lawton, F. G., Knowles, R. G., *et al.* (1994). Nitric oxide synthase activity in human gynecological cancer. *Cancer Res.* **54,** 1352–1354.

Thomsen, L. L., Miles, D. W., Happerfield, L., *et al.* (1995). Nitric oxide synthase activity in human breast cancer. *Br. J. Cancer* **72,** 41–44.

Tsang, S. S., Yin, X., Guzzo-Arkuran, C., *et al.* (1993). Loss of resolution in gel electrophoresis of RNA: A problem associated with the presence of formaldehyde gradients. *BioTechniques* **14,** 380–381.

Vedia, L. M. Y., McDonald, B., Reep, B., *et al.* (1992). Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.* **267,** 24929–24932.

Virág, L., Scott, G. S., Cuzzocrea, S., *et al.* (1998). Peroxynitrite-induced thymocyte apoptosis: The role of caspases and poly (ADP-ribose) synthetase (PARS) activation. *Immunology* **94,** 345–355.

von Knethen, A., and Brüne, B. (1997). Cyclooxygenase-2: An essential regulator of NO-mediated apoptosis. *FASEB J.* **11,** 887–895.

Walker, M. W., Kinter, M. T., Roberts, R. J., *et al.* (1995). Nitric oxide-induced cytotoxicity: Involvement of cellular resistance to oxidative stress and the role of glutathione in protection. *Pediatr. Res.* **37,** 41–49.

Walker, P. R., Kwast-Welfeld, J., Gourdeau, H., *et al.* (1993). Relationship between apoptosis and the cell cycle in lymphocytes: Roles of protein kinase C, tyrosine phosphorylation, and AP1. *Exp. Cell Res.* **207,** 142–151.

Wang, Z. Q., Stingl, L., Morrison, C., *et al.* (1997). PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev.* **11,** 2347–2358.

Wink, D. A., Nims, R. W., Darbyshire, J. F., *et al.* (1994). Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol.* **7,** 519–525.

Xie, K., Huang, S., Wang, Y., *et al.* (1996). Bcl-2 protects cells from cytokine-induced nitric-oxide-dependent apoptosis. *Cancer Immunol. Immunother.* **43,** 109–115.

Yamazaki, A., and Birnboim, H. C. (1995). Potentiation of retinoic acid-induced U-937 differentiation into respiratory burst-competent cells by nitric oxide donors. *Leukemia Res.* **19,** 325–335.

Zhang, J., Dawson, V. L., Dawson, T. M., *et al.* (1994). Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science* **263,** 687–689.

Zhao, Z., Francis, C. E., Welch, G., *et al.* (1997). Reduced glutathione prevents nitric oxide-induced apoptosis in vascular smooth muscle cells. *Biochim. Biophys. Acta* **1359,** 143–152.

CHAPTER 45

Physiological and Pathophysiological Roles of Nitric Oxide in Gastrointestinal Function

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DIVERSE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES FOR NITRIC OXIDE (NO) HAVE BEEN IDENTIFIED IN MANY TISSUES, INCLUDING THOSE OF A VASODILATOR, NEUROTRANSMITTER, ANTIMICROBIAL AGENT, IMMUNOMODULATOR, AND INTRACELLULAR SIGNALLING MOLECULE, ALL OF WHICH MAY OPERATE IN THE GUT. THUS, IN THE GASTROINTESTINAL TRACT, NO CAN AFFECT GASTRIC ACID SECRETION AND BLOOD FLOW AS WELL AS MUCUS AND BICARBONATE SECRETION. NO ALSO AFFECTS INTESTINAL MOTILITY AND SPHINCTER TONE, MICROCIRCULATION, ELECTROLYTE SECRETION, AND WATER ABSORPTION. MANY OF THESE CHANGES IN RESPONSE TO STIMULATION BY NO ARE TRANSDUCED VIA ACTIVATION OF SOLUBLE GUANYLATE CYCLASE. NO AND NO DONORS CAN BRING ABOUT MUCOSAL PROTECTION AGAINST CHALLENGE BY AFFECTING SUCH PROCESSES. NO CAN ALSO AFFECT ADHESION OF WHITE CELLS TO THE MICROVASCULAR ENDOTHELIUM, AN EVENT THAT CAN MODULATE TISSUE INJURY. IN THIS CHAPTER, THE ROLE OF NO FORMED BY THE THREE ISOFOMS OF NOS IN THE GASTROINTESTINAL TRACT IN THE MAINTENANCE OF PHYSIOLOGICAL FUNCTION AND MUCOSAL INTEGRITY, AS WELL AS THE INVOLVEMENT OF NO IN PATHOLOGICAL PROCESSES INCLUDING INTESTINAL CYTOTOXICITY, TISSUE INJURY, AND INFLAMMATION, HAS BEEN REVIEWED.

Introduction

Nitric oxide (NO) has been shown to play a diverse range of roles in biological tissues and organs, including those in the gastrointestinal tract. Diverse physiological and pathophysiological roles for NO have been identified, including those of a vasodilator, neurotransmitter, antimicrobial agent, immunomodulator, and intracellular signaling molecule, all of which may operate in the gut. NO, which is formed by the actions of three distinct isoforms of the synthetic enzyme NO synthase (NOS) [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)], can be detected in a wide range of cell types, including endothelial cells, macrophages, neurons, hepatocytes, fibroblasts, epithelial cells, and smooth muscle cells. In the gastrointestinal tract, NO acts on several of these cell types to bring about changes in secretion, motility, blood flow, electrolyte and water absorption, mucosal protection, and inflammation. Many of these changes in response to stimulation by NO are transduced via activation of soluble guanylate cyclase.

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This chapter reviews studies on the role of NO formed by the three isoforms of NOS in the gastrointestinal tract in the maintenance of physiological function and mucosal integrity. In addition, the involvement of NO in pathological processes such as inflammation will be reviewed.

Physiological Actions of NO in the Gut

Blood Flow

Nitric oxide has been shown to play an important role in modulating vascular tone within the gastrointestinal circulation. Different physiological stimuli such as shear stress, changes in Ca^{2+} concentration, or changes in oxygen tension may stimulate NO release.

Administration of the isoform-nonselective NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) reduces resting gastric mucosal blood flow in experimental studies, suggesting the involvement of endogenous NO in modulating the resting gastric microcirculation. Similarly, L-NMMA and N^G-nitro-L-arginine methyl ester (L-NAME) have been shown to increase vascular resistance in the mesenteric circulation and can reduce blood flow in the small and large intestine.

In the stomach, the increase in gastric mucosal blood flow caused by stimulants such as pentagastrin can be reduced without any influence on acid secretion by administration of L-NAME, an effect which can be reversed by coadministration of L-arginine. The back-diffusion of H^+ leads to an increase in gastric blood flow, and this microvascular response can be inhibited by L-NAME treatment, including a protective hyperemic response. These findings suggest that NO, which may interact with endogenous prostaglandins, is involved in the regulation of gastric blood flow.

The esophagus, like the stomach, is also able to resist damage due to refluxed acid by increasing mucosal blood flow. This hyperemic response is preceded by luminal histamine release and can be attenuated by mast cell stabilization, histamine H_1 receptor blockade, and NOS inhibition. NOS inhibition also prevents the histamine release, suggesting that mast cell-derived histamine acts via an NO-dependent mechanism in this response.

In the intestine NO also plays an interactive role in the regulation of blood flow. Sympathetic neurogenically mediated vasoconstriction of intestinal arterioles increases during NO synthase inhibition, and this augmented response can be reversed by exposure to L-arginine. The most likely source of this NO released during increased sympathetic nerve activity is the microvascular endothelium.

NO also appears to be involved in the intestinal hyperemia in response to luminal instillation of certain foodstuffs. Thus, glucose administration into the ileal lumen results in an increase in blood flow, which was blocked by L-NAME. It is not yet known if glucose causes a direct production of NO or whether epithelial cells transduce a vasodilator signal through vascular endothelium-derived NO during postprandial intestinal hyperemia.

NO and Cell Adhesion

Within the vasculature, NO can exert a variety of effects apart from its influence on vasomotion, including the inhibition of platelet aggregation and the attenuation of leukocyte and endothelial interaction.For a leukocyte to adhere to the endothelial lining, it must initially make a brief tethering interaction with endothelium, which then develops into a rolling interaction. It has been demonstrated experimentally that inflammation following administration of a NOS inhibitor is associated with enhanced leukocyte and endothelial interaction and with increased leukocyte adherence and emigration.

Furthermore, inhibition of NO synthesis promotes the adhesion molecule P-selectin-dependent leukocyte rolling, suggesting that NO may be a homeostatic regulator of leukocyte rolling under normal conditions. Administration of exogenous NO decreases leukocyte rolling in acute inflammation induced by a variety of conditions including ischemia–reperfusion and reactive oxidant administration.

NO and Gastric Acid Secretion

Whereas inhibition of NO formation does not directly modulate resting or stimulated gastric acid secretion, endogenous NO appears to be involved in the acute antisecretory action of agents such as lipopolysaccharide and cytokines or in moderate hypothermia, acting through mechanisms in the brain. It has also been observed that NO synthase inhibition could reduce acid secretion in response to a protein meal or gastrin administration.

Direct administration of NO donor agents can reduce vagally mediated acid secretion as well as histamine-stimulated acid secretion. It has also been demonstrated that NO donor agents can inhibit pentagastrin-stimulated acid secretion in doses that did not affect blood flow. Such an effect may reflect, in part, suppression of histamine release from enterochromaffin-like cells. Furthermore, high concentrations of NO donor agents can inhibit parietal cell activity *in vitro,* suggesting a direct action of NO on acid secretory activity.

NO and Gastroduodenal Secretion of Mucus and Bicarbonate

The continuous viscoelastic layer of gastric mucus over the mucosa has been shown to play an important role in mucosal protection against topical irritation by noxious agents in the lumen. Furthermore HCO_3^- secreted from the duodenal epithelial cells into the mucous gel has been shown to establish a pH gradient in the gel, thereby providing a line of defense against gastric acid. NO donor agents such as isosorbide dinitrate and *S*-nitroso-*N*-acetyl-penicillamine (SNAP) have been shown to increase mucus gel thickness. NO synthase inhibitors have also been shown to reduce the ability of mucosal cells to secrete and synthesize mucus. Furthermore, treatment with the NO substrate L-arginine can

reverse the reduction in gastric cellular mucous secretion in response to hypoxia–reoxygenation that has been observed in some *in vitro* situations.

It has been demonstrated that NO mediates mucus secretion in response to a number of stimulatory agents such as gastrin, histamine, and acetylcholine. Both acute and chronic inhibition of NO synthesis can reduce mucus secretion *in vivo.*

Inhibition of NO synthase activity has been shown to increase duodenal alkaline secretion, suggesting that endogenous NO may modulate the basal secretion of HCO_3^-. This inhibition may reflect suppression of a stimulatory nicotinic receptor-dependent neural mechanism.

Luminal acid formation can increase mucosal NO output, and this response can be inhibited by L-NMMA treatment. Furthermore, L-NMMA may reduce the acid-stimulated increase in alkaline secretion. Similarly, luminal administration of a NO donor agent has been shown to increase mucosal HCO_3^- secretion in the dog. The acid-induced increase in HCO_3^- secretion can be inhibited by an extract of *Helicobacter pylori,* and this effect could be overcome by L-arginine.

Mucosal damage in response to a hyperosmotic challenge also increased gastroduodenal HCO_3^- secretion, and this effect was attenuated by treatment with a NO synthase inhibitor. Thus, NO appears to mediate the alkaline secretory response to luminal acid and as a consequence of an injurious challenge to the stomach.

NO and Intestinal Electrolyte and Water Secretion

It is now well established that L-arginine and NO synthase inhibitors are able to influence intestinal secretion. However, the findings are contradictory and vary depending on the segment of intestine studied, the animal species involved, and other experimental conditions. The latter include *in vivo* or *in vitro* conditions, intact or stripped tissues, and the type of challenge used. Overall, given the data it can be suggested that under physiological conditions a NO-dependent proabsorptive tone exists. This conclusion is based on a number of findings that NO synthase inhibitors induce net fluid and electrolyte secretion *in vivo* in several animal species. However, some reports suggest that an increase in NO formation may also be capable of evoking net secretion in the gut. A notable exception is the diarrhea produced by bacterial enterotoxin, in which NO may exert a proabsorptive action. Because NO may mediate both secretagogue and proabsorptive actions within the intestine, it will be necessary to identify more precisely the conditions under which NO exerts these diverse actions.

Administration of NO-generating compounds can stimulate intestinal water and electrolyte absorption. Thus luminal administration of L-arginine or SNAP increases water and electrolyte absorption. Furthermore a NO donor agent can attenuate the secretion and permeability changes induced in the rat ileum by administration of *Clostridium difficile* toxin A. Similarly, sodium nitroprusside treatment was shown to inhibit jejunal secretion in response to *Escherichia coli* enterotoxin.

These data support the potential therapeutic use of NO-generating compounds in the treatment of some pathological hypersecretory states. Intrarectal administration of NO donor agents in animals can induce increases in insulin absorption as well as high-molecular-weight dextran. Such information thus suggests that NO donor agents may have the potential to act as potent absorption enhancers in a clinical setting.

NOS Inhibition and Diarrheal Disorders

Under physiological conditions an NO-dependent proabsorptive tone exists in the intestine. However, in some pathological states, including many different experimental models of inflammatory bowel disease in mouse, rat, guinea pig, and monkey, as well as in tissue from patients with Crohn's disease and ulcerative colitis, NO can be produced at higher concentrations capable of evoking net secretion. Such NO appears to contribute to the diarrhea associated with a number of models of intestinal inflammation.

Furthermore, elevated activity of NOS is associated with the laxative action of several intestinal secretagogues, including castor oil, phenolphthalein, bisacodyl, magnesium sulfate, bile salts, senna, and cascara. Studies using isoform-selective and -nonselective inhibitors have indicated that inhibition of NOS effectively ameliorates the intestinal secretion depending on the model evaluated. Although L-NAME or L-NMMA effectively reduces the diarrheal response to the laxatives listed, inhibitors of iNOS are only effective against bile salt- and cascara-mediated secretion.

NO and Gastrointestinal Motility

Nitric oxide has been shown to exert some potent actions on enteric smooth muscle, particularly relaxation. Strong evidence demonstrating that NO is one of the key transmitters of nonadrenergic noncholinergic (NANC) nerve-induced relaxation of gastrointestinal smooth muscle has accumulated. Indeed, such nerves have been referred to as nitrergic neurons. Evidence for such nitrergic innervation includes the demonstration that NOS immunoreactivity can be detected in gastrointestinal myenteric and submucosal neurons; also, NO release in response to electrical field stimulation can be inhibited by neural toxins including tetrodotoxin. NO, when acting as a mediator of NANC relaxation of basal tone as well as following neuronal stimulation, has been demonstrated in various segments of the gastrointestinal tract, including duodenum, ileum, ileocecal junction, colon, esophagus, and lower esophageal sphincter.

Gastric and Intestinal Motility

NO has been proposed as a physiological mediator of the regulation of gastric emptying in response to various luminal nutrients in the stomach and duodenum. In the esophagus, NO plays an important role in the mediation of propulsive activity by regulating the period and gradient between the

onset of a swallow and contractions of the esophageal circular muscle. Similarly, NO has been suggested to be involved in the coordination of peristaltic activity in guinea pig intestine via a dual excitatory and inhibitory effect on intestinal motility. The excitatory effect may involve cholinergic motor neurons, whereas the inhibitory effect may reflect direct relaxation of intestinal muscle.

NO has also been linked with the slowly migrating, cyclic peristaltic wave associated with the interdigestive state in most mammals. This interdigestive migrating complex involves a number of components, including contraction originating in the stomach and propagating to the terminal colon that is regulated by the gut hormone motilin. NO has been shown to influence these contractions, as NO synthase inhibition results in their appearance even after feeding. Inhibition of NO synthesis was also found to stimulate motilin release via cholinergic pathways independent of the vagus. The contractions induced by NO synthase inhibition appear to involve activation of 5-hydroxytryptamine (5-HT) receptors.

The mechanisms of NO-induced relaxation of gastrointestinal smooth muscle are a subject of much investigation. NO may have some association with vasoactive intestinal peptide (VIP), a signaling peptide that has also been designated as a NANC mediator. However, it has also been demonstrated that although vagal activation evokes both VIP and NO release, there is no evidence for any interaction, and the relaxation induced by VIP does not involve NO.

The contractile response of intestinal tissue to a NO synthase inhibitor can be inhibited by tetrodotoxin or hexamethonium, indicating a neural involvement. Furthermore, NO relaxes circular muscle of the rat forestomach both directly at a muscular site and indirectly via inhibition of acetylcholine release. A similar neural mechanism has been proposed for NO-mediated inhibition of duodenal motility. In contrast, it has been shown that NO does not inhibit acetylcholine release in guinea pig ileal circular muscle and may inhibit the release of tachykinins that act on NK1 receptors.

In pathological conditions associated with ileus, complete or partial gastrointestinal motor dysfunction occurs. Studies of canine gastrointestinal motility during periods of endotoxemia have shown that gastric emptying and colonic transit are slowed. Similarly, the interdigestive migrating complex is delayed. These changes have been attributed to NO production induced by endotoxin. The lipopolysaccharide-induced changes in intestinal transit can be reversed by administration of a NO synthase inhibitor, and the effects on the interdigestive complex are negated.

NO donor agents have been used in the treatment of functional disturbances of gastric motility and emptying. NO can suppress fundic, antral, pyloric, and duodenal contractions and inhibit pyloric motility in response to intraduodenal triglyceride. Similarly, NO release following glyceryl trinitrate administration slows the rate of gastric emptying and decreases antral motor activity. Similarly, L-arginine administration can reduce antral motility changes in response to a liquid test meal.

Gallbladder and Sphincter of Oddi

Release of bile into the duodenum is controlled by hepatic bile secretion, gallbladder contraction, and the sphincter of Oddi. The hormone cholecystokinin is considered to be the physiological stimulant for postprandial gallbladder contraction and sphincter relaxation. Studies have shown that the action of cholecystokinin on the sphincter of Oddi is mediated through NANC nerves, and NO is an important component of the pathway.

In humans, administration of a NO donor agent can produce gallbladder dilation in the fasting state, reduce postprandial emptying, and inhibit the motility of the sphincter of Oddi as well. It has been proposed that glyceryl trinitrate would be useful during endoscopic retrograde cholingiopancreatography cannulation and gallstone extraction. Although it has been considered that long-term nitrate therapy and the resultant inhibition of gallbladder motility may be a risk factor in the accelerated genesis of gallstones, the relaxant effect of glyceryl trinitrate and other NO donor agents, including isorbide dinitrate, has been shown to reduce the pain associated with acute biliary colic.

Esophagus and Lower Esophageal Sphincter

NO may play a role in the regulation of esophageal motility. Experimental findings suggest that, at least in part, NO may be responsible for the onset of the swallow and coordinated occurrence of contractions in the circular smooth muscle. In the esophagus, endogenous NO has been implicated in the development of a latency period and latency gradient and in the regulation of the amplitude of esophageal body peristalsis. Furthermore, NO has been shown to be a mediator of lower esophageal sphincter relaxation.

In clinical studies, NO donor agents have been utilized in the treatment of diffuse esophageal spasm and achalasia, where there are disturbances of normal esophageal motility. Diffuse esophageal spasm is a functional disorder in which the mechanisms responsible for the physiological timing of contractions are dysfunctional. Administration of NO to these patients significantly prolonged the latency period, restored the normal peristaltic contractions, and reduced their duration, thus abolishing the adverse clinical symptoms. Treatment with L-arginine was ineffective, but this may be anticipated if a functional disruption of the NO pathway exists.

Achalasia is a motor disorder characterized by abnormal peristalsis, elevated pressure of the lower esophageal sphincter, and failure of sphincter relaxation in deglutition. A reduction in NOS-containing neurons has been identified in this disorder in humans. In an animal model of achalasia, the NO donor agent sodium nitroprusside has been shown to reduce resting lower esophageal pressure in the achalasia group as well as in the control group of animals. Furthermore, in patients with achalasia, NO donor agents can induce relaxation of esophageal muscle. Thus, NO donor agents may have important therapeutic benefits in the treatment of esophageal motor disorders.

NOS INHIBITION AND MOTILITY

Because excessive NO may play a role in functional disturbances of gastric and intestinal motility, it is feasible that such disorders may respond to therapy with inhibitors of NOS. The most common disorder in this respect is the rapid transit and disrupted gastrointestinal motility of endotoxemia. This may result from the induction of iNOS, producing excessive NO. Challenge with *E. coli* lipopolysaccharide increased tissue and iNOS activity and the rate of intestinal transit, which was reversed by NOS inhibition. In contrast to the intestine, endotoxin treatment delayed gastric emptying. This effect could be reversed by dexamethasone, perhaps suggesting the involvement of iNOS in this response.

The NO produced by eNOS, although not a disorder of excess NO, facilitates gastric emptying, and this response can be inhibited by isoform-nonselective NOS inhibitors. This may be clinically useful in the treatment of conditions associated with accelerated gastric emptying, such as those associated with gastroduodenal resective surgery.

Mucosal Protection

Intragastric administration of the glyceryl trinitrate sodium nitroprusside and SNAP can protect against experimentally induced acute gastric mucosal injury induced by topical irritants such as aspirin and by intravenous infusions of endothelin-1, endotoxin, or platelet-activating factor.

In the intestine, administration of NO donor agents provides significant protection against the mucosal and microvascular dysfunction associated with ischemia–reperfusion. Prophylactic administration of L-arginine has been shown to improve intestinal barrier function in experimental animals after mesenteric ischemia. NO administration can also diminish thermal injury-associated venule constriction and irregularity within the gastrointestinal tract. However, local infusion of high doses of an NO donor agent has been shown to cause gastric mucosal damage.

A nitro-derivative of the nonsteroidal anti-inflammatory drug flurbiprofen, which contains a moiety similar to the NO-releasing moieties found in many organic nitrates, not only suppresses gastric prostaglandin synthesis but also causes significantly less hemorrhagic damage than flurbiprofen itself. Similarly, a nitroxy butyl ester derivative of ketoprofen also results in less acute gastric mucosal injury than does its parent compound, while demonstrating comparable anti-inflammatory activity. Nitric oxide-releasing derivatives of aspirin have also been shown to reduce the susceptibility of the stomach to shock-induced damage by inhibiting neutrophil adherence to the vascular endothelium. This class of NO-containing compounds has also been found effective in reducing the extent of intestinal damage in response to endotoxic shock, although the changes in blood pressure and hematocrit were similar to that produced by the parent compound itself.

Pathological Actions of NO in the Gut

Cytotoxic Actions of NO

A wide array of cell types found in the gut, including inflammatory and epithelial cells, can be induced to express iNOS, which then produces large amounts of NO (Fig. 1). Such concentrations of NO may interact with various target molecules such as oxygen, thiol groups, and metals within the prosthetic groups of various enzymes, resulting in their activation or inactivation. NO can react with nonsulfur centers of aconitase of the tricarboxylic acid cycle as well as with complexes I and II of the electron transport chain within target molecules. The inhibition of these essential components of cellular respiration results in depletion of cellular ATP stores with consequent cytotoxicity.

NO may also mediate cytotoxicity by a number of other mechanisms, including impairment of DNA synthesis, direct toxicity through deamination reactions, and possibly more importantly via the potentiation of the toxicity of oxygen radicals. NO can combine with superoxide anion (O_2^-) to form the intermediate peroxynitrite, which is then protonated to peroxynitrous acid under acidic conditions; peroxynitrous acid yields the cytotoxic products hydroxyl radical (·OH) and nitrogen dioxide (NO_2). Peroxynitrite can initiate toxic oxidative reactions *in vivo* and *in vitro*. These include initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceralderhyde-3-phosphate dehydrogenase, inhibition of membrane Na^+,K^+-ATPase, and other oxidative protein modifications. In addition, peroxynitrite is a potent trigger of DNA strand breakage, with subsequent activation of the nuclear enzyme poly (ADP-ribosyl) synthetase and eventual severe energy depletion of the cells.

High concentrations of NO donor agents have been shown to exacerbate the cytotoxic effect of hydrogen perox-

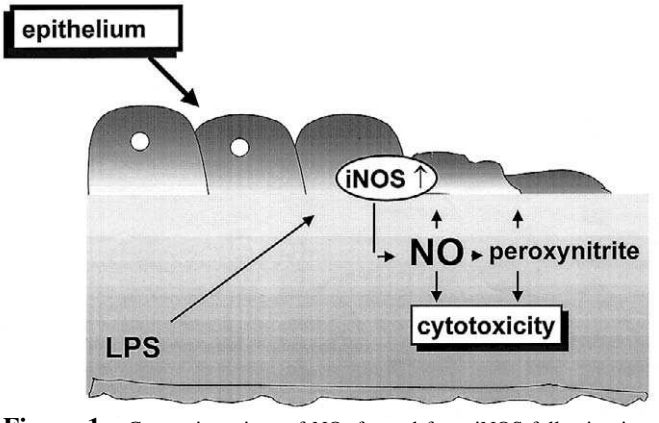


Figure 1 Cytotoxic actions of NO, formed from iNOS following its induction by lipopolysaccharide (LPS), on gastrointestinal epithelial cells. The cellular injury is brought about either by high concentrations of NO or by the combination of NO with the superoxide radical anion to form the cytotoxic moiety peroxynitrite.

ide in gastric mucosal cells and to be necessary for induction of mucosal injury by various types of intestinal bacteria. High levels of NO have also been shown to injure rat intestinal epithelial cells; this damage can be ameliorated by inhibitors of guanylate cyclase. Excessive levels of NO from endogenous or exogenous sources reduce gastric cellular viability, and this response appears to be related causally to an increase in intracellular Ca^{2+}. In isolated cells, the cytotoxic effect of NO has been found to be due to increased generation of oxidants as a result of a decrease in glutathione production. Furthermore, it has been demonstrated in isolated cells that high levels of NO can inactivate glutatione peroxidase, leading to increased accumulation of peroxides.

Infusion of NO donor agents to produce high local concentrations of NO can produce injury to the gastric mucosa. This damage is related to peroxynitrite formation and lipid peroxidation.

In the intestinal mucosa, excessive NO production is associated with increased vascular permeability (Fig. 2). The mechanism of this increased permeability appears to be associated with decreases in cellular cAMP levels, reduced levels of glutathione, and the formation of peroxynitrous acid.

In the gastrointestinal tract, induction of iNOS is associated with a reduction in cell viability. Furthermore, administration of extracts of *Helicobacter pylori* result in the induction of NOS and injury in duodenal cells.

Inducible NO Synthase and Inflammation

A large number of studies have identified increased expression and activity of iNOS in experimental models of inflammatory bowel disease as well as in human inflamed colonic, gastric, and esophageal tissue. The increase in NO synthase activity in experimentally induced colonic inflammation is related to an increase in neutrophil infiltration and subsequent tissue lipid peroxidation.

The inflammatory effect of increased levels of iNOS appears to be related to increased peroxynitrite activity. Administration of superoxide or peroxyl scavengers reduces the extent of iNOS-mediated mucosal inflammation. Furthermore, nitrotyrosine, an index of peroxynitrite and nitrosylated proteins, can be detected in inflamed tissue from experimental animals and from humans with ulcerative colitis.

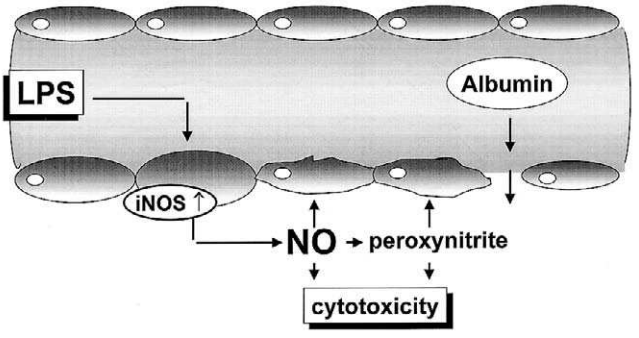


Figure 2 Induction of iNOS following challenge with lipopolysaccharide (LPS) leads to microvascular injury throughout the gastrointestinal mucosa, either as a result of high concentrations of NO or by a combination of NO and the superoxide radical anion to form the cytotoxic moiety peroxynitrite. The endothelial cell injury leads to the leakage of plasma proteins such as albumin into the interstitium.

iNOS Inhibition and Inflammation

Concurrent administration of NO synthase inhibitors that are not isoform selective with endotoxin challenge has been shown to substantially augment the early phase of microvascular injury in the gastrointestinal tract. In contrast, delay of administration of L-NMMA until 3 hours after endotoxin challenge reduced the subsequent vascular leakage at a time when inducible NOS activity becomes apparent. Thus the beneficial effect of delayed administration of L-NMMA and L-NAME is due to their ability to inhibit iNOS activity. The therapeutic efficacy of delayed administration of NOS inhibitors in intestinal injury models has been firmly established. Aminoguanidine, a putative iNOS inhibitor, while reducing the late phase vascular leakage induced by endotoxin, also enhances the early phase of mucosal and microvascular dysfunction in a manner similar to that seen after administration of nonisoform selective NOS inhibitors. Thus, the development of highly selective inhibitors of iNOS in the therapeutic control of intestinal inflammation is extremely important.

The putative iNOS-selective inhibitor *S*-methylisothiourea has been shown to ameliorate endotoxin-induced reductions in intestinal hyperpermeability and mucosal mitochondrial function, and the newly developed agent 1400W was found to be a potent and highly selective inhibitor of iNOS in intestinal dysfunction. In these studies 1400W had no detrimental effects on the early phase following endotoxin challenge while inhibiting the late phase injury. Administration of nonselective NOS inhibitors such as L-NAME or selective iNOS inhibitors such as *S*-(2-aminoethyl)isothiouronium bromide ameliorated experimentally induced colitis.

In contrast to the evidence that iNOS is cytotoxic and contributes to gut inflammation, a number of studies oppose this notion. In these studies elimination of high levels of NOS via knockout of the iNOS gene or via the use of selective iNOS antagonists did not reduce experimentally induced colitis. Similarly, other experimental studies have demonstrated that high levels of NO released from various NO donor agents did not cause breakdown of mucosal or microvascular barrier integrity under normal or inflammatory conditions. These are in contrast to a number of other studies demonstrating a marked injurious action of NO donor agents on the gastrointestinal mucosa.

It has been shown that NO released via iNOS expression following lipopolysaccharide administration can protect the gastric mucosa from damage in response to luminal irritants or stress and can protect the intestinal mucosa from an inflammatory challenge via a reduction in leukocyte inflitration and adhesion. This iNOS-mediated protection has also been attributed to an increase in cytoprotective release of PGE_2, which acts as an antioxidant either directly for maintaining cellular glutathione or by activating other antioxidants such as manganese superoxide dismutase.

In contrast to these results, the purified lipopolysaccharide derived from *Helicobacter pylori* can stimulate the expression of iNOS in duodenal cells; this event is associated with a cytotoxic action on these cells. This cellular injury can be ameliorated by iNOS inhibition as well as oxidant scavengers, suggesting the involvement of NO, superoxide, and subsequently peroxynitrite in the epithelial injury associated with *Helicobacter pylori* infection.

The involvement of NO in the tissue injury associated with infectious diseases of the gut has been explored. The intestinal inflammation associated with *Trichinella spiralis* infection can be reduced by L-NAME treatment, and the mucosal inflammation associated with shigellosis is closely related to epithelial iNOS expression. Furthermore, NO has been proposed as a primary etiologic factor in neonatal necrotizing enterocolitis. There is also involvement of iNOS in the chronic microvascular leakage and tissue inflammatory injury in the small intestine following administration of nonsteroidal anti-inflammatory agents. This slowly developing enteropathy involves indigenous bacteria and is attenuated by inhibition of iNOS, suggesting that following ingress of bacteria, lipopolysaccharide is liberated in the intestinal mucosa, bringing about the induction of iNOS. It is possible that such inflammation will respond to the therapeutic use of selective iNOS inhibitors.

Conclusions

From this review, it is apparent that the involvement of NO in the physiology and pathology of the gastrointestinal tract is multifaceted. Despite the complexity, however, knowledge of the roles of NO in the gut may identify novel clinical utilities. The use of NO donor agents in a range of motility problems is currently under investigation, as is their use in protecting the gut mucosa from injury. Indeed, the therapeutic NO-containing nonsteroidal anti-inflammatory drugs are being actively pursued in experimental and clinical studies.

A wealth of literature supports the cytotoxic actions of NO in many different inflammatory situations, both in and beyond the gastrointestinal tract. Inhibitors of NO production are also being evaluated as anti-inflammatory agents in the gut. However, it is likely that NO produced from iNOS or its subsequent products are not involved in all aspects of the inflammatory response in the gut. In addition, the role of NO may well differ with the nature of the insult, the tissue, and environment involved. As with many other inflammatory mediators, it is entirely possible that a low level of expression of iNOS will reflect a possible host defense response to challenge, but that exaggerated or uncontrolled expression of iNOS itself becomes detrimental. It is also feasible that the products of iNOS may have a greater role in the early phase of inflammation, such as following relapse in patients with inflammatory bowel disease, whereas lower levels of iNOS activity could have a beneficial action in the process of resolution in the inflammatory response. The NO pathway thus offers a number of diverse therapeutic opportunities for treating diseases of the gut.

Additional Reading

Boughton-Smith, N. K., Evans, S. M., Hawkey, C. J., Cole, A. T., Balsitis, M., Whittle, B. J. R., and Moncada, S. (1993). Differential changes in nitric oxide synthase in ulcerative colitis and Crohn's disease. *Lancet* **342,** 338–340.

Hällgren, A., Flemström, G., Sababi, M., and Nylander, O. (1995). Effect of nitric oxide inhibition on duodenal function in the rat. Involvement of neural mechanisms. *Am J. Physiol.* **269,** G246–G254.

Izzo, A. A., Masolo, N., and Capasso, F. (1998). Nitric oxide as a modulator of intestinal water and electrolyte transport. *Dig. Dis. Sci.* **43,** 1605–1620.

Konturek, S. J., Thor, P., and Domschke, W. (1995). Effects of nitric oxide in antral motility and gastric emptying in humans. *Eur. J. Gastroenterol. Hepatol.* **7,** 97–102.

Lamarque, D., and Whittle, B. J. R. (1995). Role of oxygen-derived metabolites in the rat gastric mucosal injury induced by nitric oxide donors. *Eur. J. Pharmacol.* **277,** 187–194.

Laszlo, F., Whittle, B. J. R., and Moncada, S. (1995). Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. *Br. J. Pharmacol.* **111,** 1309–1315.

Moilanen, E., Whittle, B. J. R., and Moncada, S. (1999). Nitric oxide as a factor in inflammation. *In* ''Inflammation: Basic Principles and Clinical Correlates'' (L. Gallin and R. Snyderman, eds.), pp. 787–800. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania.

Moncada, S., and Higgs, E. A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* **9,** 1319–1330.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide accounts for the biological activity of endothelium-dervied relaxing factor. *Nature* **327,** 524–526.

Pique, J. M., Esplugues, J. V., and Whittle, B. J. R. (1992). Endogenous nitric oxide as a mediator of gastric mucosal vasodilatation during acid secretion. *Gastroenterology* **102,** 168–174.

Rees, D. D., Collek, S., Palmer, R. M. J., and Moncada, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and associated effects on vascular tone: An insight in endotoxin shock. *Biochem. Biophys. Res. Commun.* **173,** 541–547.

Tepperman, B. L., and Whittle, B. J. R. (1992). Endogenous nitric oxide and sensory neuropeptides interact in the modulation of the rat gastric microcirculation. *Br. J. Pharmacol.* **105,** 171–175.

Tepperman, B. L., Brown, J. F., Korolkiewicz, R., and Whittle, B. J. R. (1994). Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: Effect of endotoxin challenge. *J. Pharmacol. Exp. Ther.* **271,** 1477–1482.

Whittle, B. J. R. (1997). Nitric oxide—a mediator of inflammation or mucosal defence. *Eur. J. Gastroenterol. Hepatol.* **9,** 1026–1032.

Whittle, B. J. R. (1994). Nitric oxide in gastrointestinal physiology and pathology. *In* ''Physiology of the Gastrointestinal Tract'' (L. R. Johnson, ed.), Vol. 1, Chap. 6, pp. 267–294. Raven, New York.

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CHAPTER 46

Pathophysiological Effects of High-Output Production of Nitric Oxide

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IN THIS CHAPTER WE FOCUS OUR DISCUSSION ON THE PATHOPHYSIOLOGICAL EFFECTS OF NITRIC OXIDE (NO), OXYGEN FREE RADICALS, AND REACTIVE NITROGEN OXIDE SPECIES IN BIOLOGICAL SYSTEMS. WE PLACE PARTICULAR EMPHASIS ON HOST RESPONSES TO VARIOUS VIRAL AND BACTERIAL INFECTIONS AND TO SOLID TUMORS, IN VIEW OF CONSEQUENT PATHOLOGICAL MANIFESTATIONS THAT RESULT FROM REACTIVE NITROGEN OXIDE DERIVATIVES. THE DISCUSSION IS BASED PRIMARILY ON BIOCHEMICAL AND IMMUNOLOGICAL DATA, INCLUDING INDUCTION OF INDUCIBLE NO SYNTHASE AND PRODUCTION OF SUPEROXIDE ANION RADICAL AND PEROXYNITRITE. FURTHERMORE, THE DISCOVERY OF AN ACCELERATED VIRAL MUTATION RATE IN THE HOST DURING VIRAL INFECTION IS DISCUSSED FROM THE PERSPECTIVE OF NO-INDUCED OXIDATIVE STRESS. MECHANISMS OF CARCINOGENESIS INVOLVING FREE RADICALS FORMED IN THE COURSE OF CHRONIC INFECTIOUS DISEASES ARE ALSO DESCRIBED. IT APPEARS THAT THE MOST CRITICAL COMMON DENOMINATOR OF CARCINOGENESIS IS FREE RADICALS, WHICH ARE FORMED DURING MICROBIAL INFECTIONS, AFTER EXPOSURE TO CHEMICAL CARCINOGENS, AND BY ELECTROMAGNETIC RADIATION.

Introduction

Increasing attention has been paid to the pathogenic roles of nitric oxide (NO) produced in excess in various pathological settings. Both NO and reactive oxygen species such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorite (HClO) are generated in inflamed tissues; in sites infected with viruses, bacteria, fungi, or parasites; and in cancerous tissues. These reactive species are generated mostly by inflammatory cells (macrophages and neutrophils), as well as by damaged tissue expressing xanthine oxidase (XO). Superoxide from macrophages, neutrophils, or various tissues will react extremely rapidly with NO to form reactive nitrogen oxides such as peroxynitrite ($ONOO^-$), which is more involved in pathogenesis than is NO per se (Beckman and Koppenol, 1996; Rubbo *et al.,* 1996). The pathophysiological action of peroxynitrite, among various reactive nitrogen oxides, is of great interest for the pathogenesis of various diseases (Beckman *et al.,* 1990). Peroxynitrite is not only a potent oxidant but also a nitrating agent of various biological molecules such as proteins and nucleic acids as well as membrane lipids (Beckman and Koppenol, 1996; Rubbo *et al.,* 1996; Ischiropoulos, 1998). Thus, endogenously formed reactive nitrogen oxides appear to have a potential impact on mutagenesis and carcinogenesis as well as having other pathological consequences (Ohshima and Bartsch, 1994; Beckman and Koppenol, 1996; Rubbo *et al.,* 1996; Szabó and Ohshima, 1997).

In this chapter we describe various pathological effects of NO in view of the pathogenic potential of high-output

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production of NO in inflammatory and infectious diseases and cancer, with the discussion based on experimental evidence obtained with animal models of inflammation, infection, and solid tumors (Maeda *et al.,* 1994; Akaike *et al.,* 1998; Maeda and Akaike, 1998; Wu *et al.,* 1998). We also discuss formation of reactive nitrogen species, particularly peroxynitrite, and their contribution to mutagenic and carcinogenic processes from the perspective of the unique and potent chemical reactivity of reactive nitrogen oxide species.

NO in Inflammation and Infections

It is now well known that superoxide and NO production is elevated in inflamed tissues. Generation of superoxide and active oxygen species is brought about by two components of the host response, namely, cellular reactions of leukocytes expressing NADPH oxidase (Clark, 1990) and humoral responses involving XO (Akaike *et al.,* 1990). The host reactions are in response to foreign matter, microorganisms, and damage caused by trauma, radiation, or ischemia–reperfusion injury. Because deficiency of an O_2^--generating enzyme, NADPH oxidase, of phagocytic leukocytes is frequently associated with severe chronic bacterial infections (termed chronic granulomatous disease, CGD) (Tauber *et al.,* 1983), it is apparent that oxygen radical formation is important in front line host defense.

Overproduction of NO that is mainly caused by inducible NO synthase (iNOS), which is usually expressed by inflammatory phagocytic cells and other types of cells (e.g., epithelial and neuronal cells), has a similar defense function (Nathan and Hibbs, 1991; Nathan, 1997). Among the three types of nitric oxide synthase (NOS), iNOS produces a much larger amount of NO for a longer time (i.e., 10- to 100-fold more) than do the other two constitutive enzymes, neuronal NOS and endothelial NOS (Stuehr and Griffith, 1992; Moncada and Higgs, 1993). Although NO per se seems to have a limited bactericidal effect, suppression or lack of NO production results in impairment of bacterial clearance by the host (Yoshida *et al.,* 1993; de Groote *et al.,* 1995; Nathan, 1997; Akaike *et al.,* 1998). As a primary host defense mechanism, these free radical molecular species interact, and the reaction products of NO and superoxide indeed have a crucial role in various infections (Akaike *et al.,* 1998).

However, NO is not always beneficial to the host; it becomes detrimental to cells and tissues as well as to microbes. As discussed in more detail later, NO generated in inflamed tissue can cause injury through peroxynitrite formation. Not only is NO toxic to microbes, it is also hazardous to normal parenchymal tissue, possibly via cytotoxicity arising, for example, from the apoptotic and necrotic effects of peroxynitrite and other nitrogen oxides (Estevez *et al.,* 1995). Evidence of the pathophysiological effects of NO is illustrated in two different settings: granulomatous inflammation and microbial infections (Fig. 1).

Granulomatous Inflammation in the Lung

Experimental granulomatosis or fibrosis in the lung can be produced by intratracheal instillation of silica (Lugano *et al.,* 1982; Reiser *et al.,* 1982; Setoguchi *et al.,* 1996) or of various types of beads, such as dextran beads (Tsuji *et al.,* 1995), Sephadex G-50 beads (Kasahara *et al.,* 1988), or latex beads (Kasahara *et al.,* 1988), and by intravenous infusion of glucan (Jones and Warren, 1992; Flory *et al.,* 1993) or of microbes, such as *Mycobacterium tuberculosis* (Orrell *et al.,* 1992), bacille Calmette-Guérin (BCG) (Shellito and Sniezek, 1990), or *Schistosoma mansoni* (Lukacs *et al.,* 1993). Pulmonary granulomatous inflammation occurs predominantly as a focal collection of mononuclear phagocytes in response to a variety of microbes, foreign particulate matter, and etiologically unknown factors. In the acute phase of inflammation, infiltration of neutrophils into the lungs is one of the earliest events, followed by infiltration of monocytes, differentiation into exudate macrophages, and activation of these macrophages (Hunninghake *et al.,* 1984). In the chronic phase, tissue or resident macrophages show a marked proliferative reaction (Van Oud Albas *et al.,* 1983).

iNOS is upregulated in lung lesions via induction of proinflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and γ-interferon (IFN-γ), when silica or zymosan (mainly composed of β-glucan) is instilled intratracheally (Setoguchi *et al.,* 1996). Overproduction of NO in zymosan- or silica-instilled rat lungs is clearly observed by using electron spin resonance (ESR) spectroscopy. Because silica particles are toxic to and kill neutrophils and macrophages, the phagocytes containing the silica particles disintegrate, and the material is rephagocytosed by macrophages. This vicious circle is repeated and becomes a strong stimulus for granulomatosis and subsequent fibrosis in the lung. During these processes, silica particles remain undigested and further stimulate iNOS production in infiltrating leukocytes, macrophages (Setoguchi *et al.,* 1996; Blackford *et al.,* 1994), and bronchiolar epithelial cells (Tsuji *et al.,* 1995). Asbestos would produce similar effects *in vivo.*

Inhibition of NO synthesis results in reduced infiltration of monocyte-derived exudate macrophages into lung lesions in silica- and zymosan-induced granulomatosis, and zymosan particles remain undigested in zymosan-instilled lungs because of NOS suppression (Setoguchi *et al.,* 1996). Similarly, in a model of carrageenan sponge implantation, granuloma formation is potentiated by treatment with L-arginine, and granuloma growth is markedly reduced by an NOS inhibitor (N^{ω}-nitro-L-arginine methyl ester, L-NAME) (Iuvone *et al.,* 1994). Certain chemokines such as monocyte chemoattractant protein-1 (MCP-1) are expressed in granulomatous inflammation (Jones and Warren, 1992; Flory *et al.,* 1993), and MCP-1 induces infiltration of monocyte-derived exudate macrophages. It is noteworthy that in lung lesions induced by silica and zymosan NOS inhibition reduces MCP-1 expression at the protein and messenger RNA (mRNA) levels, suggesting that NO induces MCP-1 in lung granulomas

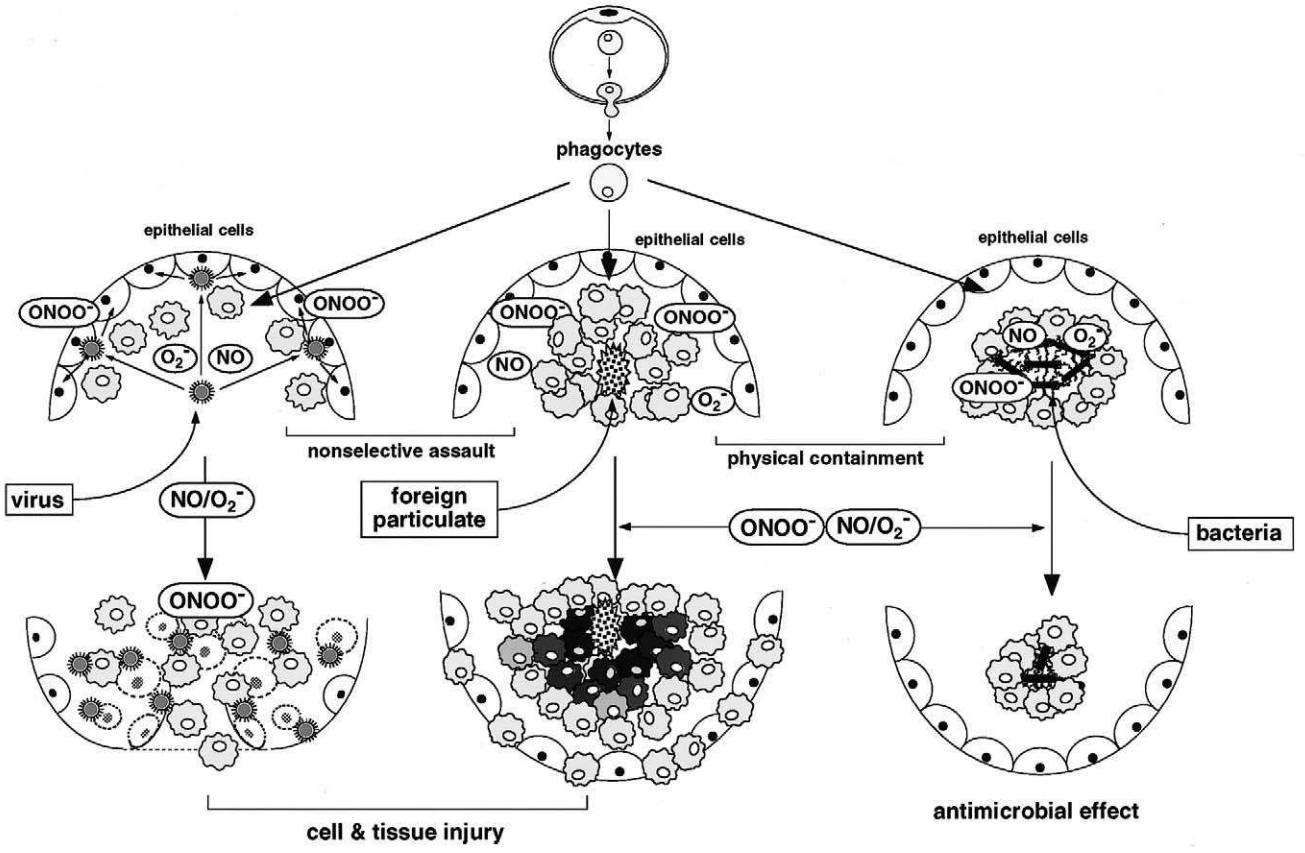


Figure 1 Biological effects of high output of NO in granulomatous inflammation and microbial infections. Foreign particulate matter, such as silica and bacteria, is physically contained in the local area of inflammation, as shown in the middle and right, respectively. Nonselective cell and tissue injury tends to occur in viral infections and granulomatous lesions, whereas bacteria can be eradicated by NO-dependent host defense actions.

via NO-dependent putative transcription or translation of MCP-1 (Setoguchi *et al.,* 1996).

Macrophages produce superoxide anion after stimulation with zymosan and silica (Berton and Gordon, 1983; Vallyathan *et al.,* 1988), and thus simultaneous generation of superoxide anion and NO, leading to peroxynitrite formation, occurs in granuloma tissues. This result is supported by intense positive immunostaining directed at nitrotyrosine in neutrophils, macrophages, and intra-alveolar inflammatory exudate in granulomas (Setoguchi *et al.,* 1996). We found that peroxynitrite activates human neutrophil procollagenase (matrix metalloproteinase 8, MMP-8), which is critical in tissue disintegration and remodeling under physiological as well as pathological conditions such as inflammation and infection (Okamoto *et al.,* 1997a, b). In addition to activating MMP-8, peroxynitrite readily inactivates both tissue inhibitor of metalloproteinase (TIMP) and α_1-proteinase inhibitor (α_1-PI), the major proteinase inhibitor of neutrophil elastase, which exists in large quantity (i.e., 2–3 mg/ml) in human plasma (Moreno and Pryor, 1992; Frears *et al.,* 1996; Whiteman *et al.,* 1996). Thus, peroxynitrite seems to accelerate tissue degradation and contribute to the pathogenesis of various diseases. It is intriguing that peroxynitrite activates cyclooxygenase, a key enzyme in the production of the potent inflammatory mediators, the prostaglandins (Landino *et al.,* 1996). Our experiment shows that activation of proMMP also appears to trigger formation of bradykinin, which is a potent inflammatory mediator (T. Akaike and H. Maeda, 2000, unpublished observation). Therefore, peroxynitrite may sustain granuloma extension via both tissue-remodeling actions on pulmonary connective tissues and proinflammatory effects.

These data indicate that excessive production of NO mediated through the action of iNOS induced by foreign particulates probably contributes to the development of pulmonary granulomatosis (Fig. 1). More importantly, NO produced in excess by iNOS seems to function as an inflammatory mediator (Clancy and Abramson, 1995; Kubes, 1995), possibly through the diverse biological actions of peroxynitrite. In this context, it is interesting that peroxynitrite exacerbates airway hyperpermeability during the allergic response of bronchial asthma (Sugiura *et al.,* 1999).

Overproduction of NO in Microbial Infections

NO in Viral Infections

iNOS is induced in a variety of experimental viral infections in rats and mice, including those with neuroviruses, such as Borna disease virus, herpes simplex virus type 1

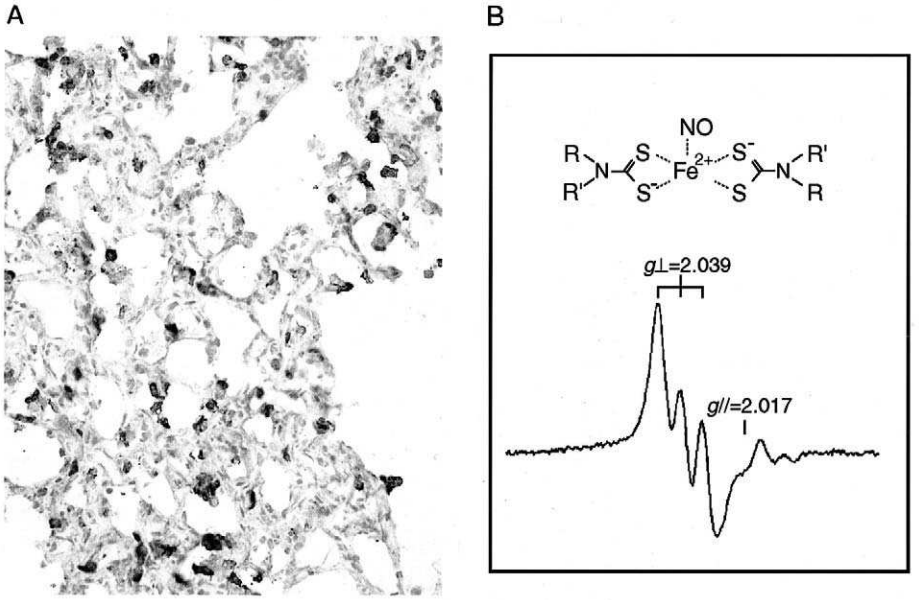


Figure 2 Typical pathological changes in virus-infected tissues expressing iNOS and NO. (A) iNOS immunostaining of lung infected with Sendai virus (2 LD_{50}; 8 days after infection; magnification, ×230). Intensive staining is observed mainly in phagocytic cells such as macrophages infiltrating the alveoli and interstitial tissues. (B) NO signal as identified by ESR spectroscopy at 110 K as an NO dithiocarbamate–iron complex adduct generated in the same virus-infected lung. Approximately 10–20 μ*M* of the NO adduct is usually obtained from the infected lung tissues. The chemical structure of the adduct is also shown.

(HSV-1), and rabies virus, and pneumotropic and cardiotropic viruses, such as influenza virus, Sendai virus, and Coxsackie virus (Koprowski *et al.,* 1993; Zheng *et al.,* 1993; Campbell *et al.,* 1994; Akaike *et al.,* 1995, 1996, 1998; Bi *et al.,* 1995; Kreil and Eibl, 1996; Mikami *et al.,* 1996).

In the case of pneumotropic virus infections in mice, iNOS is expressed by exudate macrophages and bronchial epithelial cells in lung tissues, and the high output of NO in virus-infected lesions is evident by ESR spin trapping with the use of a dithiocarbamate–iron complex (Fig. 2) (Akaike *et al.,* 1996; Akaike and Maeda, 1999). In general, the time profile of iNOS induction in the lung correlates well with that of pulmonary consolidation rather than that of virus replication in the lung. Virus replication reaches a maximum 3 to 4 days after infection, whereas NO production reaches a maximum on days 7 to 8, when the viral count is almost undetectable. Pathological manifestations as determined by the consolidation score parallel the NO production. Lethality in the mice starts to increase on day 8 after viral infection.

iNOS induction in this pneumotropic virus infection is mediated by proinflammatory cytokines such as IFN-γ, as in many other inflammatory diseases as mentioned above. Induction of IL-4 becomes detectable in virus-infected lung as early as 2 days after infection, and it increases steadily, attaining a maximum value 6 days after infection. The level of IL-4 in plasma increases rapidly more than 8 days after infection. NO production in the lung is seen only 6 to 9 days after infection, corresponding to the appearance of pathological changes. It is also important to note that induction of arginase I mRNA has been identified in virus-infected lung, paralleling IL-4 induction in the plasma (T. Akaike and H. Maeda, 2000, unpublished observation).

Downregulation of iNOS expression is reported for some cytokines, for example, IL-4, IL-10, and transforming growth factor β (Cunha *et al.,* 1992; Vodovotz *et al.,* 1993; Bogdan *et al.,* 1994); a suppressive effect of IL-4 and IL-10 on iNOS mRNA induction has been shown in murine macrophages in culture. In addition, these suppressor cytokines may reduce NO production indirectly via induction of arginase (Corraliza *et al.,* 1995; Gotoh *et al.,* 1996; Sonoki *et al.,* 1997), which diminishes the supply of substrate (L-arginine) for this enzyme. It is known that IL-4 and IL-10 are involved in the stimulation and differentiation of B cells so that they produce antibody, through a Th2 response pathway (Wright, 1997). It seems, therefore, that suppressor cytokines for iNOS induction switch the NO-dependent host response to a virus-specific humoral immune response against the intruding virus.

NO has an antiviral action, although its selective antiviral toxicity is still controversial. A number of experiments have verified that simultaneous production of NO and superoxide is common in influenza virus pneumonia in mice (Akaike *et al.,* 1996, 1998). For example, induction of iNOS correlates with XO upregulation in infected lung. When superoxide generated in the lung is removed by intravenous administration of superoxide dismutase (SOD) derivatized with a synthetic polymer to remain in circulation more than 10 times longer than native SOD, NO production in virus-infected tissue apparently increases, as identified by ESR spectroscopy (Akaike *et al.,* 1996). In addition, extensive immunostaining of virus-infected lung with antinitrotyrosine antibody

suggests that peroxynitrite is generated in inflammatory tissue during pneumonia (Akaike *et al.,* 1996).

Overproduction of NO together with superoxide production yielding peroxynitrite nonselectively impairs the physiological functions of the host cells regardless of the source of infection. In fact, we showed that neither inhibition of NO biosynthesis nor scavenging of superoxide and inhibition of its production affects virus yield in lungs infected with influenza virus. However, a significant improvement in lung pathology and survival rate of the influenza virus-infected animals is obtained by treatment with the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA) or superoxide antidotes such as pyran-conjugated SOD, or with an inhibitor of XO, allopurinol. The contribution of high-output NO to influenza pathogenesis was also verified by Karupiah *et al.* (1998) using mice lacking iNOS: virus-induced pneumonia in iNOS-deficient mice is much less severe than that in wild-type mice. A similar result was obtained by Kreil and Eibl (1996), who used an NOS inhibitor in tick-borne encephalitis virus infection in mice. Although an antiproliferative action of NO against HSV has been described for cells in culture (Karupiah *et al.,* 1993; Croen, 1993), our results *in vivo* indicate that suppression of excessive production of NO by L-NMMA in the central nervous system of HSV-1-infected animals results in improvement of neuronal damage, but suppression of NO generation does not affect virus propagation (Fujii *et al.,* 1999). Similarly, improvement of HSV-1-induced pneumonia by L-NMMA was reported by Adler *et al.* (1997).

Moreover, iNOS expression has been found in brain tissue of a patient with severe human immunodeficiency virus-1 (HIV-1) encephalitis (Bukrinsky *et al.,* 1995). A viral envelope glycoprotein of HIV, gp41, triggered iNOS expression in human astrocytes and murine cortical brain cells in culture (Adamson *et al.,* 1999; Hori *et al.,* 1999). Of considerable importance is the finding that NO produced by iNOS causes neuronal cell death, suggesting involvement of NO in the pathogenesis of HIV-associated dementia and its related central nervous system injury (Bukrinsky *et al.,* 1995; Adamson *et al.,* 1999; Hori *et al.,* 1999; Boven *et al.,* 1999).

All the results just described suggest that the overproduction of NO induced by iNOS in virus infections is critical in the pathogenesis of these infections, rather than the functioning of NO as a specific antiviral molecule in host defense (Fig. 1). Viruses usually attack tissue indiscriminantly, and many types of viruses propagate and spread not only from cell to cell but also by free diffusion in tissues. Thus, the physical containment strategy in host defense is mostly ineffective against viral pathogens. Free radical effector molecules such as NO and superoxide produced by the host in defense will assault both normal cells and tissues and virus-infected cells, and thus the detrimental effects of NO production and peroxynitrite formation outweigh any benefits to the host (Fig. 1).

ANTIMICROBIAL EFFECT OF NO

The pathogenic effect of NO and superoxide in virus infections appears to be in clear contrast to the antimicrobial actions of NO and superoxide observed in bacterial, fungal, and parasitic infections (Granger *et al.,* 1988; Nathan and Hibbs, 1991; Doi *et al.,* 1993; James, 1995; Umezawa *et al.,* 1995, 1997; Nathan, 1997). NO overproduction is suggested to cause lipopolysaccharide (LPS)-induced lung injury (Kristof *et al.,* 1998) and neurological damage in bacterial meningitis in children (Kornellisse *et al.,* 1996). Bacterial infections are also known to induce iNOS expression in septic foci and sites of inflammation. In this case, bacterial cell components, namely, LPS and teichoic acid, may be involved directly or indirectly through induction of a series of proinflammatory cytokines during the infection.

Although the antibacterial action of NO was proposed by Hibbs and co-workers a long time ago (Granger *et al.,* 1988), NO per se seems rather inert up to the millimolar concentration (Yoshida *et al.,* 1993; de Groote *et al.,* 1995). This effect may be more pronounced in phagocytosed cells. In our experiments, peroxynitrite or NO_2 exhibited far greater (100-fold more) bactericidal action, whereas NO released from an NONOate [1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene] or authentic NO dissolved in water showed only a marginal effect at 1 m*M* (Yoshida *et al.,* 1993; Kuwahara *et al.,* 2000). It is interesting in this context that S-nitrosylated α_1-PI, a major protease inhibitor in human plasma, becomes 10- to 100-fold more bacteriostatic than NO or *S*-nitroglutathione on a molar basis (Miyamoto *et al.,* 2000a, b).

The antimicrobial effect of NO and superoxide *in vivo* is most clearly observed in a study of *Salmonella typhimurium* infection in mice, in which XO and iNOS are strongly upregulated as part of the host defense reaction, similar to the situation in virus infections (Umezawa *et al.,* 1997). The most primitive host defense response is physical containment of the intruding pathogens to a confined area. Containment of the pathogen is typically characterized as a pathological change, that is, abscess or granuloma formation. In the case of murine salmonellosis, multiple microabscesses showing a clear contour are observed in livers infected with *S. typhimurium* (Umezawa *et al.,* 1995, 1997). The bacteria are contained by phagocytes (such as neutrophils and macrophages) in localized septic lesions. As a result, reactive molecular species, for example, NO, superoxide, OCl^-, hydrogen peroxide (H_2O_2), and peroxynitrite, directly affect invading pathogens only in the confined area, and most commonly intracellularly, so that effective cytotoxic action against pathogens but minimal tissue injury in the surrounding area will occur during the defense process of the host (Fig. 1).

Mutagenesis and Carcinogenesis Caused by High-Output NO

The mutagenic potential of free radicals and peroxynitrite has been reported (Vuillaume, 1987; Ohshima and Bartsch, 1994; Liu and Hotchkiss, 1995; Szabó and Ohshima, 1997).

It is thus of paramount importance to explore a link between excessive generation of NO and superoxide and carcinogenesis.

NO and Carcinogenesis

Oxidation of NO by any mechanism results in generation of various nitrogen oxide derivatives. It has been known since the 1960s that nitrous acid damages nucleic acids by deamination (Fraenkel-Conrat, 1964); therefore, it follows that mutation and hence carcinogenesis are initiated by free radicals and nitrogen oxides. Peroxynitrite formed by the reactions of NO and superoxide and reactive nitrogen oxides including nitrogen dioxide (NO_2) are the most crucial molecular species.

Classic textbooks in oncology or pathology describe three completely different causes of cancer: chemical carcinogens (or chemicals such as heterocyclic amines), infections with tumor viruses such as simian virus 40 (SV40), and exposure to radiation such as X rays, γ rays, and ultraviolet light (see Fig. 3). The last one, radiation, is the oldest known carcinogenic factor involving the generation of free radicals in radiation physics. Hydroxyl radical, superoxide, and singlet oxygen are generated by electromagnetic interaction between water or oxygen molecules, and they react with nucleic acids, proteins, membrane lipids, and other biomolecules. The damage to nucleic acids causes mutation, with the consequent development of cancer. The existence of radicals such as superoxide in biological systems has become well known. As described earlier, we now have ample evidence that microbial or parasitic infections and inflammatory conditions can generate free radicals as well as reactive oxygen and nitrogen oxide intermediates. In different settings, we have observed that chemical carcinogens, for example, heterocyclic amines, can generate superoxide in the presence of the cytochrome P-450 system or cytochrome P-450 reductase and, more recently, cytochrome b_5 reductase (Sato *et al.,* 1992; Maeda *et al.,* 1999). Thus, one of the best studied carcinogens, the heterocyclic amine found in cooked meat and fish, is a source of superoxide generation. In addition, 8-hydroxyguanine (8OHG) generation, as a result of the reaction with hydroxyl radical, is now established (Kasai and Nishimura, 1984).

Among the reactive molecular species that might injure nucleic acids, superoxide and NO are regarded as the most important. In fact, it is conceivable that peroxynitrite can oxidize DNA (Inoue and Kawanishi, 1995; Spencer *et al.,* 1996) and nitrate DNA, particularly its guanine residue to form 8-nitroguanine (Yermilov *et al.,* 1995a; Spencer *et al.,* 1996). Nitroguanine is then spontaneously released from DNA, resulting in apurinic site formation (Yermilov *et al.,* 1995b). H_2O_2 is also important unless converted to H_2O and O_2 by catalase, because H_2O_2 can be the source of the more reactive hydroxyl radical (·OH) via the Fenton reaction in the presence of transition metals in biological systems (Halliwell and Gutteridge, 1984). Another important alternative mechanism involves H_2O_2 and the myeloperoxidase-catalyzed pathway, which generates NO_2Cl and NO_2 in the presence of NO_2^-, which results in nitration or chlorination of tyrosine and guanine residues of protein and DNA (Eiserich *et al.,* 1998; Byun *et al.,* 1999).

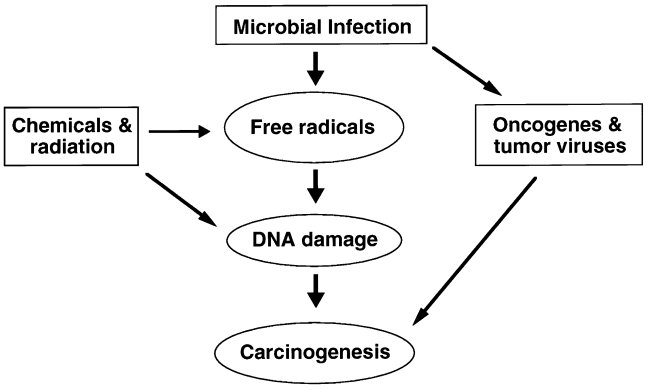


Figure 3 Central themes and universal mechanisms in carcinogenesis: Interaction of free radicals and DNA in infection and inflammation, and with carcinogens, leads to cancer.

As mentioned earlier in this chapter, not only defense-oriented phagocytic cells such as macrophages and polymorphonuclear cells but also endothelial and epithelial cells are activated to produce free radical species, most commonly in microbial infections or because of stress or other insult to the host. Once these reactive molecular species (or free radicals) are generated during the inflammatory responses of the host, they can damage DNA, enzymes and proteins, and other vital molecules. Some oxygen radicals such as the hydroxyl radical, which is highly reactive and short-lived, may be converted to more stable lipid peroxide radicals (LOO·), with longer half-lives, which would travel long distances *in vivo* and may accumulate in fat-rich organs and cellular microenvironments (Akaike *et al.,* 1992; Sawa *et al.,* 1998).

Several epidemiological studies suggest a link between infections and carcinogenesis (see Table I). The importance of free radicals in carcinogenesis is increasing as the evidence of excessive production of NO and oxygen radicals in infections and inflammation accumulates (Vuillaume, 1987; Yuspa and Poirier, 1988; Harris, 1991). Thus, although the free radical theory played a rather minor role in previous studies of cancer etiology, it now appears more realistic in view of more recent findings of the generation of superoxide, peroxynitrite, and NO in infectious diseases.

In the history of infection-related carcinogenesis, many efforts have focused on tumor viruses with the viral oncogene or v-*onc.* However, carcinogenesis induced by viruses (and also bacteria) does not involve the so-called v-*onc*; most such agents (hepatitis viruses B and C, herpes simplex type II, *Helicobacter pylori,* and *Schistosoma,* as shown in Table I) are devoid of v-*onc.* The most common factor is that these infections involve chronic inflammation, in which long-term exposure of the host organism, mostly for more than 10 years, to both nitrogen- and oxygen-related free radicals may cause mutation of the hot spot of certain genes such as p53 and other tumor suppressor genes.

Table I Infection-Related Carcinogenesis Caused by Various Infectious Agents

Agent	Cancer
Bacteria	
Helicobacter pylori	Gastric cancer (gastritis/ulcer)
Salmonella typhi, S. paratyphi A, B	Gallbladder cancer, pancreatic cancer
Viruses	
Hepatitis virus types B and C	Hepatoma (chronic hepatitis/cirrhosis)
Herpes simplex virus type 2	Cervical cancer (recurrent infection)
Epstein-Barr virus	Gastric cancer, nasopharyngeal cancer (Burkitt lymphoma)
Papillomaviruses	Cervical cancer
Polyomaviruses (e.g., SV40, JCV)	Brain cancer?, tumors at multiple sites
Human T-cell leukemia virus	Adult T-cell leukemia
Parasites	
Opisthorchis viverrini	Cholangiocarcinoma (cholangiofibrosis)
Schistosoma species	Bladder cancer, hepatoma

The cause-and-effect relationship of *H. pylori* infection and gastric cancer appears to be of great interest in this context. Gastric cancer is one of the major cancers in Japan as well as in many developing countries. It now appears to be caused by a spiral bacterium, *H. pylori* (Sugiyama *et al.,* 1998; Honda *et al.,* 1998). *Helicobacter pylori* can cause a chronic infection, manifested frequently as gastritis and gastric ulcer. Baik *et al.,* (1996) reported pronounced DNA damage, particularly formation of 8OHG, in human gastric mucosa with chronic *H. pylori* infection, providing additional *in vivo* evidence that infections cause DNA damage. Induction of iNOS and production of nitrotyrosine are also found in *H. pylori*-infected human mucosa (Mannick *et al.,* 1996; Pignatelli *et al.,* 1998). In chronic typhoid and paratyphoid carriers infected with *Salmonella typhi* or *S. paratyphi* A or B, the incidence of cancers of the gallbladder and pancreas is also reported as high (Caygill *et al.,* 1994). This may be another example of cancer involving bacterial infections.

Moreover, various agents, including hepatitis B and C viruses, Epstein-Barr virus, and parasites (*Opisthorchis viverrini* and *S. mansoni*), have been suggested to be risk factors for hepatoma, gastric cancer, and other types of cancer (Table I). The cause of this risk may be closely related to the formation of free radicals and reactive nitrogen oxides, particularly peroxynitrite (Ohshima and Bartsch, 1994).

We have presented here examples of free radical generation in viral and bacterial infections that would result in consequences similar to those of radiation and chemical carcinogens. The free radical hypothesis in carcinogenesis seems to unify all of these different carcinogenic mechanisms, as summarized in Fig. 4. Preventive measures for cancer may thus include minimizing excessive exposure to free radicals *in vivo* by dietary uptake of various antioxidants, for exam-

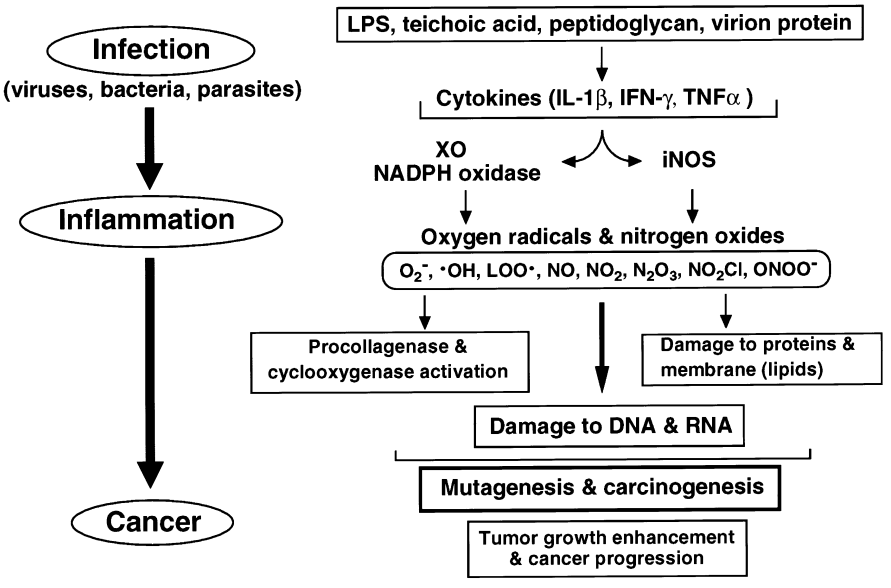


Figure 4 Various events involving free radicals in infection and inflammation that lead to cancer.

ple, a diet high in flavonoids, by adequate anti-inflammatory regimens, or by control of chronic infections.

Mutagenesis in Microbial Pathogens Induced by NO-Derived Metabolites

Among the NO-induced pathological effects, the mutagenic potential of NO in microbial pathogens is also intriguing. One study showed that human leukocytes producing superoxide, but not leukocytes from patients with CGD, are mutagenic for *S. typhimurium* TA100 (Weitzman and Stossel, 1981). Peroxynitrite is mutagenic for prokaryotic DNA, possibly via its nitration of guanine residues of DNA. A typical base substitution caused by peroxynitrite is G → T transversion, which is an indirect result of depurination of nitroguanine in DNA (Yermilov *et al.,* 1995b; Juedes and Wogan, 1996). Mutagenesis induced by a high output of NO is also documented in a study with murine macrophages expressing iNOS (Zhuang *et al.,* 1998).

As mentioned earlier, overproduction of NO and oxygen radicals appears to be a common phenomenon in various infections. The resultant reactive molecular species such as peroxynitrite nonselectively affect the cells and tissues of the host. Such host defense effectors are originally produced to injure the intruding pathogens, which will then suffer oxidative stress because of the host. Therefore, it may be that mutagenesis in various pathogens occurs naturally in biological systems during infections as a result of host defense.

For viral mutation, it is of potential interest to investigate a possible association of oxidative stress and virulence of viruses. Beck *et al.* (1995) show that the pathogenicity of coxsackie virus B3 is strongly potentiated *in vivo* in mice fed a selenium-deficient diet. More importantly, an avirulent strain of the virus is converted to a potent cardiotoxic variant during infection in selenium-depleted animals. The deficiency of selenium may result in an ineffective antioxidant system, for example, low levels of glutathione peroxidase. These studies were extended to vitamin E- and glutathione peroxidase-deficient animals, and the results suggest that oxidative stress facilitates selection and generation of virulent mutants (Beck *et al.,* 1998). Although the molecular mechanism for the conversion of avirulent to virulent strains of coxsackie virus *in vivo* is unclear, impaired immunological clearance due to reduced levels of antioxidants may cause an increased incidence of heterogeneous mutants of the virus, resulting in selection of highly pathogenic variants.

In addition, our study verifies for the first time that oxidative stress induced by high-output NO accelerates RNA virus mutations (T. Akaike *et al.,* 2000). In brief, by using a recombinant RNA virus, Sendai virus (a negative-sense and single-strand RNA virus) containing a marker gene for genetic mutation, and iNOS knockout mice, we obtained solid and direct evidence showing that overproduction of NO in the hosts (wild-type mice) *in vivo* apparently increases and accelerates viral mutation rates compared with the situation in iNOS-deficient mice. This process of accelerated mutation expands the heterogeneity of variants of the pathogen, leading to rapid evolution under selective pressure. NO and superoxide and hence peroxynitrite generation occurs universally in infected hosts. This finding therefore has great implications for RNA virus evolution in general, including the rapid generation of drug-resistant and immunologically tolerant and cell tropism-altered mutants of HIV *in vivo.*

NO and Solid Tumor Growth

Enhancement of Tumor Growth by NO

In this section, we describe the pathophysiological effects of NO in relation to tumor growth. Rapid tumor growth requires an adequate supply of nutrients and oxygen from circulating blood. This nutrient demand is sustained via enhanced vascular permeability in solid tumor tissues, which is mediated by a number of factors derived from tumor cells or from the host tissue, including NO, bradykinin, and vascular permeability factor (VPF; also called vascular endothelial growth factor, VEGF) (Senger *et al.,* 1983; Matsumura *et al.,* 1988; Vaupel *et al.,* 1989; Maeda *et al.,* 1994; Nakano *et al.,* 1996; Suzuki *et al.,* 1996; Wu *et al.,* 1998). Maintenance of regional blood flow in solid tumor tissues appears to be most important for tumor cell growth (Kallinowski *et al.,* 1989). NO released from vascular endothelial cells plays an important physiological role in the regulation of blood flow in the systemic circulation via its potent vasodilating action (Furchgott and Vanhoutte, 1989; Ignarro, 1991; Moncada and Higgs, 1993). Solid tumor growth may therefore be sustained by increased blood flow mediated by prolonged and excessive production of NO.

We have previously demonstrated that NO functions as a vascular permeability factor (Maeda *et al.,* 1994). Furthermore, a number of studies show that tumor neovasculature is highly defective in its architecture; for example, smooth muscle cells are lacking and angiotensin II receptors are missing. Vascular density in tumors is also higher than in normal tissues. All of these result in enhanced vascular permeability in solid tumors. This means that tumor blood vessels are quite leaky: even albumin and other macromolecules can move out of the vessels. The L-arginine-dependent NO pathway mediates angiogenic activity not only in normal tissues (Leibovich *et al.,* 1994) but also in solid tumors (Jenkins *et al.,* 1995). It is intriguing that Ziche *et al.* (1997) reported that VEGF (VPF) induces angiogenesis via formation of NO. Angiogenesis leading to hypervascularization is most frequently associated with rapid tumor growth. NO-aided tumor growth has been shown in tumor-bearing mice, in which human adenocarcinoma cells overexpressing iNOS were implanted and NO-induced angiogenesis led to acceleration of tumor growth *in vivo* (Jenkins *et al.,* 1995). Promotion of tumor progression and metastasis by NO is also suggested by structural evidence (Lala and Orucevic, 1998; Thomsen and Miles, 1998).

Other important factors that affect the vascular permeability of solid tumors include bradykinin, as discussed earlier

(Maeda *et al.,* 1988, 1996). We now know that the inhibition or scavenging of NO or bradykinin results in suppression of permeability and of tumor growth, whereas vascular permeability is proportional to tumor growth (Maeda *et al.,* 1994; Doi *et al.,* 1996, 1999; Nakano *et al.,* 1996; Thomsen *et al.,* 1997; Tozer *et al.,* 1997; Wu *et al.,* 1998). Because bradykinin, which triggers NO release from endothelial cells (Palmer *et al.,* 1988), is generated effectively in tumors (Matsumura *et al.,* 1988; Maeda *et al.,* 1996), the bradykinin–NO interaction may be important in tumor growth.

The tumor-infiltrating activated macrophage is a major contributor to overproduction of NO in solid tumors, although some tumor cells can also generate NO. A particularly high output of NO from iNOS is potentially cytotoxic for various tumor cells (Hibbs *et al.,* 1988; Bastian *et al.,* 1994; Lepoivre *et al.,* 1994). Therefore, NO has two opposite effects in tumor biology, i.e., suppression and promotion of tumor growth. These discrepant actions may be explained by a putative mechanism in tumor cells that protects against the cytotoxicity of excess NO.

Heme Oxygenase as a Potent Protective Effector for NO Cytotoxicity

Of considerable interest is a unique biological function of heme oxygenase (HO), which is involved in catabolic breakdown of heme compounds in iron metabolism (Tenhunen *et al.,* 1968). HO degrades heme to release carbon monoxide (CO), iron, and biliverdin (Maines, 1997). Two isoforms of HO, namely, HO-1 and HO-2, are known to exist in eukaryotic systems (Maines and Kappas, 1974). HO-1 is induced by various stimuli, such as proinflammatory cytokines and heavy metals (Shibahara *et al.,* 1985; Maines, 1997; Yet *et al.,* 1997). Induction of HO-1 has been suggested to result in an important protective response of cells against oxidative damage, because HO-1 induction may decrease the cellular heme level (pro-oxidant) and elevate the level of bilirubin (antioxidant), which is derived from biliverdin and is a potent scavenger of reactive oxygen species (Kim *et al.,* 1995a, b; Maines, 1997). Lancaster and co-workers reported that pretreatment of rat hepatocytes with a low dose of NO donor agent confers resistance to cellular oxidative damage through induction of HO-1 (Kim *et al.,* 1995a). Their report also demonstrated that HO-1 induction protects the cells from NO-mediated cytotoxicity. HO-1 has been shown to be constitutively expressed in the liver and spleen and in some tumor cells (Tenhunen *et al.,* 1968; Shibahara *et al.,* 1985; Lee and Ho, 1994; Goldman *et al.,* 1996; Hara *et al.,* 1996; Maines, 1997). In addition, it is proposed that CO, a product of HO, functions as a gaseous signal transduction molecule in a manner similar to that of NO (Suematsu *et al.,* 1995; Maines, 1997; Prabhakar *et al.,* 1997).

HO expression is highly upregulated in some experimental and human solid tumor tissues (Lee and Ho, 1994; Goldman *et al.,* 1996; Hara *et al.,* 1996; Takahashi *et al.,* 1996). For example, HO-1 is constantly upregulated in a solid tumor model in rats (Doi *et al.,* 1999). The level of HO-1 mRNA expression is apparently enhanced in AH136B tumor cells in culture by treatment with an NO (NO^+) donor agent. Regulation of HO-1 expression by NO is also verified in AH136B tumor-bearing rats *in vivo,* as revealed by suppression of HO-1 induction by treatment of the animals with an NOS inhibitor. Also, hypoxia causes HO-1 induction. Intraarterial administration of the HO inhibitor Zn-protoporphyrin IX strongly suppresses AH136B tumor growth *in vivo* (Doi *et al.,* 1999). On the basis of these findings, the upregulation of HO-1 is thought to have protective and beneficial effects for tumor cells against the antitumor actions of NO derived from the host as well as of many anticancer agents.

Concluding Remarks

The pathophysiological effects and consequences of NO produced in excess in inflammatory or infectious tissue are described in this chapter, with special emphasis on infections, cancer, and mutagenesis. The biological and biochemical activities of reactive nitrogen oxides such as peroxynitrite, via interplay of NO with superoxide and active oxygen species, are of paramount interest, because NO and its oxidized derivatives have diverse functions not only as inflammatory mediators but also as inter- and intracellular signaling molecules. Also, the nitrogen oxides produced via high-output NO may be involved in carcinogenesis as well as mutagenesis. NO-induced oxidative stress might affect the mutation and evolution of various pathogens directly, possibly through peroxynitrite formation. Improved understanding of NO-related molecular pathogenesis will help in the exploration of novel therapeutic strategies.

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References

Adamson, D. C., Kopnisky, K. L., Dawson, T. M., and Dawson, V. L. (1999). Mechanisms and structural determinants of HIV-1 coat protein, gp41-induced neurotoxicity. *J. Neurosci.* **19,** 64–71.

Adler, H., Beland, J. L., Del-Pan, N. C., Kobzik, L., Brewer, J. P., Martin, T. R., and Rimm, I. J. (1997). Suppression of herpes simplex virus type 1 (HSV-1)-induced pneumonia in mice by inhibition of inducible nitric oxide synthase (iNOS, NOS2). *J. Exp. Med.* **185,** 1533–1540.

Akaike, T., and Maeda, H. (1999). Nitric oxide in influenza. *In* ''Nitric Oxide in Infection'' (F. C. Fang, ed.), pp. 397–415. Kluwer Academic/Plenum, New York.

Akaike, T., Ando, M., Oda, T., Doi, T., Ijiri, S., Araki, S., and Maeda, H. (1990). Dependence on O_2^- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J. Clin. Invest.* **85,** 739–745.

Akaike, T., Sato, K., Ijiri, S., Miyamoto, Y., Kohno, M., Ando, M., and Maeda, H. (1992). Bactericidal activity of alkyl peroxyl radicals gener-

ated by heme-iron-catalyzed decomposition of organic peroxides. *Arch. Biochem. Biophys.* **294,** 38–43.

Akaike, T., Weihe, E., Schaefer, M., Fu, Z. F., Zheng, Y. M., Vogel, W., Schmidt, H., Koprowski, H., and Dietzschold, B. (1995). Effect of neurotropic virus infection on neuronal and inducible nitric oxide synthase activity in rat brain. *J. Neurovirol.* **1,** 118–125.

Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y. M., Dietzschold, B., and Maeda, H. (1996). Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2448–2453.

Akaike, T., Suga, M., and Maeda, H. (1998). Free radicals in viral pathogenesis: Molecular mechanisms involving superoxide and NO. *Proc. Soc. Exp. Biol. Med.* **217,** 64–73.

Akaike, T., Fujii, S., Kato, A., Yoshitake, J., Miyamoto, Y., Sawa, T., Okamoto, S., Suga, M., Asakawa, M., Nagai, Y., and Maeda, H. (2000). Viral mutation accelerated by nitric oxide production during infection *in vivo*. *FASEB J.* **14** (in press).

Baik, S. C., Youn, H. S., Chung, M. H., Lee, W. K., Cho, M. J., Ko, G. H., Park, C. K., Kasai, H., and Rhee, K. H. (1996). Increased oxidative DNA damage in *Helicobacter pylori*-infected human gastric mucosa. *Cancer Res.* **56,** 1279–1282.

Bastian, N. R., Yim, C. Y., Hibbs, J. B., Jr., and Samlowski, W. E. (1994). Induction of iron-derived EPR signals in murine cancers by nitric oxide. Evidence for multiple intracellular targets. *J. Biol. Chem.* **269,** 5127–5131.

Beck, M. A., Shi, Q., Morris, V. G., and Levander, O. A. (1995). Rapid genomic evolution of a non-virulent coxsackievirus B3 in selenium-deficient mice results in selection of identical virulent isolates. *Nat. Med.* **1,** 433–436.

Beck, M. A., Esworthy, R. S., Ho, Y.-S., and Chu, F.-F. (1998). Glutathione peroxidase protects mice from viral-induced myocarditis. *FASEB J.* **12,** 1143–1149.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Berton, G., and Gordon, S. (1983). Modulation of macrophage mannosyl-specific receptors by cultivation on immobilized zymosan. Effects on superoxide-anion release and phagocytosis. *Immunology* **49,** 705–715.

Bi, Z., Barna, M., Komatsu, T., and Reiss, C. S. (1995). Vesicular stomatitis virus infection of the central nervous system activates both innate and acquired immunity. *J. Virol.* **69,** 6466–6472.

Blackford, J. A., Antonini, J. J. M., Castranova, V., and Dey, R. D. (1994). Intratracheal instillation of silica up-regulates inducible nitric oxide synthase gene expression and increases nitric oxide production in alveolar macrophages and neutrophils. *Am. J. Respir. Cell. Mol. Biol.* **11,** 426–431.

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q., and Nathan, C. (1994). Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukocyte Biol.* **55,** 227–233.

Boven, L. A., Gomes, L., Hery, C., Gray, F., Verhof, J., Portegies, P., Tardieu, M., and Nottet, H. S. (1999). Increased peroxynitrite activity in AIDS dementia complex: Implication for the neuropathogenesis of HIV-1 infection. *J. Immunol.* **162,** 4319–4327.

Bukrinsky, M. I., Nottet, H. S. L. M., Schmidtmayerova, H., Dubrovsky, L., Flanagan, C. R., Mullins, M. E., Lipton, S. A., and Gendelman, H. E. (1995). Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HSV-1)-infected monocytes: Implications for HIV-associated neurological disease. *J. Exp. Med.* **181,** 735–745.

Byun, J., Henderson, J. P., Mueller, D. M., and Heinecke, J. W. (1999). 8-Nitro-2'-deoxyguanosine, a specific marker of oxidation by reactive nitrogen species, is generated by myeloperoxidase-hydrogen peroxide-nitrite system of activated human phagocytes. *Biochemistry* **38,** 2590–2600.

Campbell, I. L., Samimi, A., and Chiang, C.-S. (1994). Expression of the inducible nitric oxide synthase. Correlation with neuropathology and clinical features in mice with lymphocytic choriomeningitis. *J. Immunol.* **153,** 3622–3629.

Caygill, C. P., Hill, M. J., Braddick, M., and Sharp, J. C. (1994). Cancer mortality in chronic typhoid and paratyphoid carriers. *Lancet* **343,** 83–84.

Clancy, R. M., and Abramson, S. B. (1995). Nitric oxide: A novel mediator of inflammation. *Proc. Soc. Exp. Biol. Med.* **210,** 93–101.

Clark, R. C. (1990). The neutrophil NADPH oxidase system: Molecular aspects. *In* ''Microbial Determinants of Virulence and Host Response'' (E. M. Ayoub, G. H. Cassell, W. C. Branche, Jr., and T. J. Henry, eds.), pp. 253–269. American Society for Microbiology, Washington, D.C.

Corraliza, I. M., Soler, G., Eichmann, K., and Modolell, M. (1995). Arginase induction by suppression of nitric oxide synthesis (IL-4, IL-10 and PGE_2) in murine bone marrow-derived macrophages. *Biochem. Biophys. Res. Commun.* **206,** 667–673.

Croen, K. D. (1993). Evidence for an antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J. Clin. Invest.* **91,** 2446–2452.

Cunha, F. Q., Moncada, S., and Liew, F. Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. *Biochem. Biophys. Res. Commun.* **182,** 1155–1159.

Doi, T., Ando, M., Akaike, T., Suga, M., Sato, K., and Maeda, H. (1993). Resistance to nitric oxide in *Mycobacterium avium* complex and its implication in pathogenesis. *Infect. Immun.* **61,** 1980–1989.

Doi, K., Akaike, T., Horie, H., Noguchi, Y., Fujii, S., Beppu, T., Ogawa, M., and Maeda, H. (1996). Excessive production of nitric oxide in rat solid tumor and its implications in rapid tumor growth. *Cancer* **77**(Suppl.), 1598–1604.

Doi, K., Akaike, T., Fujii, S., Tanaka, S., Ikebe, N., Beppu, T., Shibahara, S., Ogawa, M., and Maeda, H. (1999). Induction of heme oxygenase-1 by nitric oxide and ischemia in experimental solid tumors and implications for tumor growth. *Br. J. Cancer* **80,** 1945–1954.

Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vilet, A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* **391,** 393–397.

Estévez, A. G., Radi, R., Barbeito, L., Shin, J. T., Thompson, J. A., and Beckman, J. S. (1995). Peroxynitrite-induced cytotoxicity in PC12 cells: Evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. *J. Neurochem.* **65,** 1543–1550.

Flory, C. M., Jones, M. L., and Warren, J. S. (1993). Pulmonary granuloma formation in the rat is partially dependent on monocyte chemoattractant protein 1. *Lab. Invest.* **69,** 396–404.

Fraenkel-Conrat, H. (1964). The genetic code of a virus. *Sci. Am.* **211,** 47–54.

Frears, E. R., Zhang, Z., Blake, D. R., O'Connell, J. P., and Winyard, P. G. (1996). Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Lett.* **381,** 21–24.

Fujii, S., Akaike, T., and Maeda, H. (1999). Role of nitric oxide in pathogenesis of herpes simplex virus encephalitis in rats. *Virology* **256,** 203–212.

Furchgott, R. F., and Vanhoutte, P. M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.* **3,** 2007–2018.

Goldman, A. I., Choudhury, M., da Silva, J. L., and Jiang, S. (1996). Quantitative measurement of heme oxygenase-1 in the human renal adenocarcinoma. *J. Cell. Biochem.* **63,** 342–348.

Gotoh, T., Sonoki, T., Nagasaki, A., Terada, K., Takiguchi, M., and Mori, M. (1996). Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. *FEBS Lett.* **395,** 119–122.

Granger, D. L., Hibbs, J. B., Jr., Perfect, J. R., and Durack, D. T. (1988). Specific amino acid (L-arginine) requirement for microbiostatic activity of murine macrophages. *J. Clin. Invest.* **81,** 1129–1136.

de Groote, M. A., Granger, D., Xu, Y., Campbell, G., Prince, R., and Fang, F. C. (1995). Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 6399–6403.

Halliwell, B., and Gutteridge, J. M. C. (1984). Oxygen toxicity, oxygen radicals, transition metals and diseases. *Biochem. J.* **219,** 1–14.

Hara, E., Takahashi, K., Tominaga, T., Kumabe, T., Kayama, T., Suzuki, H., Fujita, H., Yoshimoto, T., Shirato, K., and Shibahara, S. (1996). Expression of heme oxygenase and inducible nitric oxide synthase mRNA in human brain tumors. *Biochem. Biophys. Res. Commun.* **224,** 153–158.

Harris, C. C. (1991). Chemical and physical carcinogenesis: Advances and perspectives for the 1990s. *Cancer Res.* **51**(Suppl), 5023s–5044s.

Hibbs, J. B., Taintor, R. R., Varin, Z., and Rachlin, E. M. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94.

Honda, S., Fujioka, T., Tokieda, M., Satoh, R., Nishizono, A., and Nasu, M. (1998). Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian gerbils. *Cancer Res.* **58,** 4255–4259.

Hori, K., Burd, P. R., Furuke, K., Kutza, J., Weih, K. A., and Clouse, K. A. (1999). Human immunodeficiency virus-1-infected macrophages induce inducible nitric oxide synthase and nitric oxide (NO) production on astrocytes: Astrocytic NO as a possible mediator of neuronal damage in acquired immunodeficiency syndrome. *J. Immunol.* **93,** 1843–1850.

Hunninghake, G. W., Garrett, K. C., Richerson, H. B., Fantone, J. C., Ward, P. A., Rennard, S. I., Bitterman, P. B., and Crystal, R. G. (1984). Pathogenesis of the granulomatous lung diseases. *Am. Rev. Respir. Dis.* **130,** 476–496.

Ignarro, L. J. (1991). Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.* **41,** 485–490.

Inoue, S., and Kawanishi, S. (1995). Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Lett.* **371,** 86–88.

Ischiropoulos, H. (1998). Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356,** 1–11.

Iuvone, T., Carnuccio, R., and Rosa, M. D. (1994). Modulation of granuloma formation by endogenous nitric oxide. *Eur. J. Pharmacol.* **265,** 89–92.

James, S. L. (1995). Role of nitric oxide in parasitic infections. *Microbiol. Rev.* **59,** 533–547.

Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Baylis, S. A., Rhodes, P., Westmore, K., Emson, P. C., and Moncada, S. (1995). Role of nitric oxide in tumor growth. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4392–4396.

Jones, M. L., and Warren, J. S. (1992). Monocyte chemoattractant protein 1 in a rat model of pulmonary granulomatosis. *Lab. Invest.* **66,** 498–503.

Juedes, M. J., and Wogan, G. N. (1996). Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat. Res.* **349,** 51–61.

Kallinowski, F., Schlenger, K. H., Runkel, S., Kloes, M., Stohrer, M., Okunieff, P., and Vaupel, P. (1989). Blood flow, metabolism, cellular microenvironment, and growth rate of human tumor xenografts. *Cancer Res.* **49,** 3759–3764.

Karupiah, G., Xie, Q., Buller, R. M. L., Nathan, C., Duarte, C., and MacMicking, J. D. (1993). Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science* **261,** 1445–1448.

Karupiah, G., Chen, J. H., Mahalingam, S., Nathan, C. F., and MacMicking, J. D. (1998). Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J. Exp. Med.* **188,** 1541–1546.

Kasahara, K., Kobayashi, K., Shikama, Y., Yoneya, I., Soezima, K., Ide, H., and Takahashi T. (1988). Direct evidence for granuloma-inducing activity of interleukin-1. Induction of experimental pulmonary granuloma formation in mice by interleukin-1-coupled beads. *Am. J. Pathol.* **130,** 629–638.

Kasai, H., and Nishimura, S. (1984). Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res.* **12,** 2137–2145.

Kim, Y.-M., Bergonia, H., and Lancaster, J. R., Jr. (1995a). Nitrogen oxide-induced autoprotection in isolated rat hepatocytes. *FEBS Lett.* **374,** 228–232.

Kim, Y.-M., Bergonia, H. A., Müller, C., Pitt, B. R., Watkins, W. D., and Lancaster, J. R., Jr. (1995b). Loss and degradation of enzyme-bound heme induced by cellular nitric oxide synthesis. *J. Biol. Chem.* **270,** 5710–5713.

Koprowski, H., Zheng, Y. M., Heber-Katz, E., Fraser, N., Rorke, L., Fu, Z. F., Hanlon, C., and Dietzschold, B. (1993). *In vivo* expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3024–3027.

Kornellisse, R. F., Hoekman, K., Visser, J. J., Hop, W. C. J., Huijmans, J. G. M., van der Straaten, P. J. C., van der Heijden, A. J., Sukhai, R. N., Neijens, H. J., and de Groot, R. (1996). The role of nitric oxide in bacterial meningitis in children. *J. Infect. Dis.* **174,** 120–126.

Kreil, T. R., and Eibl, M. M. (1996). Nitric oxide and viral infection: No antiviral activity against a flavivirus *in vitro,* and evidence for contribution to pathogenesis in experimental infection *in vivo. Virology* **219,** 304–306.

Kristof, A. S., Goldberg, P., Laubach, V., and Hussain, S. N. (1998). Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am. J. Respir. Crit. Care Med.* **158,** 1883–1889.

Kubes, P. (1995). Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. *Microcirculation* **2,** 235–244.

Kuwahara, H., Miyamoto, Y., Akaike, T., Kuboto, T., Sawa, T., Okamoto, S., and Maeda, H. (2000). *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infect. Immun.* **68** (in press).

Lala, P. K., and Orucevic, A. (1998). Role of nitric oxide in tumor progression: Lessons from experimental tumors. *Cancer Metastasis Rev.* **17,** 91–106.

Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996). Peroxinitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15069–15074.

Lee, T. C., and Ho, I. C. (1994). Expression of heme oxygenase in arsenic-resistant human lung adenocarcinoma cells. *Cancer Res.* **54,** 1660–1664.

Leibovich, S. J., Polverini, P. J., Fong, T. W., Harlow, L. A., and Koch, A. E. (1994). Production of angiogenic activity by human monocytes requires an L-arginine/nitric oxide-synthase-dependent effector mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4190–4194.

Lepoivre, M., Flamn, J. M., Bobe, P., Lemaire, G., and Henry, Y. (1994). Quenching of the tyrosyl free radical of ribonucleotide reductase by nitric oxide. Relationship to cytostasis induced in tumor cells by cytotoxic macrophage. *J. Biol. Chem.* **269,** 21891–21897.

Liu, R. H., and Hotchkiss, J. H. (1995). Potential genotoxicity of chronically elevated nitric oxide: A review. *Mutat. Res.* **339,** 73–89.

Lugano, E. M., Dauber, J. H., and Daniele, R. P. (1982). Acute experimental silicosis. Lung morphology, histology, and macrophage chemotaxin secretion. *Am. J. Pathol.* **109,** 27–36.

Lukacs, N. W., Kunkel, S. L., Strieter, R. M., Warmington, K., and Chensue, S. W. (1993). The role of macrophage inflammatory protein 1α in *Schistosoma mansoni* egg-induced granulomatous inflammation. *J. Exp. Med.* **177,** 1551–1559.

Maeda, H., and Akaike, T. (1998). Nitric oxide and oxygen radicals in infection, inflammation and cancer. *Biochemistry (Moscow)* **63,** 854–865.

Maeda, H., Matsumura, Y., and Kato, H. (1988). Purification and identification of [hydroxypropyl^3]bradykinin in ascitic fluid from a patient with gastric cancer. *J. Biol. Chem.* **263,** 16051–16054.

Maeda, H., Noguchi, Y., Sato, K., and Akaike, T. (1994). Enhanced vascular permeability in solid tumor is mediated by nitric oxide and inhibited by both new nitric oxide scavenger and nitric oxide synthase inhibitor. *Jpn. J. Cancer Res.* **85,** 331–334.

Maeda, H., Akaike, T., Wu, J., Noguchi, Y., and Sakata, Y. (1996). Bradykinin and nitric oxide in infectious disease and cancer. *Immunopharmacology* **33,** 222–230.

Maeda, H., Sawa, T., Yubisui, T., and Akaike, T. (1999). Free radical generation from heterocyclic amines by cytochrome b_5 reductase in the presence of NADH. *Cancer Lett.* **143,** 117–121.

Maines, M. D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* **37,** 517–554.

Maines, M. D., and Kappas, A. (1974). Cobalt induction of hepatic heme oxygenase: With evidence that cytochrome P-450 is not essential for this enzyme activity. *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4293–4297.

Mannick, E. E., Bravo, L. E., Zarama, G., Realpe, J. L., Zhang, X. J., Ruiz, B., Fontham, E. T., Mera, R., Miller, M. J., and Correa, P. (1996). Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: Effect of antibiotics and antioxidants. *Cancer Res.* **56,** 3238–3243.

Matsumura, Y., Kimura, M., Yamamoto, T., and Maeda, H. (1988). Involvement of the kinin-generating cascade in enhanced vascular permeability in tumor tissue. *Jpn. J. Cancer Res.* **79,** 1327–1334.

Mikami, S., Kawashima, S., Kanazawa, K., Hirata, K., Katayama, Y., Hotta, H., Hayashi, Y., Ito, H., and Yokoyama, M. (1996). Expression of nitric oxide synthase in a murine model of viral myocarditis induced by coxsackievirus B3. *Biochem. Biophys. Res. Commun.* **220,** 983–989.

Miyamoto, Y., Akaike, T., Alam, M. S., Inoue, K., Hamamoto, T., Ikebe, N., Yoshitake, J., Okamoto, T., and Maeda, H. (2000a). Novel functions of human α_1-protease inhibitor after *S*-nitrosylation: Inhibition of cysteine protease and antibacterial activity. *Biochem. Biophys. Res. Commun.* **267,** 918–923.

Miyamoto, Y., Akaike, T., and Maeda, M. (2000b). *S*-Nitrosylated human α_1-protease inhibitor. *Biochem. Biophys. Acta* **1477,** 90–97.

Moncada, S., and Higgs, A. (1993). The L-arginine–nitric oxide pathway. *N. Engl. J. Med.* **329,** 2002–2012.

Moreno, J. J., and Pryor, W. (1992). Inactivation of α_1-proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol.* **5,** 425–431.

Nakano, S., Matsukado, K., and Black, K. L. (1996). Increased brain tumor microvessel permeability after intracarotid bradykinin infusion is mediated by nitric oxide. *Cancer Res.* **56,** 4027–4031.

Nathan, C. F. (1997). Inducible nitric oxide synthase: What difference does it make? *J. Clin. Invest.* **100,** 2417–2423.

Nathan, C. F., and Hibbs, J. B. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* **3,** 65–70.

Ohshima, H., and Bartsch, H. (1994). Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mutat. Res.* **305,** 253–264.

Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M., Ichimori, K., and Maeda, H. (1997a). Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: A novel mechanism of procollagenase activation involving nitric oxide. *Arch. Biochem. Biophys.* **342,** 261–274.

Okamoto, T., Akaike, T., Suga, M., Tanase, S., Horie, H., Miyajima, S., Ando, M., Ichinose, Y., and Maeda, H. (1997b). Activation of human matrix metalloproteinases by various bacterial proteinases. *J. Biol. Chem.* **272,** 6059–6066.

Orrell, J. M., Brett, S. J., Ivanyi, J., Coghill, G., Grant, A., and Beck, J. S. (1992). Morphometric analysis of *Mycobacterium tuberculosis* infection in mice suggests a genetic influence on the generation of the granulomatous inflammatory response. *J. Pathol.* **166,** 77–82.

Palmer, R. M., Ashton, D. S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333,** 664–666.

Pignatelli, B., Bancel, B., Esteve, J., Malaveille, C., Calmels, S., Correa, P., Patricot, L. M., Laval, M., Lyandrat, N., and Ohshima, H. (1998). Inducible nitric oxide synthase, anti-oxidant enzymes and *Helicobacter pylori* infection in gastritis and gastric precancerous lesions in humans. *Eur. J. Cancer Prev.* **7,** 439–447.

Prabhakar, N. R., Dinerman, J. L., Agani, F. H., and Snyder, S. H. (1997). Carbon monoxide: A role in carotid body chemoreception. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1994–1997.

Reiser, K. M., Hesterberg, T. W., Haschek, W. M., and Last, J. A. (1982). Experimental silicosis. I. Acute effects of intratracheally instilled quartz on collagen metabolism and morphologic characteristics of rat lungs. *Am. J. Pathol.* **107,** 176–185.

Rubbo, H., Darley-Usmar, V., and Freeman, B. A. (1996). Nitric oxide regulation of tissue free radical injury. *Chem. Res. Toxicol.* **9,** 809–820.

Sato, K., Akaike, T., Kojima, Y., Ando, M., Nagao, M., and Maeda, H. (1992). Evidence of direct generation of oxygen radicals from heterocyclic amines by NADPH/cytochrome P-450 reductase *in vitro*. *Jpn. J. Cancer Res.* **83,** 1204–1209.

Sawa, T., Akaike, T., Kida, K., Fukushima, Y., Takagi, K., and Maeda, H. (1998). Lipid peroxyl radicals from oxidized oils and heme-iron: Implication of a high-fat diet in colon carcinogenesis. *Cancer Epidemiol. Biomarkers Prev.* **7,** 1007–1012.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219,** 983–985.

Setoguchi, K., Takeya, M., Akaike, T., Suga, M., Hattori, R., Maeda, H., Ando, M., and Takahashi, K. (1996). Expression of inducible nitric oxide synthase and its involvement in pulmonary granulomatous inflammation in rats. *Am. J. Pathol.* **149,** 2005–2022.

Shellito, J., and Sniezek, M. (1990). Altered release of eicosanoids by rat alveolar macrophages during granulomatous pulmonary inflammation. *Am. J. Respir. Cell Mol. Biol.* **2,** 289–296.

Shibahara, S., Müller, R., Taguchi, H., and Yoshida, T. (1985). Cloning and expression of cDNA for rat heme oxygenase. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7865–7869.

Sonoki, T., Nagasaki, A., Gotoh, T., Takiguchi, M., Takeya, M., Matsuzaki, H., and Mori, M. (1997). Coinduction of nitric oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues *in vivo* by lipopolysaccharide. *J. Biol. Chem.* **272,** 3689–3693.

Spencer, J. P., Wong, J., Jenner, A., Aruoma, O. I., Cross, C. E., and Halliwell, B. (1996). Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem. Res. Toxicol.* **9,** 1152–1158.

Stuehr, D. J., and Griffith, O. W. (1992). Mammalian nitric oxide synthase. *Adv. Enzymol. Relat. Mol. Biol.* **65,** 287–346.

Suematsu, M., Goda, N., Sano, T., Kashiwagi, S., Egawa, T., Shinoda, Y., and Ishimura, Y. (1995). Carbon monoxide: An endogenous modulator of sinusoidal tone in the perfused rat liver. *J. Clin. Invest.* **96,** 2431–2437.

Sugiura, H., Ichinose, M., Oyake, T., Mashito, Y., Ohuchi, Y., Endoh, N., Miura, M., Yamagata, S., Koarai, A., Akaike, T., Maeda, H., and Shirato, K. (1999). Role of peroxynitrite in airway microvascular hyperpermeability during late allergic phase in guinea pig. *Am. J. Respir. Crit. Care Med.* **160,** 663–671.

Sugiyama, A., Maruta, F., Ikeno, T., Ishida, K., Kawasaki, S., Katsuyama, T., Shimizu, N., and Tatematsu, M. (1998). *Helicobacter pylori* infection enhances *N*-methyl-*N*-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. *Cancer Res.* **58,** 2067–2069.

Suzuki, K., Hayashi, N., Miyamoto, M., Yamamoto, M., Ohkawa, K., Ito, Y., Sasaki, Y., Yamaguchi, Y., Nakase, H., Noda, K., Enomoto, N., Arai, K., Yamada, Y., Yoshihara, H., Tsujimura, T., Kawano, K., Yoshikawa, K., and Kamada, T. (1996). Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res.* **56,** 3004–3009.

Szabó, C., and Ohshima, H. (1997). DNA damage induced by peroxynitrite: Subsequent biological effects. *Nitric Oxide* **1,** 373–385.

Takahashi, K., Hara, E., Suzuki, H., Sasano, H., and Shibahara, S. (1996). Expression of heme oxygenase isozyme mRNAs in the human brain and induction of heme oxygenase-1 by nitric oxide donors. *J. Neurochem.* **67,** 482–489.

Tauber, A. I., Borrengaard, N., Simons, E., and Wright, J. (1983). Chronic granulomatous disease: A syndrome of phagocyte oxidase deficiencies. *Medicine* **62,** 286–309.

Tenhunen, R., Marver, H. S., and Schmid, R. (1968). The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Natl. Acad. Sci. U.S.A.* **61,** 748–755.

Thomsen, L. L., and Miles, D. W. (1998). Role of nitric oxide in tumour progression: Lessons from human tumours. *Cancer Metastasis Rev.* **17,** 107–118.

Thomsen, L. L., Scott, J. M. J., Topley, P., Knowles, R. G., Keerie, A.-J., and Frend, A. J. (1997). Selective inhibition of inducible nitric oxide synthase inhibits tumor growth in vivo: Studies with 1400W, a novel inhibitor. *Cancer Res.* **57,** 3300–3304.

Tozer, G. M., Prise, V. E., and Chaplin, D. J. (1997). Inhibition of nitric oxide synthase induces a selective reduction in tumor blood flow that is reversible with L-arginine. *Cancer Res.* **57,** 948–955.

Tsuji, M., Dimov, V. B., and Yoshida, T. (1995). In vivo expression of monokine and inducible nitric oxide synthase in experimentally induced pulmonary granulomatous inflammation. Evidence for sequential production of interleukin-1, inducible nitric oxide synthase, and tumor necrosis factor. *Am. J. Pathol.* **147,** 1001–1015.

Umezawa, K., Ohnishi, N., Tanaka, K., Kamiya, S., Koga, Y., Nakazawa, H., and Ozawa, A. (1995). Granulation in livers of mice infected with *Salmonella typhimurium* is caused by superoxide released from host phagocytes. *Infect. Immun.* **63,** 4402–4408.

Umezawa, K., Akaike, T., Fujii, S., Suga, M., Setoguchi, K., Ozawa, A., and Maeda, H. (1997). Induction of nitric oxide synthesis and xanthine oxidase and their role in the antimicrobial mechanism against *Salmonella typhimurium* in mice. *Infect. Immun.* **65,** 2932–2940.

Vallyathan, V., Shi, X. L., Dalal, N. S., Irr, W., and Castranova, V. (1988). Generation of free radicals from freshly fractured silica dust. *Am. Rev. Respir. Dis.* **138,** 1213–1219.

Van Oud Albas, A. B., van der Linden-Schrever, B., and van Furth, R. (1983). Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intra-alveolar administration of aerosolized heat-killed BCG. *Am. Rev. Respir. Dis.* **128,** 276–284.

Vaupel, P., Kallinowski, F., and Okunieff, P. (1989). Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. *Cancer Res.* **49,** 6449–6465.

Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q., and Nathan, C. (1993). Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. *J. Exp. Med.* **178,** 605–613.

Vuillaume, M. (1987). Reduced oxygen species, mutation, induction and cancer initiation. *Mutat. Res.* **186,** 43–72.

Weitzman, S. A., and Stossel, T. P. (1981). Mutation caused by human phagocytes. *Science* **212,** 546–547.

Whiteman, M., Tritschler, H., and Halliwell, B. (1996). Protection against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation by oxidized and reduced lipoic acid. *FEBS Lett.* **379,** 74–76.

Wright, P. F. (1997). Respiratory diseases. *In* ''Viral Pathogenesis'' (N. Nathanson, R. Ahmed, F. Gonzalez-Scarano, D. E. Griffin, K. V. Holmes, F. A. Murphy, and H. L. Robinson, eds.), pp. 703–711. Lippincott-Raven, Philadelphia, Pennsylvania.

Wu, J., Akaike, T., and Maeda, H. (1998). Modulation of enhanced vascular permeability in tumor by a bradykinin antagonist, a cyclooxygenase inhibitor, and an NO scavenger. *Cancer Res.* **58,** 159–165.

Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995a). Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. *FEBS Lett.* **376,** 207–210.

Yermilov, V., Rubio, J., and Ohshima, H. (1995b). Formation of 8-nitroguanine in DNA treated with peroxynitrite *in vitro* and its rapid removal from DNA by depurination. *Carcinogenesis* **16,** 2045–2050.

Yet, S.-F., Pellacani, A., Patterson, C., Tan, L, Folta, S. C., Foster, L., Lee, W.-S., Hsieh, C.-M., and Perella, M. A. (1997). Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *J. Biol. Chem.* **272,** 4295–4301.

Yoshida, K., Akaike, T., Doi, T., Sato, K., Ijiri, S., Suga, M., Ando, M., and Maeda, H. (1993). Pronounced enhancement of ·NO-dependent antimicrobial action by an ·NO-oxidizing agent, imidazolineoxyl *N*-oxide. *Infect. Immun.* **61,** 3552–3555.

Yuspa, S. H., and Poirier, M. C. (1988). Chemical carcinogenesis: From animal models to molecular models in one decade. *Adv. Cancer Res.* **50,** 25–70.

Zheng, Y. M., Martin, K.-H., Schäfer, M. K. H., Weihe, E., Sheng, H., Corisdeo, S., Fu, Z. F., Koprowski, H., and Dietzschold, B. (1993). Severity of neurological signs and degree of inflammatory lesions in the brains of the rats with Borna disease correlate with the induction of nitric oxide synthase. *J. Virol.* **67,** 5786–5791.

Zhuang, J. C., Lin, C., Lin, D., and Wogan, G. N. (1998). Mutagenesis associated with nitric oxide production in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 8286–8291.

Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H.-T., Donnini, S., Granger, H. J., and Bicknell, R. (1997). Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J. Clin. Invest.* **99,** 2625–2634.

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CHAPTER 47

Nitric Oxide and Septic Shock

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NITRIC OXIDE (NO) IS GENERATED BY THREE DIFFERENT ISOFORMS OF NO SYNTHASE (NOS). TWO ISOFORMS ARE EXPRESSED CONSTITUTIVELY (IN ENDOTHELIUM, eNOS; IN BRAIN, nNOS), WHEREAS ONE IS INDUCED (iNOS) BY ENDOTOXIN OR CYTOKINES. EXPRESSION OF iNOS IN MANY ORGANS AND TISSUES IN SEPTIC SHOCK (CAUSED BY GRAM-NEGATIVE OR GRAM-POSITIVE BACTERIA) RESULTS IN AN ENHANCED FORMATION OF NO, WHICH CONTRIBUTES TO HYPOTENSION, VASCULAR HYPOREACTIVITY TO VASOCONSTRICTORS, AND POSSIBLY ORGAN INJURY/DYSFUNCTION AND HOST DEFENSE. INHIBITION OF THE EXPRESSION OF iNOS PROTEIN WITH, FOR EXAMPLE, DEXAMETHASONE, INHIBITORS OF THE ACTIVATION OF PROTEIN TYROSINE KINASES, OR INHIBITORS OF THE TRANSCRIPTION FACTOR NF-κB, REDUCES THE CIRCULATORY AND MULTIPLE ORGAN FAILURE CAUSED BY ENDOTOXIN IN ANIMALS. ALTHOUGH ALL OF THESE INTERVENTIONS PREVENT THE EXPRESSION OF iNOS, THEY NEED TO BE GIVEN AS A PRETREATMENT (PRIOR TO THE ONSET OF SHOCK), AND THEY EXERT MANY OTHER EFFECTS (PREVENTION OF THE EXPRESSION OF OTHER PROTEINS) AND/OR SIDE EFFECTS. THIS CHAPTER REVIEWS THE EFFECTS AND SIDE EFFECTS OF VARIOUS INHIBITORS OF THE ACTIVITY OF NOS, WHICH CAN BE ADMINISTERED AT A STAGE WHEN HYPOTENSION HAS ALREADY DEVELOPED. THERE IS NOW GOOD EVIDENCE THAT INHIBITION OF iNOS ACTIVITY REDUCES THE HYPOTENSION CAUSED BY ENDOTOXIN OR SEPTIC SHOCK IN ANIMALS AND HUMANS. IN CONTRAST, INHIBITION OF eNOS ACTIVITY MAY LEAD TO EXCESSIVE VASOCONSTRICTION (ADVERSE EFFECTS). THERE IS LIMITED EVIDENCE REGARDING THE DEGREE OF iNOS INDUCTION IN CELLS/TISSUES OF HUMANS WITH SEPTIC SHOCK. DATA FROM CLINICAL TRIALS (PHASE III) INDICATE THAT NONSELECTIVE INHIBITORS OF NOS ACTIVITY [E.G., N^G-MONOMETHYL-L-ARGININE (L-NMMA)] DO NOT REDUCE MORTALITY IN PATIENTS WITH SEPTIC SHOCK.

An Introduction to the Pathophysiology and Therapy of Septic Shock

Septic shock is the life-threatening complication of an overwhelming systemic infection in which the immune system releases inflammatory mediators, resulting in pathophysiological vasodilation, hematological abnormalities, and organ dysfunction and failure. Sepsis affects 300,000–500,000 patients annually in the United States (Parrillo, 1989). The prevalence of sepsis in hospitalized patients has significantly increased since the 1980s. Data from the National Hospital Discharge Survey of the Centers for Disease Control (1977–1987) show an increase in the discharge diagnosis of sepsis of 139% (*Morbidity and Mortality Weekly Report,* 1988). The increase was especially marked in patients over 65 years of age (162%). Despite improvements in intensive care management of critically ill patients, new antibiotics, and extensive research into the pathophysiology of sepsis, the mortality of septic shock still ranges from 20 to 55% (Dunn, 1987; Young, 1990). Most notably, mortality

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increases to over 70% when shock is associated with multiple organ failure (Parker and Parrillo, 1983; Sprung *et al.,* 1984).

The first clinical presentation of sepsis often consists of fever, tachycardia, peripheral vasodilation, hypotension, and oliguria. However, the key symptom of shock is a severe fall in blood pressure, which is often associated with the dysfunction or failure of several important organs, including lung, kidney, liver, and brain. Despite the observed increase in cardiac output, blood pressure is not maintained because of excessive vasodilation. Treatment of septic shock includes respiratory support to optimize tissue oxygenation, intravenous fluid administration, broad-spectrum antimicrobial therapy, and vasopressor support.

The definition of septic shock is independent of the presence or absence of a multiple organ dysfunction syndrome (MODS), which is defined as impaired organ function such that homeostasis cannot be maintained without intervention (Baue, 1993). Primary MODS is a direct result of a well-defined insult to a specific organ. Secondary MODS occurs as a consequence of an exaggerated host response, termed systemic inflammatory response syndrome (SIRS). Approximately 75% of deaths from septic shock occur within hours to days after the onset of shock and are due to therapy-resistant hypotension, leading to the conclusion that peripheral vascular failure is the predominant factor determining outcome (Groeneveld *et al.,* 1988). The rest of the deaths occur days or weeks after the patient has recovered from hypotension, and the cause of death is multiple organ failure (Dal Nogare, 1991). Adult respiratory distress syndrome (ARDS), followed by renal and hepatic failure, is the most common sequence of events.

Septic shock is primarily initiated by components of the cell wall of gram-positive or gram-negative bacteria (Rietschel and Brade, 1992), but structural components of many other microorganisms generate a very similar biological response when they enter the circulation. Gram-positive organisms do not contain endotoxin (lipopolysaccharide or LPS), which is the cell wall component of gram-negative bacteria responsible for the initiation of septic shock. The cell wall of gram-positive bacteria contains lipoteichoic acid (LTA) and peptidoglycan (PepG). LTA is a macroamphiphile, containing a substituted poly(glycerophosphate) backbone attached to a glycolipid. PepG is a large polymer, which provides stress resistance and shape-determining properties to bacterial cell walls (Springer, 1990). LTA and PepG act in synergy to release tumor necrosis factor α (TNF-α) and γ-interferon, to induce iNOS (inducible nitric oxide synthase), and to cause shock and multiple organ failure in anesthetized rats (DeKimpe *et al.,* 1995). Interestingly, a specific fragment of PepG, namely, NAG-NAM-L-Ala-D-isoglutamine, is the moiety within the PepG polymer that acts synergistically with LTA to induce NO formation in macrophages (Kengatharan *et al.,* 1998). This moiety is also present in the PepG of the nonpathogenic bacterium *Bacillus subtilis.* Interestingly, LTA from *Staphylococcus aureus* but not that from *B. subtilis* works in synergy with PepG from either bacterium. Thus, it has been proposed (Kengatharan *et al.,* 1998) that it is the structure of LTA which determines pathogenicity, whereas PepG amplifies the response to LTA.

Cytokines and Septic Shock

Cytokines are a heterogeneous group of hormonelike proteins, produced by all organs and many cell types, that establish a communication network between various cells of each organ. Activation of the cytokine network follows a lag phase and is preceded by the activation of, for example, the complement and kallikrein system. The study and the understanding of the cytokine network in septic shock is complicated by the following factors: (1) cytokines often induce the secretion of additional cytokines; (2) cytokines modulate each other's actions, resulting in additional, synergistic, or inhibitory effects, or even a novel effect not seen with individual cytokines alone; (3) the sequence of cytokine exposure can influence the response of a specific target cell; and (4) the effects of cytokines may be dose-related, with quantitatively different biological effects seen at different doses (Deitsch, 1992).

The proinflammatory cytokines TNF-α and interleukin-1β (IL-1β) have been implicated in the pathophysiology of many cardiovascular disorders, including circulatory shock (Okusawa *et al.,* 1988; Tracy, 1991; Billiau and Vandekerckhove, 1991; Mozes *et al.,* 1991; Hewett *et al.,* 1993; Dinarello, 1996). Administration of TNF-α alone or in combination with low doses of endotoxin mimics several features of the pathophysiology of circulatory shock, including hypotension and organ injury (Tracy, 1991; Billiau and Vandekerckhove, 1991). Intravenous administration of IL-1, either alone or in combination with low doses of LPS or TNF-α, also produces a shocklike state (Okusawa *et al.,* 1988). Pronounced rises in the serum levels of TNF-α and IL-1β occur in experimental endotoxemia (Beutler *et al.,* 1985) and in humans with sepsis and septic shock (Friedland *et al.,* 1996; Stuber *et al.,* 1996). Higher concentrations of TNF-α and IL-1β are associated with an increase in the risk for ARDS, MODS, and death (Roumen *et al.,* 1993). Antibodies directed against TNF-α or IL-1β exert protective effects in various animal models of endotoxin shock (Tracy *et al.,* 1987; Wakabayashi *et al.,* 1991).

Nitric Oxide and Septic Shock

Discovery of the Role of Nitric Oxide in the Pathophysiology of Shock

In July of 1990, Thiemermann and Vane reported that the acute hypotension caused by injection of endotoxin into the rat was attenuated by intravenous infusion of the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) (1 mg/kg/min i.v. for 20 min). This beneficial effect of L-NMMA was reversed by L-arginine (6 mg/kg/min

i.v. for 20 min). One could argue that the increase in blood pressure [due to inhibition of endothelial NOS (eNOS) activity] afforded by infusion of L-NMMA may be sufficient to cause a functional antagonism of any hypotensive effect of endotoxin. This is, however, unlikely, as an infusion of the α_1-receptor agonist phenylephrine (in a dose that caused a degree of hypotension similar to that produced by L-NMMA) did not affect the fall in blood pressure caused by endotoxin in the anesthetized rat. The authors, therefore, proposed that a stimulation of the formation of NO contributes to the endotoxin-induced hypotension *in vivo* (Thiemermann and Vane, 1990). In November of the same year, Kilbourn and colleagues (1990) reported that a single injection of L-NMMA (20 mg/kg i.v. at 1.5 min after endotoxin) reversed the acute fall in blood pressure and peripheral vascular resistance caused by injection of endotoxin in the anesthetized dog. Although L-NMMA caused a pressor effect in dogs without endotoxemia, the increase in blood pressure afforded by the NOS inhibitor in dogs with endotoxemia was significantly greater than in the respective control animals. In addition, L-NMMA did not reduce the hypotension caused by an organic nitrate (glyceryl trinitrate). These findings again suggested that endotoxin stimulates the formation of NO, which in turn contributes to the hypotension afforded by this wall fragment of gram-negative bacteria (Kilbourn *et al.,* 1990). Taken together, these two studies suggested that inhibitors of NOS activity may be useful in the therapy of the severe hypotension in septic shock.

One key feature of the severe hypotension in septic shock was the, then unexplained, phenomenon that this hypotension is difficult to overcome by infusion of vasopressor agents such as norepinephrine. Although this ''vascular hyporeactivity'' or ''vasoplegia'' had been known for a long time, the underlying pathology was unknown. In a series of elegant studies published between September and November of 1990, the group of Dr. Stoclet (Strassbourg, France) reported that the vascular hyporeactivity caused by endotoxin *in vitro* and *in vivo* was also secondary to and enhanced formation of NO. For instance, incubation of (endothelium-denuded) rat aortic rings for 5 hours with endotoxin attenuated the vasoconstrictor response elicited by norepinephrine. Inhibition of the activities of NOS and guanylate cyclase with L-NMMA and methylene blue restored the contractile responses to norepinephrine. The observed effect of L-NMMA was reversed by L-arginine but not by D-arginine. Thus, the hyporeactivity to norepinephrine caused by endotoxin in vascular smooth muscle is secondary to activation both of the L-arginine pathway and of soluble guanylate cyclase (Fleming *et al.,* 1990). Similarly, the vascular hyporeactivity to calcium was also attenuated by L-NMMA in an L-arginine-reversible fashion (Gray *et al.,* 1990). Most notably, L-NMMA restored the pressor responses to norepinephrine in rats subjected to endotoxemia *in vivo* (Julou-Schaeffer *et al.,* 1990). The impairment of the contractile responses elicited by phenylephrine in rat aortic rings obtained from rats subjected to endotoxemia was attenuated by NOS inhibitors, cycloheximide, and glucocorticoids, suggesting that the observed overproduction of NO is secondary to the induction of iNOS in the vasculature (Rees *et al.,* 1990). An enhanced formation of NO by eNOS (endothelium) and iNOS (vascular smooth muscle) contributes to the early and late hypotension (Szabo *et al.,* 1993) as well as the vascular hyporeactivity to vasoconstrictor agents caused by endotoxin *in vivo.* We now know that the circulatory failure associated with the administration of TNF-α (Kilbourn *et al.,* 1990) and IL-2 (Kilbourn *et al.,* 1994) and with hemorrhagic shock (Thiemermann *et al.,* 1993), gram-positive shock (De Kimpe *et al.,* 1995; Kengatharan *et al.,* 1998), and traumatic shock (Szabo and Thiemermann, 1995) is, at least in part, due to an enhanced formation of NO by iNOS. Taken together, these studies form the basis of the hypothesis developed between 1990 and 1993 that agents which reduce the formation of NO (particularly by iNOS) may be useful to overcome the ''therapy-refractory'' circulatory failure observed in patients with shock of different etiologies. This conclusion is supported by the discovery that mice deficient in iNOS (iNOS knockout mice) exhibit only a minor fall in blood pressure when challenged with endotoxin (MacMicking *et al.,* 1995; Wei *et al.,* 1995). Most of the results published between 1990 and 1995 supported the view that reducing the enhanced formation of NO (e.g., by iNOS) may become a useful therapeutic approach in sepsis/septic shock. In principle, there are two approaches to achieve this goal, namely, inhibition of iNOS induction and/or inhibition of the activity of iNOS by inhibiting the enzyme itself or one of its cofactors.

Inhibition of the Induction of iNOS in Experimental Endotoxemia

The mechanism of iNOS induction is not fully understood. It clearly involves the transcription of mRNA and novel protein biosynthesis. Sequencing of the DNA regions upstream of the NOS gene (i.e., the promoter region) revealed separate promoter regions for the induction of iNOS by LPS and γ-interferon (Xie *et al.,* 1993). There is increasing evidence for the involvement of the nuclear transcription factor NF-κB (Sherman *et al.,* 1993; Mulsch *et al.,* 1993; Griscavage *et al.,* 1995), tyrosine kinase activation (Dong *et al.,* 1993; Marczin *et al.,* 1993; Kengatharan *et al.,* 1996), microtubule depolymerization (Marczin *et al.,* 1993), and protein kinase Cϵ (Diaz-Guerra *et al.,* 1996) in the induction process (Fig. 1).

Induction of iNOS can be inhibited by numerous agents including glucocorticoids (Radomski *et al.,* 1990), thrombin (Schini *et al.,* 1992), ethanol (Militante *et al.,* 1997), as well as macrophage deactivation factor and transforming growth factor β, platelet-derived growth factor, endothelin-1, IL-4, IL-8, IL-10, and IL-13 (Green and Nacy, 1993; Schneemann *et al.,* 1993; Doyle *et al.,* 1994; Hirahashi *et al.,* 1996; Saura *et al.,* 1996). Inhibitors of protein kinase C (Okuda *et al.,* 1997), inhibitors of protein tyrosine kinase (Kengatharan *et al.,* 1996; Salzman *et al.,* 1996; Joly *et al.,* 1997), or inhibitors of the activation of NF-κB (Xie *et al.,* 1994; Griscavage *et al.,* 1995; Kengatharan *et al.,* 1996; Schini-Kerth *et al.,*

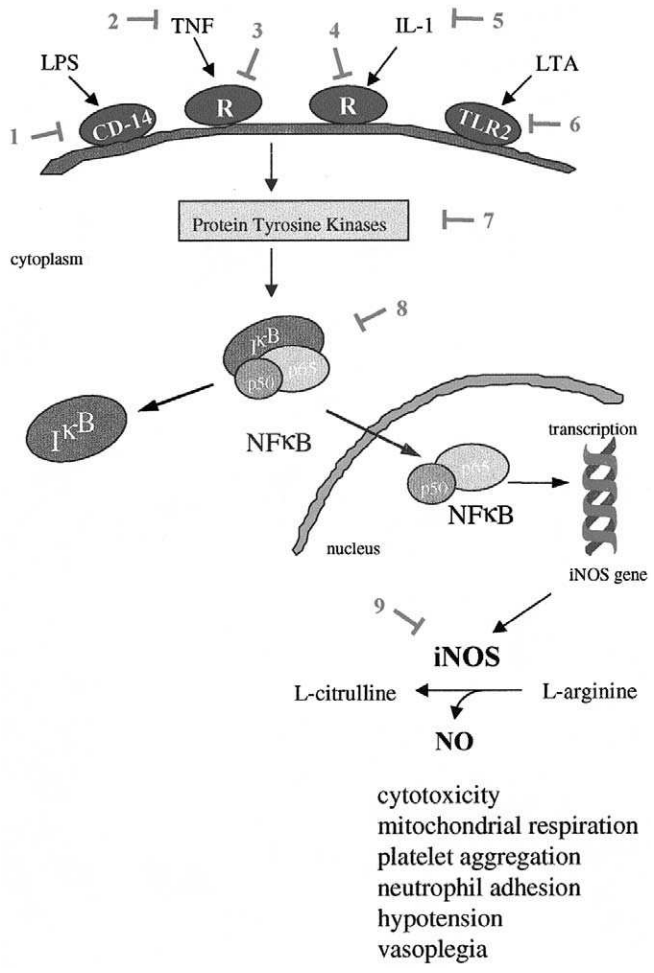


Figure 1 Hypothetical signal transduction pathway leading to the induction of iNOS and possibilities for reduction of NO formation. Possibilities to reduce NO formation include the following: 1, CD-14 antibody; 2, tumor necrosis factor-α (TNF) antibody; 3, TNFα receptor antagonist; 4, interleukin-1β (IL-1β) receptor antagonist; 5, IL-1β antibody; 6, toll-like receptor 2 (TLR2) receptor antagonist; 7, protein tyrosine kinase inhibitors (e.g., genistein, tyrphostins); 8, inhibitors of the activation of the nuclear transcription factor NF-κB [e.g., pyrrolidine dithiocarbamate (PDTC), dexamethasone, calpain inhibitor-1]; 9, inhibitors of iNOS activity [e.g., N^G-methyl-L-arginine (L-NMMA), aminoguanidine, aminoethyl-isothiourea (AE-ITU)].

1997) can also inhibit the induction of iNOS. An increase in cAMP induces iNOS in vascular smooth muscle cells and rat renal mesangial cells (Koide *et al.,* 1993; Kunz *et al.,* 1996), whereas prolonged elevation of intracellular cAMP levels in macrophages inhibits iNOS induction (Bulut *et al.,* 1993). NO itself can also regulate its activity, both by inhibiting iNOS activity (Assreuy *et al.,* 1993) and by downregulating iNOS mRNA (Nussler and Billiar, 1993). It should be noted that some of the above interventions (e.g., inhibitors of the activities of protein tyrosine kinases and of NF-κB) will also prevent the expression of many other genes. Thus, it is impossible to determine if, and to what extent, the reported beneficial effects of tyrosine kinase inhibitors (Novogrodsky *et al.,* 1994; Vanichkin *et al.,* 1996; Ruetten and Thiemermann, 1997a), IL-10 (Gerard *et al.,* 1997), IL-13 (Muchamuel *et al.,* 1997; Nicoletti *et al.,* 1997), and dexamethasone (Wright *et al.,* 1992; Ruetten and Thiemermann, 1997a) and of inhibitors of NF-κB (Ruetten and Thiemermann, 1997b; Liu *et al.,* 1999) in animal models of shock are due to the prevention of iNOS induction (Figs. 2 and 3). The molecular mechanism by which dexamethasone exerts beneficial effects in endotoxin shock is not well understood, but there is evidence that glucocorticoids inhibit the action of the transcription factors AP-1 (activator protein 1) and NF-κB (Barnes and Karin, 1997). For instance, a protein–protein interaction between the activated glucocorticoid receptor and NF-κB, resulting in prevention of its binding to the κB consensus motif on the promoter of its target genes, has been reported. In addition, glucocorticoids enhance the formation of IκBα, which results in an excess of this inhibitory factor in the nucleus and cytosol. Thus, activated NF-κB, when ''traveling'' to the nucleus, meets with and binds to IκB to form its ''dormant'' (inactive) cytosolic form (for a detailed review, see Barnes and Karin, 1997). Agents that selectively prevent the expression of iNOS or result in the expression of an inactive protein are currently being developed.

It should be stressed that all of the above interventions must be administered prior to the application of endotoxin or at least prior to the induction of iNOS to exert beneficial effects in animal models of shock. For instance, the administration of dexamethasone, calpain inhibitor I, or tyrosine kinase inhibitors to rats at 2 hours after the injection of endotoxin neither exerts beneficial effects on hemodynamic or organ injury nor inhibits the induction of iNOS (Wright *et al.,* 1992; Paya *et al.,* 1993; Ruetten and Thiemermann, 1997a, b) (Figs. 2 and 3). Similar to the clinical administration of antibodies against proinflammatory cytokines, the timely administration of inhibitors of the induction of iNOS will be crucial to achieve beneficial effects in patients with severe sepsis. Practically, one needs to determine the time between the induction of iNOS (or early phases of the syndrome) and the administration of the inhibitors of iNOS induction. This supports the view that drugs that directly inhibit iNOS activity are more likely to exert beneficial effects in the therapy of septic shock than agents that inhibit the induction of iNOS. The latter compounds may, however, be used in chronic conditions (e.g., of an inflammatory nature) in which iNOS has been reported to play a role.

Inhibition of NOS Activity: Effects and Side Effects in Animals with Septic Shock

Although there is good evidence that endotoxemia or sepsis in rodents results in the induction of iNOS (in various tissues), leading to an increase in the plasma levels of nitrite/nitrate (from 20 up to 600 μ*M*), there is limited information regarding the time course of iNOS induction, the degree of iNOS activity (in tissues), or even the plasma levels of nitrite/nitrate in large animal models (pig, dog, sheep, baboon) of shock or in humans with sepsis and septic shock. Clearly, sepsis (or endotoxemia) results in an increase in the plasma

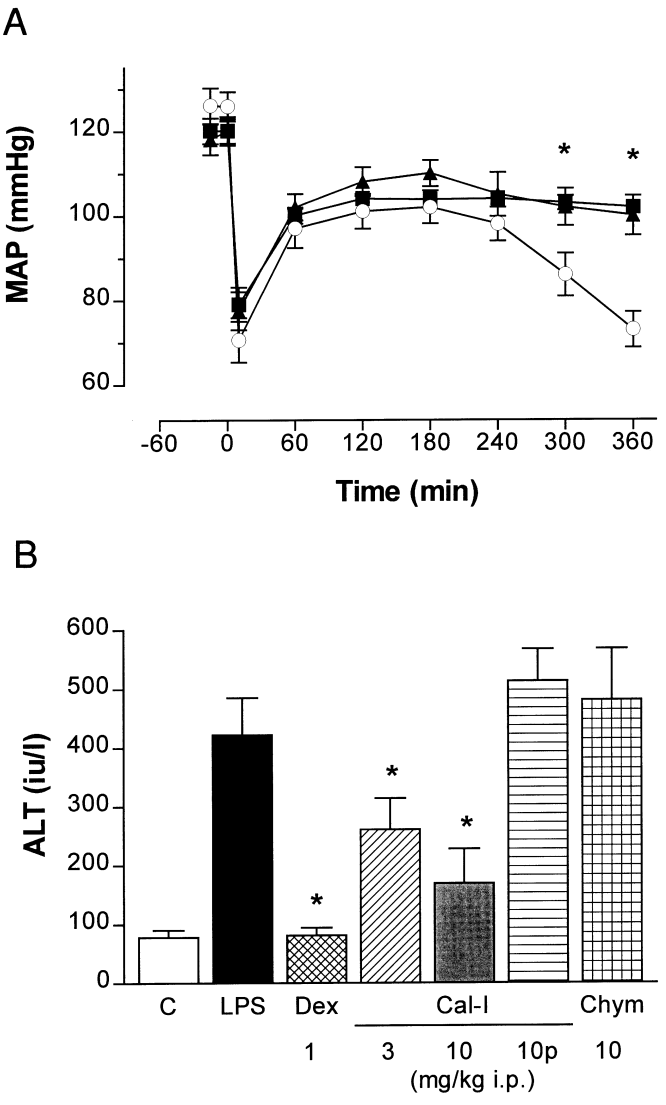


Figure 2 Calpain inhibitor I or dexamethasone prevent (A) the delayed circulatory failure (fall in mean arterial blood pressure; MAP) and (B) liver failure (increase in alanine aminotransferase; ALT) in rats with septic shock. Different groups of animals received vehicle for *Escherichia coli* lipopolysaccharide (LPS) (C, $n = 4$), LPS alone (LPS, 10 mg/kg i.v., $n = 10$, open circles), LPS plus 1 mg/kg i.p. dexamethasone (Dex, $n = 6$; filled squares), LPS plus 3 mg/kg i.p. of calpain inhibitor I (Cal-I, 3; $n = 6$), LPS plus 10 mg/kg i.p. of calpain inhibitor I (Cal-I, 10; $n = 7$, filled triangles), LPS plus late administration at 2 hours after LPS of 10 mg/kg i.p. of calpain inhibitor I (Cal-I, 10p; $n = 4$), or LPS plus chymostatin (Chym; $n = 5$). Data are expressed as means ± SEM of n observations. $*p < 0.05$ represents significant difference when compared to LPS controls.

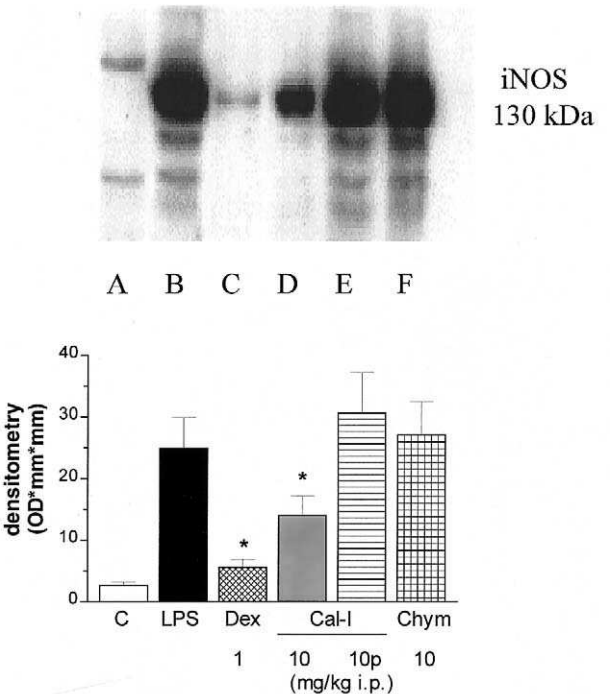


Figure 3 Effect of calpain inhibitor I on the expression of iNOS protein in lung homogenates at 6 hours after administration of endotoxin. Different groups of animals received vehicle (C) for *E. coli* lipopolysaccharide (LPS; lane A), LPS alone (LPS; lane B), LPS plus 1 mg/kg i.p. dexamethasone (Dex; lane C), LPS plus 10 mg/kg i.p. of calpain inhibitor I (Cal-I; lane D), LPS plus late administration at 2 hours after LPS of 10 mg/kg i.p. of calpain inhibitor I (Cal-I; lane E), or LPS plus chymostatin (Chym; lane F). Similar results of the Western blots were seen using tissue extracts from two other animals with the same treatment. $*p < 0.05$ represents significant difference when compared to LPS controls.

levels of nitrite/nitrate in these species. Thus, when evaluating the role of NO or elucidating the effects of NOS inhibitors in animal models of shock, one needs to consider that (i) many of the models used are acute, nonresuscitated, hypodynamic models of shock, (ii) the effects (and side effects) of nonselective inhibitors of NOS activity will greatly vary depending on the degree of iNOS induction in the species, and (iii) any observed effects of the respective NOS inhibitor used will obviously depend on the chosen dose regimen and timing of the intervention (Table I).

L-NMMA AND OTHER ANALOGS OF L-ARGININE

Inhibition of iNOS activity can be achieved with competitive inhibitors of NOS enzyme activity. The discovery of L-arginine analogs that inhibit NOS activity, including L-NMMA, provided the first tool to explore the beneficial or side effects of NOS inhibitors in animals and humans with septic shock. The subsequent discovery of the NOS

Table I Possible Effects of Administration of NOS Inhibitors in Septic Shock

Beneficial effects	Adverse effects
Increased blood pressure	Excessive vasoconstriction
Restores responsiveness to pressor agents	Pulmonary hypertension
Cardiac output return to baseline values	Fall in cardiac output
Decreased production of peroxynitrite	Increased platelet adhesiveness
Attentuation of inhibition of mitochondrial respiration	Increased neutrophil adhesion
Improved organ function	Worsened organ function
Improved survival	Reduction in survival

inhibitors N^G-nitro-L-arginine (L-NA) and its methyl ester (L-NAME; Moore *et al.,* 1990), which in contrast to L-NMMA were inexpensive and readily available, stimulated numerous studies aimed at evaluating the role of NO in septic shock by using (high doses of) L-NAME. This was somewhat unfortunate, as L-NAME is a more potent inhibitor of eNOS than iNOS activity. Thus, L-NAME caused many adverse effects resulting from the inhibition of eNOS activity, including excessive vasoconstriction with a fall in cardiac output, pulmonary hypertension, and enhanced adhesion of platelets and neutrophils to the endothelium. Thus, it was not surprising that high doses of L-NAME or L-NMMA increased mortality in mice and rabbits with endotoxic shock (see Thiemermann, 1994, for review). The hypothesis that the basal release of NO by eNOS has an important role in the regulation of regional blood flow (beneficial effects of NO), while the excessive generation of NO by iNOS ''in the wrong place at the wrong time'' contributes to some aspects of the pathophysiology of shock (harmful effects of NO), has stimulated the search for selective inhibitors of iNOS activity (Fig. 4).

AMINOGUANIDINE

Aminoguanidine was the first relatively selective inhibitor of iNOS activity discovered (Corbett *et al.,* 1992; Nilsson, 1999). Although aminoguanidine is a more potent inhibitor of iNOS than eNOS activity *in vitro* and *in vivo* (Misko *et al.,* 1993; Wu *et al.,* 1995), aminoguanidine is not a very potent inhibitor of iNOS activity (IC_{50}, approximately 100–150 μM). In addition, aminoguanidine is not a very specific inhibitor of iNOS activity, as this guanidine has many other pharmacological properties, including inhibition of (1) histamine decarboxylase, (2) polyamine catabolism, (3) the formation of advanced glycosylation end products, and (4) catalase activity (Wu *et al.,* 1995). Interestingly, aminoguanidine also prevents the expression of iNOS protein by a hitherto unknown mechanism (Ruetten and Thiemermann, 1996). Thus, aminoguanidine (like aminoethyl-isothiourea, see later) has to be regarded as an agent which affects iNOS activity by two distinct mechanisms, namely, prevention of the expression of iNOS protein and inhibition of iNOS activity.

S-SUBSTITUTED ISOTHIOUREAS

S-Substituted isothioureas (ITUs) are non-amino acid analogs of L-arginine and also potent inhibitors of iNOS activity with variable isoform selectivity (Garvey *et al.,* 1994; Szabo *et al.,* 1994; Southan *et al.,* 1995). The most potent isothioureas are those with only short alkyl chains on the sulfur atom and no substituents on the nitrogen atoms. For instance, *S*-ethyl-ITU is a potent competitive inhibitor of human iNOS, eNOS, and nNOS (brain NOS) activity. In contrast to *S*-ethyl-ITU, aminoethyl-ITU, and *S*-methyl-ITU are more selective inhibitors of iNOS than of eNOS activity *in vitro* and *in vivo* (Southan *et al.,* 1995). Interestingly, aminoethyl-ITU is metabolized to mercaptoethyl-guanidine (and possibly to the isothiourea 2-aminothiazoline), which

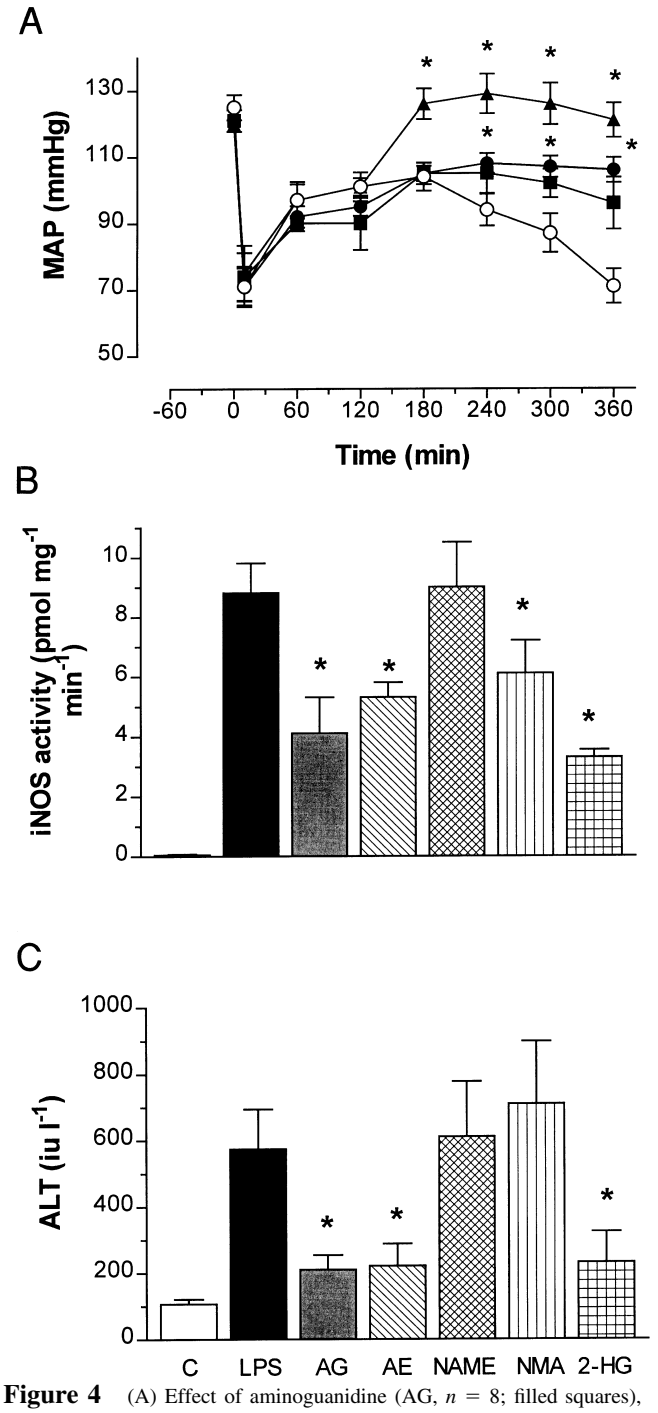


Figure 4 (A) Effect of aminoguanidine (AG, $n = 8$; filled squares), aminoethyl-isothiurea (AE, $n = 8$; filled circles), or N^{ω}-nitro-L-arginine methyl ester (L-NAME, $n = 6$; filled triangles) on the fall in mean arterial blood pressure (MAP) caused by *E. coli* lipopolysaccharide (LPS; 10 mg/kg i.v.) in the anesthetized rat. The alterations in MAP (over time) of rats that were pretreated with vehicle (for the drugs) and then received LPS are also shown (LPS control, $n = 10$; open circles). (B) Effect of different NOS inhibitors on the increase in iNOS activity and (C) the serum concentration of alanine aminotransferase (ALT) in rats with septic shock. Different groups of LPS rats were infused for 4 hours with vehicle ($n = 10$), AG ($n = 8$), AE ($n = 8$), N^{ω}-nitro-L-arginine methyl ester (NAME, $n = 6$), N^G-methyl-L-arginine (NMA, $n = 6$), or 1-amino-2-hydroxy-guanidine (2-HG, $n = 10$). The infusion of drug or vehicle was started at 2 hours after LPS. Data are expressed as means ± SEM of n observations. $^*p < 0.05$ represents a significant reduction in concentration/activity when compared to LPS rats.

may represent the active principle of aminoethyl-ITU. Indeed, mercaptoalkyl-guanidines, in particular mercaptoethyl- and mercaptopropyl-guanidines, are the most potent (guanidino) inhibitors of iNOS activity (see Southan and Szabo, 1996 for review).

AMIDINES

S-Substituted ITUs and guanidines contain the amidine function [$-CH(-NH)NH_2$], a feature that they have in common with O-substituted isoureas and amidines themselves. Indeed, amidines including 2-iminopiperidine, butyramidine, 2-aminopyridine, propioamidine, and acetamidine inhibit NOS activity. Interestingly, both 2-iminopiperidine and butyramidine are more potent inhibitors of iNOS activity than L-NMMA in murine macrophages. Recently, an analog of acetamidine termed 1400W {*N*-[3-(aminomethyl)benzyl] acetamidine} has been reported to be a slow, tight-binding inhibitor of human iNOS. The inhibition by 1400W of the activity of human iNOS was (1) extremely potent, (2) dependent on the cofactor NADPH, and (3) either irreversible or extremely slowly reversible. Most notably, 1400W was approximately 5000-fold more potent as an inhibitor of iNOS activity than of eNOS activity. Moreover, the inhibition by this agent of the activity of eNOS or nNOS activity was reversible by L-arginine, whereas iNOS inhibition was not. In a rat model of vascular injury caused by endotoxin, 1400W was 50-fold more potent as an inhibitor of iNOS than eNOS activity (Garvey *et al.,* 1997). Thus, 1400W appears to be the most potent and selective inhibitor of iNOS activity known to date and, hence, will be an ideal tool to elucidate the role of NO from iNOS in shock and other diseases associated with the induction of iNOS.

We have more recently discovered that 1400W (3–10 mg/kg/hour) attenuates both iNOS activity as well as the delayed hypotension caused by endotoxin in the rat in a dose-related fashion. Most notably, infusion of 10 mg/kg/hour of 1400W (starting 2 hours after endotoxin) abolished both the increase in nitrite-plus-nitrate as well as the delayed hypotension caused by LPS. These beneficial hemodynamic effects of 1400W did not, however, translate into a protection of organs such as the kidney, liver, and pancreas against the multiple organ injury caused by endotoxin in the rat. These results suggest that an enhanced formation of NO from iNOS contributes to the circulatory failure but not the organ dysfunction associated with endotoxic shock (Wray *et al.,* 1998). In addition to exerting beneficial effects in animal models of shock, 1400W also reduces the tissue injury caused by ischemia–reperfusion of the brain (Parmentier *et al.,* 1999).

Reduction of NO Formation: Effects and Side Effects in Patients with Septic Shock

Although our understanding of the role of NO in animal models of circulatory shock has improved substantially over the past years, our knowledge regarding the biosynthesis and importance of NO in the pathophysiology of patients with shock (of various etiologies) is still very limited. Indeed, a Medline search covering the period from 1987 to November 1995 revealed that only 8 to 14% of all of the publications which included the key term ''nitric oxide'' also included the key word ''human'' (Preiser and Vincent, 1996). What, then, is the evidence that septic shock in humans is associated with an enhanced formation of NO? Elevated plasma and urine levels of nitrite plus nitrate have been reported in adults and children with severe septic shock as well as in patients with burn injuries who subsequently developed sepsis. Moreover, elevated plasma levels of nitrite plus nitrate occur in patients receiving IL-2 chemotherapy. In contrast, there is also evidence that the plasma levels of nitrite plus nitrate are lower in patients after trauma, surgery, and in patients with HIV infections (see Preiser and Vincent, 1996). Interestingly, the increase in iNOS activity in leukocytes obtained from patients with sepsis appears to correlate with the number of failing organs but not with blood pressure. Taken together, these studies support the conclusion that septic shock in humans is associated with an enhanced formation of NO. It should, however, be stressed that the increase in the plasma levels of nitrite plus nitrate elicited by endotoxin, cytokines, or bacteria in rodents (10-fold) is substantially higher than the observed increases in the plasma levels of these metabolites of NO in other animal species (pig, sheep, etc.) or humans. Moreover, our understanding of (i) the biosynthesis of NO, (ii) the regulation of and the mechanism involved in the expression of iNOS, and (iii) the role of NO in MODS in shock are largely based on animal experiments of endotoxic shock in rodents. In contrast, we know relatively little about the role of NO in patients with septic and other forms of circulatory shock.

There is evidence that endotoxin and cytokines (TNF-α, IL-1, and γ-interferon, when given in combination) cause the expression of iNOS as well as the formation of NO in various human cells (primary or cell lines), including hepatocytes, mesangial cells, retinal pigmented epithelial cells, and lung epithelial cells (Morris and Billiar, 1994; Preiser and Vincent, 1996). Interestingly, IL-1 causes the hyporeactivity of human hand veins (*in situ*) to the constrictor effects elicited by exogenous or endogenous norepinephrine, and this vascular hyporeactivity is largely attenuated by L-NMMA, suggesting that it is mediated by NO (P. Vallance, personal communication). Although the clinical trials aimed at reducing the effects of TNF-α and IL-1 in patients with septic shock were not designed to prevent the formation of NO, some of the key findings of these trials are summarized in the following paragraph.

There is no convincing evidence that interventions aimed at reducing the effects of TNF-α (e.g., antibodies against TNF-α, soluble TNFα receptors) cause a significant reduction in 28-day mortality in patients with septic shock (Abraham *et al.,* 1995; Dhainaut *et al.,* 1995; Cohen and Carlet, 1996; Reinhart *et al.,* 1996). Most notably, there is one report documenting that the treatment of septic patients

with the TNF receptor:Fc fusion protein causes a dose-related increase in mortality (Fisher *et al.,* 1996). Similarly, clinical trials evaluating the effects of the IL-1 receptor antagonists have not resulted in a significant reduction in 28-day mortality (Fisher *et al.,* 1994; Knaus *et al.,* 1996). Although the trials failed to provide evidence that any of the anticytokine interventions used caused a significant reduction in 28-day mortality, these studies nevertheless support the view that both TNF-α as well as IL-1 play a role in the pathophysiology of septic shock and indicate that anticytokine therapy may well be of benefit for certain groups of patients. The IL-1ra Phase III Sepsis Syndrome Group has reported that (i) there is a direct relationship between the predicted risk of mortality of a patient at study entry and the efficacy of the IL-1 receptor antagonist (Il-1ra) in that (ii) patients with a predicted risk of mortality of <24% derived little benefit, whereas (iii) IL-1ra reduced the risk of death in the first 2 days for patients with a predicted risk of mortality of >24% (Knaus *et al.,* 1996). There is, however, some evidence that the prevention of the formation of both TNF-α and IL-1β (e.g., with γ-interferon or IL-10) is superior to prevention of the formation of either one of these cytokines in reducing mortality in rodent models of endotoxemia (Smith *et al.,* 1993). Moreover, the reduction in survival afforded by a combination immunotherapy (antibody against TNF-α, J5 antiserum against endotoxin, and a *Pseudomonas* O-serotype-specific opsonophagocytic monoclonal antibody) was greater than the one afforded by any combination of two antibodies or single antibody therapy (Gross *et al.,* 1993).

What, then, is the clinical experience with inhibitors of the activity of NOS? Early reports of beneficial hemodynamic effects of L-NMMA in humans with septic shock (Petros *et al.,* 1994) stimulated a phase I, multicenter, open-label, dose-escalation (1, 2.5, 5, 10, or 20 mg/kg/hour for up to 8 hours) study using L-NMMA (546C88) in 32 patients with septic shock. In this study, L-NMMA sustained blood pressure and enabled a reduction in vasopressor (norepinephrine) support. The cardiac index fell (possibly due to an increase in peripheral vascular resistance), and left ventricular function was well maintained. Moreover, L-NMMA increased oxygen extraction, while pulmonary shunt was not worsened (Watson *et al.,* 1995). This first study was followed by a placebo-controlled multicenter study involving 312 patients with septic shock, which evaluated the effects of L-NMMA on the resolution of shock at 72 hours (primary endpoint) (Grover *et al.,* 1999). The severity of illness according to the SAPS II score was similar between placebo and the L-NMMA group. Infusion of L-NMMA enhanced mean arterial blood pressure and systemic vascular resistance index and decreased cardiac output (from elevated toward normal levels). L-NMMA had no effect on left ventricular systolic work index, indicating that the fall in cardiac output was not due to an impairment in cardiac contractility. In patients treated with L-NMMA, there was a transient increase in mean pulmonary artery pressure. Interestingly, L-NMMA did not affect the thrombocytopenia or renal dysfunction caused by sepsis. Most notably, 41% of patients treated with L-NMMA, but only 21% of patients treated with placebo, recovered from shock within 72 hours. There was a strong trend for a reduction in mortality (at day 14) in patients treated with L-NMMA.

In June 1997, Glaxo Wellcome started a phase III clinical trial evaluating the effect of 546C88 (targinine, L-NMMA) in patients with septic shock. The trial was stopped by the company in spring 1998, because of concerns about a higher mortality in the treated group than in the placebo group at an interim analysis. The trial, which involved 177 centers from 26 countries, started in June 1997. It had enrolled 797 patients at the time of suspension. The interim analysis included data from 522 patients, 309 of whom had received 546C88. The data and safety monitoring committee reported a trend toward increased mortality in the active treatment group and thus recommended stopping the trial because of patients' safety concerns. The trial will not be resumed, and it is unlikely that development of the drug will continue (SCRIP, 1998; No. 2330, p. 21).

The reasons for the discrepancy in outcome between animal experiments (with agents that prevent the induction or the activity of NOS) and clinical trials are not entirely clear, but they may include (i) relatively late intervention in clinical trials (compared to pretreatment in animal studies), (ii) inhomogeneity of patients (e.g., differences in age, gender, causes of shock, severity of disease), or (iii) the pharmacology (dose regimen, time of intervention, length of treatment) of the intervention chosen. In addition, it is possible, if not likely, that inhibitors of NOS activity, such as L-NMMA, that are not selective for iNOS may exert side effects arising from excessive vasoconstriction. Clearly, the pathophysiology leading to the circulatory failure, organ dysfunction, and ultimately death in patients with septic shock is multifactorial.

Concluding Remarks

More than a decade has passed since the discovery that an enhanced formation of NO contributes to the circulatory failure in patients with septic shock. Although the effects of selective inhibitors of iNOS activity have not been evaluated in controlled clinical trials, there is good evidence that an enhanced formation of NO by iNOS contributes to the circulatory failure associated with septic shock in humans. A therapy-refractory fall in blood pressure (in the absence of MODS) is—only in some cases—the underlying cause of mortality of patients with septic shock. Thus, it appears likely that interventions aimed at eliminating the detrimental effects of a single mediator (''single-bullet approach to the therapy of shock''), although useful in some acute animal models, are less likely (if not unlikely) to cause a significant reduction in 28-day mortality in patients with septic shock.

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References

Abraham, E., Wunderink, R., Silverman, H., Perl, T. M., Nasraway, S., Levy, H., *et al.* (1995). Efficacy and safety of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter trial. TNF-alpha Mab Sepsis Study Group. *JAMA* **273,** 934–941.

Assreuy, J., Cunha, F. Q., Liew, F. Y., and Moncada, S. (1993). Feedback inhibition of nitric oxide synthase activity by nitric oxide. *Br. J. Pharmacol.* **108,** 833–837.

Barnes, P. J., and Adcock, I. M. (1997). NF-κB: A pivotal role in asthma and a new target for therapy. *Trends Pharmacol. Sci.* **18,** 46–50.

Barnes, P. J., and Karin, M. (1997). Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **336,** 1066–1071.

Baue, A. E. (1993). The multiple organ or system failure syndrome. *In* ''Pathophysiology of Shock, Sepsis, and Organ Failure'' (G. Schlag and H. Redl, eds.), pp. 1004–1018. Springer-Verlag, Berlin.

Beutler, B., Milsark, I. W., and Cerami, A. C. (1985). Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* **229,** 869–871.

Billiau, A., and Vandekerckhove, F. (1991). Cytokines and their interactions with other inflammatory mediators in the pathogenesis of sepsis and septic shock. *Eur. J. Clin. Invest.* **21,** 559–573.

Bulut, V., Severn, A., and Liew, F. Y. (1993). Nitric oxide production by murine macrophages is inhibited by prolonged elevation of cyclic AMP. *Biochem. Biophys. Res. Commun.* **195,** 1134–1138.

Cohen, J., and Carlet, J. (1996). INTERSEPT: An international, multicenter, placebo-controlled trial of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis. International Sepsis Trial Study Group. *Crit. Care Med.* **24,** 1431–1440.

Corbett, J. A., Tilton, R. G., Chang, K., Hasan, K. S., Ido, Y., Wang, J. L., *et al.* (1992). Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* **41,** 552–554.

Cross, A. S., Opal, S. M., Palardy, J. E., Bodmer, M. W., and Sadoff, J. C. (1993). The efficacy of combination immunotherapy in experimental *Pseudomonas* sepsis. *J. Infect. Dis.* **167,** 112–118.

Dal Nogare, A. R. (1991). Southwestern internal medicine conference: Septic shock. *Am. J. Med. Sci.* **302,** 50–65.

Deitsch, E. A. (1992). Multiple organ failure: Pathophysiology and potential future therapy. *Ann. Surg.* **216,** 117–134.

De Kimpe, S. J., Kengatharan, M., Thiemermann, C., and Vane, J. R. (1995). The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10359–10363.

Dhainaut, J. F., Vincent, J. L., Richard, C., Lejeune, P., Martin, C., Fierobe, L., *et al.* (1995). CDP571, a humanized antibody to tumor necrosis factor-alpha safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock. CPD571 Sepsis Study Group. *Crit. Care Med.* **23,** 1461–1469.

Diaz-Guerra, M. J., Bodelon, O. G., Velasco, M., Whelan, R., Parker, P. J., and Bosca, L. (1996). Up-regulation of protein kinase C-epsilon promotes the expression of cytokine-inducible nitric oxide synthase in RAW 264.7 cells. *J. Biol. Chem.* **271,** 32028–32033.

Dinarello, C. A. (1996). Cytokines as mediators in the pathogenesis of septic shock. *Curr. Top. Microbiol. Immunol.* **216,** 133–165.

Dong, Z., Qi, X., and Fidler, I. J. (1993). Protein tyrosine kinase inhibitors decrease the induction of nitric oxide synthase activity in lipopolysaccharide-responsive and non-responsive murine macrophages. *J. Immunol.* **151,** 2717–2724.

Doyle, A. G., Herbein, G., Montaner, L. J., Minty, A. J., Caput, D., Ferrara, P., *et al.* (1994). Interleukin-13 alters the activation state of murine macrophages in vitro: Comparison with interleukin-4 and interferon-gamma. *Eur. J. Immunol.* **24,** 1441–1445.

Dunn, D. (1987). Immunotherapeutic advances in the treatment of gram-negative sepsis. *World J. Surg.* **11,** 233–240.

Fisher, C. J., Jr., Dhainaut, J. F., Opal, S. M., Pribble, J. B., Balk, R. A., Slotman, G. J., *et al.* (1994). Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. *JAMA* **271,** 1836–1843.

Fisher, C. J., Jr., Agosti, J. M., Opal, S. M., Lowry, S. F., Balk, R. A., Sadoff, J. C., *et al.* (1996). Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The soluble TNF Receptor Sepsis Study Group. *N. Engl. J. Med.* **334,** 1697–1702.

Fleming, I., Gray, G. A., Julou-Schaeffer, G., Parratt, J. R., and Stoclet, J. C. (1990). Incubation with endotoxin activates the L-arginine pathway in vascular tissue. *Biochem. Biophys. Res. Commun.* **171,** 562–568.

Friedland, J. S., Porter, J. C., and Daryanani, S. (1996). Plasma proinflammatory cytokine concentrations, Acute Physiology and Chronic Health Evaluation (APACHE) III scores and survival in patients in an intensive care unit. *Crit. Care Med.* **24,** 1775–1781.

Garvey, E. P., Oplinger, J. A., Tanoury, G. J., Sherman, P. A., Fowler, M., Marshall, S., *et al.* (1994). Potent and selective inhibition of human nitric oxide synthases. Inhibition by non-amino acid isothioureas. *J. Biol. Chem.* **269,** 26669–26676.

Garvey, E. P., Oplinger, J. A., Furfine, E. S., Kiff, R. J., Laszlo, F., Whittle, B. J., *et al.* (1997). 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J. Biol. Chem.* **272,** 4959–4963.

Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., Fiers, W., Goldman, M., and Velu, T. (1993). Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J. Exp. Med.* **177,** 547–550.

Gray, G. A., Julou-Schaeffer, G., Oury, K., Fleming, I., Parratt, J. R., and Stoclet, J. C. (1990). An L-arginine-derived factor mediates endotoxin-induced vascular hyposensitivity to calcium. *Eur. J. Pharmacol.* **191,** 89–92.

Green, S. F., and Nacy, C. A. (1993). Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis. *Curr. Opin. Infect. Dis.* **6,** 384–396.

Griscavage, J. M., Wilk, S., and Ignarro, L. J. (1995). Serine and cysteine proteinase inhibitors prevent nitric oxide production by activated macrophages by interfering with transcription of the inducible NO synthase gene. *Biochem. Biophys. Res. Commun.* **215,** 721–729.

Groeneveld, A. B., Nauta, J. J., and Thijs, L. G. (1988). Peripheral vascular resistance in septic shock: Its relation to outcome. *Intensive Care Med.* **14,** 141–147.

Grover, R., Zaccardelli, D., Colice, G., Guntupalli, K., Watson, D., and Vincent, J. L. (1999). An open-label dose escalation study of the nitric oxide synthase inhibitor, *N*(G)-methyl-L-arginine hydrochloride (546C88), in patients with septic shock. Glaxo Wellcome International Septic Shock Study Group. *Crit. Care Med.* **27,** 913–922.

Hewett, J. A., Jean, P. A., Kunkel, S. L., and Roth, R. A. (1993). Relationship between tumor necrosis factor-alpha and neutrophils in endotoxin-induced liver injury. *Am. J. Physiol.* **265,** G1011–G1015.

Hirahashi, J., Nakaki, T., Hishikawa, K., Marumo, T., Yasumori, T., Hayashi, M., *et al.* (1996). Endothelin-1 inhibits induction of nitric oxide synthase and GTP cyclohydrolase I in rat mesangial cells. *Pharmacology* **53,** 241–249.

Joly, G. A., Ayres, M., and Kilbourn, R. G. (1997). Potent inhibition of inducible nitric oxide synthase by geldanamycin, a tyrosine kinase inhibitor, in endothelial, smooth muscle cells, and in rat aorta. *FEBS Lett.* **403,** 40–44.

Julou-Schaeffer, G., Gray, G. A., Fleming, I., Schott, C., Parratt, J. R., and Stoclet, J. C. (1990). Loss of vascular responsiveness induced by endotoxin involves the L-arginine pathway. *Am. J. Physiol.* **259,** H1038–H1043.

Kengatharan, M., De-Kimpe, S. J., and Thiemermann, C. (1996). Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages. SO. *Br. J. Pharmacol.* **117,** 1163–1170.

Kengatharan, K. M., De-Kimpe, S., Robson, C., Foster, S. J., and Thiemermann, C. (1998). Mechanism of gram-positive shock: Identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. *J. Exp. Med.* **188,** 305–315.

Kilbourn, R. G., Jubran, A., Gross, S. S., Griffith, O. W., Levi, R., Adams, J., *et al.* (1990). Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.* **172,** 1132–1138.

Kilbourn, R. G., Owen-Schaub, L. B., Cromeens, D. M., Gross, S. S., Flaherty, M. J., Santee, S. M., Alak, A. M., and Griffith, O. W. (1994). N^G-methyl-L-arginine, an inhibitor of nitric oxide formation, reverses IL-2-mediated hypotension in dogs. *J. Appl. Physiol.* **76,** 1130–1137.

Knaus, W. A., Harrell, F. E., Jr., LaBrecque, J. F., Wagner, D. P., Pribble, J. P., Draper, E. A., *et al.* (1996). Use of predicted risk of mortality to evaluate the efficacy of anti-cytokine therapy in sepsis. The rhIL-1ra Phase III Sepsis Syndrome Study Group. *Crit. Care Med.* **24,** 46–56.

Koide, M., Kawahara, Y., Nakayama, I., Tsuda, T., and Yokoyama, M. (1993). Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells. Synergism with the induction elicited by inflammatory cytokines. *J. Biol. Chem.* **268,** 24959–24966.

Kunz, D., Walker, G., Wiesenberg, I., and Pfeilschifter, J. (1996). Inhibition by tetranactin of interleukin 1 beta- and cyclic AMP-induced nitric oxide synthase expression in rat renal mesangial cells. *Br. J. Pharmacol.* **118,** 1621–1626.

Liu, S. F., Ye, X., and Malik, A. B. (1997). In vivo inhibition of nuclear factor-kappa B activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. *J. Immunol.* **159,** 3976–3983.

MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., *et al.* (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81,** 641–650.

Marczin, N., Papapetropoulos, A., and Catravas, J. D. (1993). Tyrosine kinase inhibitors suppress endotoxin- and IL-1 beta-induced NO synthesis in aortic smooth muscle cells. *Am. J. Physiol.* **265,** H1014–H1018.

Militante, J. D., Feinstein, D. L., and Syapin, P. J. (1997). Suppression by ethanol of inducible nitric oxide synthase expression in C6 glioma cells. *J. Pharmacol. Exp. Ther.* **281,** 558–565.

Misko, T. P., Moore, W. M., Kasten, T. P., Nicklos, G. A., Corbett, J. A., Tilton, R. G., McDaniel, M. L., Williamson, J. R., and Currie, M. G. (1993). Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.* **233,** 119–126.

Morbidity and Mortality Weekly Report (1988). Increase in national hospital discharge survey rates for septicemia—United States. 1979–1987. *MMWR* **89,** 31–34.

Morris, S. M., Jr., and Billiar, T. R. (1994). New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.* **266,** E829–E839.

Mozes, T., Ben-Efraim, S., Tak, C. J., Heiligers, J. P., Saxene, P. R., and Bonta, I. L. (1991). Serum levels of tumor necrosis factor determine the fatal or non-fatal course of endotoxic shock. *Immunol. Lett.* **27,** 157–162.

Muchamuel, T., Menon, S., Pisacane, P., Howard, M. C., and Cockayne, D. A. (1997). IL-13 protects mice from lipopolysaccharide-induced lethal endotoxemia: Correlation with downmodulation of TNF-alpha, IFN-gamma, and IL-12 production. *J. Immunol.* **158,** 2898–2903.

Nicoletti, F., Mancuso, G., Cusumano, V., Di-Marco, R., Zaccone, P., Bendtzen, K., and Teti, G. (1997). Prevention of endotoxin-induced lethality in neonatal mice by interleukin-13. *Eur. J. Immunol.* **27,** 1580–1583.

Nilsson, B. O. (1999). Biological effects of aminoguanidine: An update. *Inflammation Res.* **48,** 509–515.

Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N., and Levitzki, A. (1994). Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science* **264,** 1319–1322.

Nussler, A. K., and Billiar, T. R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. *J. Leukocyte Biol.* **54,** 171–178.

Okuda, S., Kanda, F., Kawahara, Y., and Chihara, K. (1997). Regulation of inducible nitric oxide synthase expression in L6 rat skeletal muscle cells. *Am. J. Physiol.* **272,** C35–C40.

Okusawa, S., Gelfand, J. A., Ikejima, T., Connolly, R. J., and Dinarello, C. A. (1988). Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *J. Clin. Invest.* **81,** 1162–72.

Parker, M., and Parrillo, J. E. (1983). Septic shock: Hemodynamics and pathogenesis. *J. Am. Med. Assoc.* **250,** 3324–3327.

Parmentier, S., Bohme, G. A., Lerouet, D., Damour, D., Stutzmann, J. M., Margaill, I., and Plotkine, M. (1999). Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury. *Br. J. Pharmacol.* **127,** 546–552.

Parrillo, J. E. (1989). Septic shock in humans: Clinical evaluation, pathogenesis, and therapeutic approach. *In* ''Textbook of Critical Care'' (W. Shoemaker, S. Ayres, A. Grenuik, P. Holbrook, and W. Thompson, eds.), 2nd Ed., p. 1006. Saunders, Philadelphia, Pennsylvania.

Paya, D., Gray, G. A., Fleming, I., and Stoclet, J. C. (1993). Effect of dexamethasone on the onset and persistence of vascular hyporeactivity induced by *E. coli* lipopolysaccharide in rats. *Circ. Shock* **41,** 103–112.

Petros, A., Lamb, G., Leone, A., Moncada, S., Bennett, D., and Vallance, P. (1994). Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovasc. Res.* **28,** 34–39.

Preiser, J. C., and Vincent, J. L. (1996). Nitric oxide involvement in septic shock: Do human beings behave like rodents? *In* ''Yearbook of Intensive Care and Emergency Medicine'' (J. L. Vincent, ed.), pp. 358–365. Springer-Verlag, Berlin.

Radomski, M. W., Palmer, R. M., and Moncada, S. (1990). Characterization of the L-arginine: Nitric oxide pathway in human platelets. *Br. J. Pharmacol.* **101,** 325–330.

Rees, D. D., Cellek, S., Palmer, R. M., and Moncada, S. (1990). Dexamethasone prevents the induction by endotoxin of nitric oxide synthase and the associated effects on vascular tone: An insight into endotoxin shock. *Biochem. Biophys. Res. Commun.* **173,** 541–547.

Reinhart, K., Wiegand-Lohnert, C., Grimminger, F., Kaul, M., Withington, S., Treacher, D., *et al.* (1996). Assessment of the safety and efficacy of the monoclonal anti-tumor necrosis factor antibody-fragment, MAK 195F, in patients with sepsis and septic shock: A multicenter, randomized, placebo-controlled, dose-ranging study. *Crit. Care Med.* **24,** 733–42.

Rietschel, E. T., and Brade, H. (1992). Bacterial endotoxins. *Sci. Am.* **267,** 54–61.

Roumen, R. M., Hendriks, T., van der Ven-Jongekrijg, J., Nieuwenhuijzen, G. A., Sauerwein, R. W., van der Meer, J. W., *et al.* (1993). Cytokine patterns in patients after major vascular surgery, hemorrhagic shock, and severe blunt trauma. Relation with subsequent adult respiratory distress syndrome and multiple organ failure. *Ann. Surg.* **218,** 769–76.

Ruetten, H., and Thiemermann, C. (1996). Prevention of the expression of inducible nitric oxide synthase by aminoguanidine or aminoethyl-isothiourea in macrophages and in the rat. *Biochem. Biophys. Res. Commun.* **225,** 525–530.

Ruetten, H., and Thiemermann, C. (1997a). Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: A possible role for protein tyrosine kinase. *Br. J. Pharmacol.* **122,** 59–70.

Ruetten, H., and Thiemermann, C. (1997b). Attenuation by calpain inhibitor I, an inhibitor of the proteolysis of IκB, of the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. *Br. J. Pharmacol.* **121,** 695–704.

Salzman, A., Denenberg, A. G., Ueta, I., O'Conner, M., Linn, S. C., and Szabo, C. (1996). Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *Am. J. Physiol.* **270,** G565–G573.

Saura, M., Martinez-Dalmau, R., Minty, A., Perez-Sala, D., and Lamas, S. (1996). Interleukin-13 inhibits inducible nitric oxide synthase expression in human mesangial cells. *Biochem. J.* **313,** 641–646.

Schini, V. B., Catovski, S., Scott-Burden, T., and Vanhoutte, P. (1992). The inducible nitric oxide synthase is impaired by thrombin in vascular smooth muscle cells. *J. Cardiovasc. Pharmacol.* **20,** S142–S144.

Schini-Kerth, V. B., Boese, M., Busse, R., Fisslthaler, B., and Mulsch, A. (1997). *N*-alpha-tosyl-L-lysine chloromethylketone prevents expression of iNOS in vascular smooth muscle by blocking activation of NF-kappa B. *Arterioscler. Thromb. Vasc. Biol.* **17,** 672–679.

Schneemann, M., Schoedon, G., Frei, K., and Schaffner, A. (1993). Immunovascular communication: Activation and deactivation of murine endothelial cell nitric oxide synthase by cytokines. *Immunol. Lett.* **35,** 159–162.

Sherman, M. P., Aeberhard, E. E., Wong, V. Z., Griscavage, J. M., and Ignarro, L. J. (1993). Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **191,** 1301–1308.

Smith, S. R., Calzetta, A., Bankowski, J., Kenworthy-Bott, L., and Terminelli, C. (1993). Lipopolysaccharide-induced cytokine production and mortality in mice treated with *Corynebacterium parvum. J. Leukocyte Biol.* **54,** 23–29.

Southan, G. J., and Szabo, C. (1996). Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem. Pharmacol.* **51,** 383–394.

Southan, G. J., Szabo, C., and Thiemermann, C. (1995). Isothioureas: Potent inhibitors of nitric oxide synthases with variable isoform selectivity. *Br. J. Pharmacol.* **114,** 510–516.

Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature* **346,** 425–434.

Sprung, C. L., Caralis, P. V., Marcial, E. H., Pierce, M., Gelbard, M. A., Long, W. M., *et al.* (1984). The effects of high-dose corticosteroids in patients with septic shock. A prospective, controlled study. *N. Engl. J. Med.* **311,** 1137–1143.

Stuber, F., Petersen, M., Bokelmann, F., and Schade, U. A. (1996). Genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. *Crit. Care Med.* **24,** 381–384.

Szabo, C., and Thiemermann, C. (1995). Regulation of the expression of the inducible isoform of nitric oxide synthase. *Adv. Pharmacol.* **34,** 113–153.

Szabo, C., Mitchell, J. A., Thiemermann, C., and Vane, J. R. (1993). Nitric-oxide-mediated hyporeactivity to norepinephrine precedes the induction of nitric oxide synthase in endotoxin shock. *Br. J. Pharmacol.* **108,** 786–792.

Szabo, C., Southan, G. J., and Thiemermann, C. (1994). Beneficial effects and improved survival in rodent models of shock with *S*-methylisothiourea, a novel, potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12472–12476.

Thiemermann C. (1994). The role of the L-arginine:nitric oxide pathway in circulatory shock. *Adv. Pharmacol.* **28,** 45–79.

Thiemermann, C., and Vane, J. R. (1990). Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharide in the rat in vivo. *Eur. J. Pharmacol.* **182,** 591–595.

Thiemermann, C., Szabo, C., Mitchell, J. A., and Vane, J. R. (1993). Vascular hyporeactivity to vasoconstrictor agents and hemodynamics decompensation in hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 267–271.

Tracey, K. J. (1991). Tumor necrosis factor (cachectin) in the biology of septic shock syndrome. *Circ. Shock* **35,** 123–128.

Tracey, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G. C., *et al.* (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* **330,** 662–664.

Vanichkin, A., Patya, M., Gazit, A., Levitzki, A., and Novogrodsky, A. (1996). Late administration of a lipophylic tyrosine kinase inhibitor prevents lipopolysaccharide and *Escherichia coli*-induced lethal toxicity. *J. Infect. Dis.* **173,** 927–933.

Wakabayashi, G., Gelfand, J. A., Burke, J. F., Thompson, R. C., and Dinarello, C. A. (1991). A specific receptor antagonist for interleukin 1 prevents *Escherichia coli*-induced shock in rabbits. *FASEB J.* **5,** 338–343.

Watson, D., Donaldson, J., Grover, R., Mottola, D., Guntipalli, K., and Vincent, J. L. (1995). The cardiopulmonary effects of 546C88 in human with septic shock. *Intensive Care Med.* **21,** S117.

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., *et al.* (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375,** 408–411.

Wray, G. M., Millar, C. G., Hinds, C. J., and Thiemermann, C. (1998). Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. *Shock* **9,** 329–335.

Wright, C. E., Rees, D. D., and Moncada, S. (1992). Protective and pathological roles of nitric oxide in endotoxin shock. *Cardiovasc. Res.* **26,** 48–57.

Wu, C. C., Chen, S. J., Szabo, C., Thiemermann, C., and Vane, J. R. (1995). Aminoguanidine attenuates the delayed circulatory failure and improves survival in rodent models of endotoxic shock. *Br. J. Pharmacol.* **114,** 1666–1672.

Xie, Q., Whisnant, R., and Nathan, C. (1993). Promotor of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J. Exp. Med.* **177,** 1779–1784.

Xie, Q., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-B/Rel in induction of nitric oxide. *J. Biol. Chem.* **269,** 4705–4708.

Young, L. (1990). Gram negative sepsis. *In* ''Principles and Practice of Infectious Disease'' (G. L. Mandell, *et al.,* eds), pp. 611–636. Churchill Livingston, New York.

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CHAPTER 48

Nitric Oxide and Vascular Disease

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NITRIC OXIDE (NO) IS A POTENT REGULATOR OF VESSEL TONE, STRUCTURE, AND INTERACTION WITH CIRCULATING BLOOD ELEMENTS. IN HEALTHY INDIVIDUALS, THE VASODILATION INDUCED BY NO ENHANCES BLOOD FLOW BY INCREASING VASCULAR COMPLIANCE AND REDUCING VASCULAR RESISTANCE. NO INHIBITS THE PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELLS AND PREVENTS ADHERENCE OF PLATELETS AND LEUKOCYTES TO THE VESSEL WALL. NO ALSO PLAYS AN IMPORTANT ROLE IN ANGIOGENESIS. IN INDIVIDUALS WITH RISK FACTORS FOR ATHEROSCLEROSIS (E.G., HYPERTENSION, HYPERCHOLESTEROLEMIA, DIABETES MELLITUS, TOBACCO EXPOSURE, AND AGING) NO ACTIVITY AND/OR SYNTHESIS ARE IMPAIRED. THE IMPAIRMENT IS MULTIFACTORIAL AND DEPENDENT UPON ASSOCIATED CONDITIONS AND THE STAGE OF VASCULAR DISEASE, BUT THE END RESULT IS PATHOPHYSIOLOGICAL INCREASES IN VASCULAR RESISTANCE AND CHANGES IN VASCULAR STRUCTURE THAT RESULT IN ATHEROSCLEROSIS, MEDIAL HYPERTROPHY, MYOINTIMAL HYPERPLASIA, AND/OR THROMBUS FORMATION, AS WELL AS IMPAIRED ANGIOGENIC RESPONSE TO OCCLUSIVE ARTERIAL DISEASE. ONE OF THE DERANGEMENTS OF THE NO SYNTHASE PATHWAY THAT CONTRIBUTES TO THESE PATHOPHYSIOLOGIES IS COMPETITIVE INHIBITION OF NO SYNTHESIS BY THE ENDOGENOUS ANTAGONIST ASYMMETRIC DIMETHYLARGININE. NEW INSIGHTS INTO THE MECHANISMS OF THIS DERANGEMENT MAY LEAD TO A NEW THERAPEUTIC AVENUE IN THE TREATMENT OF VASCULAR DISEASE.

Introduction

The endothelium is a delicate and diaphanous film of tissue that lines all blood vessels. Despite its apparent fragility, this tissue exerts significant influence over vessel tone, structure, and interaction with circulating blood elements. The endothelium elaborates a remarkable panoply of paracrine products to serve these functions and to preserve a balance between vasoconstriction and vasodilation, between cell proliferation and quiescence, and between coagulation and blood fluidity. Generally, within the healthy vessel wall the endothelial factors that predominate are those that induce vasodilation, prevent coagulation, and suppress cell adherence and growth. However, under certain conditions, the balance may be shifted. For example, the endothelium synthesizes tissue plasminogen activator, thrombomodulin, and heparans that favor fluidity of the blood. However, the endothelium also is capable of producing von Willebrand factor, plasminogen activator inhibitor, and adhesive glycoproteins that participate in coagulation and cell adherence. This diversity of endothelial synthetic function maintains blood flow but provides the physiological flexibility for the endothelium to respond to conditions that demand hemostasis.

The same delicate balance exists for the endothelial regulation of vascular growth. Ordinarily the endothelium suppresses the proliferation of the underlying vascular smooth muscle cells by its elaboration of nitric oxide (NO), prostacyclin, and transforming growth factor β (TGF-β). However, the endothelium is also capable of producing agents

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that stimulate vascular growth, including platelet-derived growth factor, basic fibroblast growth factor (bFGF), and insulin-like growth factor.

Finally, the endothelium regulates vascular tone. By its production of nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factor, natriuretic peptide, and adrenomedullin, the endothelium maintains the systemic circulation in a vasodilated state, although it is also capable of producing vasoconstrictors such as endothelin and angiotensin II.

In the healthy vessel, the endothelial factors that predominate are those that favor vasodilation, blood fluidity, and cell quiescence. Endothelium-derived nitric oxide is paradigmatic of an endothelial factor that induces vasodilation, and which opposes inflammation, thrombosis, and cellular proliferation. Disorders that disturb the synthesis or activity of nitric oxide result in vasculopathies characterized by vasoconstriction, inflammation, thrombosis, and/or initiation of the cell cycle. Hypercholesterolemia, hypertension, hyperglycemia, hyperhomocysteinemia, tobacco exposure, and aging each cause endothelial dysfunction that contributes to the vascular disease associated with these conditions. The endotheliopathy that precedes and contributes to vascular disease is multifactorial, and it is dependent on the underlying metabolic disorder. Insights have been obtained regarding the mechanisms of these endotheliopathies which may lead to new therapeutic strategies.

Derangement of the NO Synthase Pathway in Atherosclerosis

In animal models of atherosclerosis, and in human patients, endothelium-mediated NO-dependent vasodilation is impaired (Bossaller *et al.,* 1987; Celermajer *et al.,* 1992; Ludmer *et al.,* 1986; Verbeuren *et al.,* 1986). The impairment in endothelium-dependent vasodilation occurs early in the course of disease, and it affects the peripheral and myocardial conduit and resistance vessels of animals and humans exposed to risk factors for atherosclerosis (Cohen *et al.,* 1988; Creager *et al.,* 1990; Zeiher *et al.,* 1991). The loss of normal endothelium-dependent vasodilation results in abnormal vasomotion. This has clinical consequences. For example, the normal coronary vasodilation to an increase in blood flow is reversed to a vasoconstriction in individuals with coronary artery disease, which undoubtedly contributes to exercise-induced angina (Nabel *et al.,* 1990). Furthermore, in hypercholesterolemic individuals with coronary artery disease, aggressive antilipid therapy improves coronary endothelial function and reduces myocardial ischemia, as documented by Holter monitoring of ST segment changes (Treasure *et al.,* 1995; Andrews *et al.,* 1997).

The impairment of endothelium-dependent vasodilation is multifactorial and dependent on the vessel and species studied, the stage of atherosclerosis, and the associated metabolic disorders (Förstermann *et al.,* 1989; Tesfamariam *et al.,* 1992; Ohara *et al.,* 1993; Kikuta *et al.,* 1998; Lerman *et al.,* 1995; Girerd *et al.,* 1990; Pritchard *et al.,* 1995; Cosentino and Katusic, 1995; Stroes *et al.,* 1995; Vallance *et al.,* 1992). The mechanism of impairment may include endothelial generation of superoxide anion (O_2^-) and increased degradation of NO, elaboration of vasoconstrictor prostanoids and endothelin, reduced elaboration of prostacyclin, and/or impaired biosynthesis of NO (Förstermann *et al.,* 1989; Tesfamariam *et al.,* 1992; Ohara *et al.,* 1993; Lerman *et al.,* 1995; Girerd *et al.,* 1990). Impaired biosynthesis of NO may be due to (1) alterations in NOS affinity for L-arginine, (2) lipid-induced impairment of the high-affinity cationic amino acid transporter (CAT), (3) reduced availability of the cofactor tetrahydrobiopterin, or (4) increased levels of asymmetric dimethylarginine (ADMA), the competitive inhibitor of nitric oxide synthase (NOS) (Vallance *et al.,* 1992; Cosentino and Katusic, 1995; Stroes *et al.,* 1995; Pritchard *et al.,* 1995; Kikuta *et al.,* 1998). Our group and others have accumulated extensive data to indicate that ADMA is a major determinant of endothelial vasodilator dysfunction in humans at risk for atherosclerosis.

We and others have shown that endothelial vasodilator dysfunction in hypercholesterolemic animals or humans could be reversed by administration of L-arginine (Cooke *et al.,* 1991a; Creager *et al.,* 1992; Drexler *et al.,* 1991). We found that in hypercholesterolemic humans, the impaired forearm blood flow response to the endothelial agonist methacholine is rapidly normalized by intravenous infusion of L-arginine but not D-arginine (Creager *et al.,* 1992). Similarly, in patients with atherosclerotic or transplant coronary artery disease, the impairment of acetylcholine-induced vasodilation of the epicardial and coronary resistance vessels is reversed by an intravenous infusion of L-arginine (Drexler *et al.,* 1991, 1994).

Our initial reports regarding the reversibility by L-arginine of endothelial vasodilator dysfunction were met with some skepticism. By the time our work was published, Förstermann and colleagues had characterized the kinetics of NOS and found that, *in vitro,* the K_m of NOS for L-arginine was in a micromolar range (Pollock *et al.,* 1991). Because human plasma L-arginine levels are 50–100 μM, L-arginine should not be rate limiting. However, our studies were confirmed and extended by a number of other investigators. In animal models or in patients with hypercholesterolemia and/or atherosclerosis, the studies are remarkably concordant (Bode-Böger *et al.,* 1996a; Maxwell *et al.,* 1998; Böger *et al.,* 1995; Clarkson *et al.,* 1996; Davies *et al.,* 1995; Drexler *et al.,* 1991; Dubois-Rande *et al.,* 1992; Goode and Heagerty, 1995; Hutchison *et al.,* 1997; Mehta *et al.,* 1996; Randall *et al.,* 1994; Rossitch *et al.,* 1991; Schushke *et al.,* 1994). There are several explanations for the ''arginine paradox,'' including the following: effects of hypercholesterolemia and/or atherosclerosis on NOS affinity (Pritchard *et al.,* 1995), the effect of glutamine to inhibit activation of NOS (Arnal *et al.,* 1995), a reduction in the availability of the cofactor tetrahydrobiopterin (Cosentino and Katusic, 1995), the colocalization to the caveolar membrane of NOS and CAT (the

cationic amino acid transporter that mediates L-arginine influx) (McDonald *et al.,* 1997), and reduced arginine transport due to lipid-induced impairment of the high-affinity CAT (Kikuta *et al.,* 1998) or to elevation in plasma levels of ADMA (asymmetric dimethylarginine). Localization of NOS and CAT to the caveolar membrane may explain the sensitivity of the NOS pathway to reductions in extracellular L-arginine levels (Hutchison *et al.,* 1997).

ADMA is a competitive inhibitor of NOS. ADMA is derived from the methylation of internal arginine residues in proteins; hydrolysis of these proteins produces ADMA (note that ADMA is not produced by methylation of free arginine, and the plasma ADMA level is not affected by L-arginine intake) (Bode-Böger *et al.,* 1996b). ADMA is excreted by the kidney or metabolized by DDAH (dimethylarginine dimethylaminohydrolase) to citrulline and dimethylamine (MacAllister *et al.,* 1996). Normal plasma levels are 0.5 to 1 μM (Vallance *et al.,* 1992). Intriguingly, plasma levels of ADMA are elevated in a number of conditions associated with endothelial vasodilator dysfunction including renal failure, hypercholesterolemia, hyperhomocysteinemia, hypertension, diabetes mellitus, and heart failure (Vallance *et al.,* 1992; Usui *et al.,* 1998; Matsuoka *et al.,* 1997; Böger *et al.,* 1998a). In patients with renal failure, ADMA levels are increased 10-fold (Vallance *et al.,* 1992). Plasma from renal failure patients induces vasoconstriction of vascular rings, which is reversible by addition of L-arginine to the medium (Vallance *et al.,* 1992). Dialysis normalizes plasma ADMA levels and improves endothelium-dependent relaxation of peripheral vessels in these individuals (Hand *et al.,* 1998).

Luscher and colleagues have previously shown that flow-mediated vasodilation (FMVD) of the brachial artery is largely mediated by endothelium-derived NO (Joannides *et al.,* 1995). Flow-mediated vasodilation is impaired in individuals with atherosclerosis or risk factors for atherosclerosis. The degree of impairment is related to the number of cardiovascular risk factors present, among which the plasma low density lipoprotein (LDL) cholesterol level may be one of the most important (Celermajer *et al.,* 1994). The mechanism(s) leading to this defect may include an increased degradation and/or reduced synthesis of NO (Minor *et al.,* 1990; Cooke and Dzau, 1997a). In young asymptomatic hypercholesterolemic subjects, we have shown that plasma ADMA levels are doubled in association with an arginine-reversible impairment of FMVD of the brachial artery (Figs. 1 and 2). Intriguingly, the impairment of FMVD is better correlated to the arginine/ADMA ratio than it is to LDL cholesterol levels (Böger *et al.,* 1997a).

We measured plasma levels of L-arginine and ADMA by high-performance liquid chromatography (HPLC) in 49 hypercholesterolemic and 31 normocholesterolemic individuals (Boger *et al.,* 1998b). ADMA levels were elevated twofold in hypercholesterolemic individuals (2.2 $\pm$ 0.2 versus 1.0 $\pm$ 0.1 μmol/liter), whereas L-arginine levels were similar. In a segment of the entire study group, measurements of flow-mediated vasodilation of the brachial artery were made using duplex ultrasonography. Flow-mediated vasodilation and urinary nitrate excretion, which are indicators of endothelium-derived NO activity or biosynthesis, were inversely correlated to plasma ADMA levels. This study suggests that elevated plasma ADMA levels in hypercholesterolemia impair NO biosynthesis in humans.

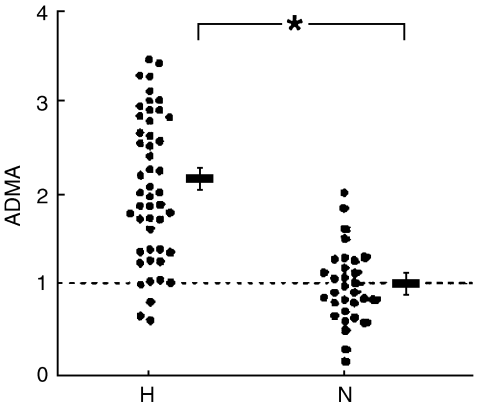


Figure 1 Plasma ADMA in hypercholesterolemic (H) and normal (N) subjects (modified from Böger *et al.,* 1998b, *Circulation* 98(18), 1842–1847). *Values are slightly different.

The two- to threefold elevation of plasma ADMA that is observed in patients with hypercholesterolemia and/or atherosclerosis is physiologically significant. Faraci *et al.* (1995) found that concentrations of ADMA at these levels inhibit endothelium-dependent relaxation of basilar arteries in the hamster. Added to crude purified preparations of endothelial NOS (eNOS), ADMA in a physiological or pathophysiological range (1 to 10 μM) induces a dose-dependent inhibition of NO synthesis. Accordingly, at the concentrations observed in hypercholesterolemic patients, ADMA inhibits NO biosynthesis. Furthermore, when the arginine/ADMA ratios are mimicked in *in vitro* experiments, cultured endothelial cells increase their production of superoxide anions and reduce the activity of NO.

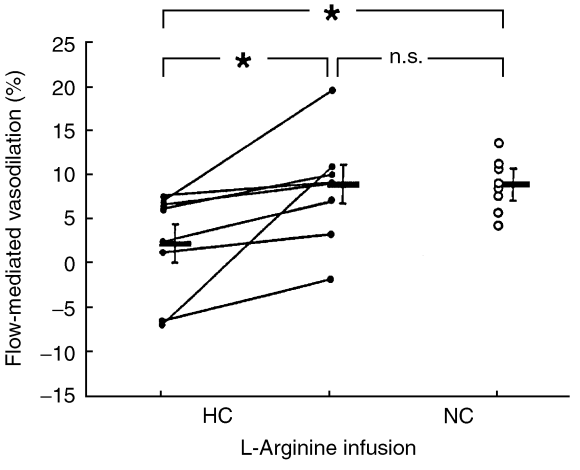


Figure 2 L-Arginine infusion restores endothelium-dependent vasodilation in hypercholesterolemic (HC) subjects (modified from Böger *et al.,* 1998b, *Circulation* 98(18), 1842–1847). NC, normal control. *Values are slightly different; n.s., not significantly different.

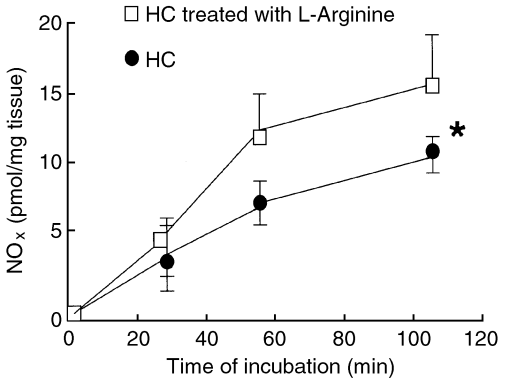


Figure 3 Vascular NO synthesis is increased by L-arginine (modified from Tsao *et al.,* 1994).

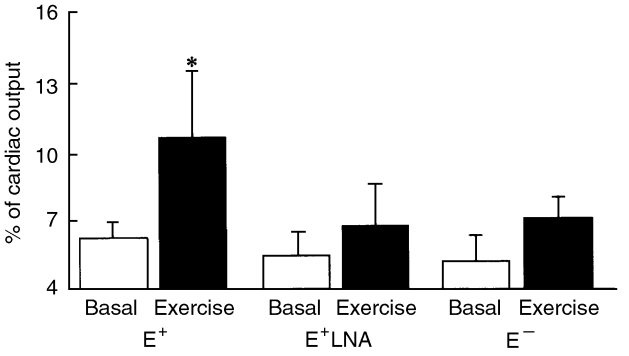


Figure 4 Basal (open bars) and peak exercise (solid bars) distribution of cardiac output to gastrocnemius and quadriceps muscles expressed as percentage of total cardiac output in E^+, E^+ L-NA, and E^- mice (Maxwell *et al.,* 1998, *Circulation* 98(4), 369–374).

The doubling of ADMA plasma concentrations in hypercholesterolemic (HC) subjects may reflect an even greater level of this endogenous NO synthase inhibitor within endothelial cells. In experimental models of vascular injury, there is attenuated endothelium-dependent vasodilation even after the intimal lining has been fully regenerated (Weidinger *et al.,* 1990). It has been shown that in regenerated endothelial cells, levels of ADMA (as well as another NO synthase inhibitor, L-monomethylarginine) are elevated threefold compared with normal cells (Azumi *et al.,* 1995).

In hypercholesterolemic New Zealand White (NZW) rabbits, plasma ADMA levels are elevated twofold (Böger *et al.,* 1995; Yu *et al.,* 1994). In these animals, endothelium-dependent NO-mediated vasodilation of the aorta and resistance vessels is impaired. We have directly measured the vascular synthesis of NO by the rabbit thoracic aorta. Animals were fed a high cholesterol diet for 2 weeks and were given L-arginine (2.5%) or vehicle in the drinking water *ad libitum,* which resulted in a doubling of plasma arginine levels. The production of nitrogen oxides by the thoracic aortas (as measured by chemiluminescence) was increased in animals receiving supplemental L-arginine (Fig. 3) (Tsao *et al.,* 1994). This study demonstrates directly that administration of L-arginine enhances vascular NO synthesis.

The endothelial dysfunction induced by hypercholesterolemia has adverse effects at the level of the resistance vessels. As mentioned earlier, prior to the development of obstructive disease in the conduit vessels, hypercholesterolemia impairs the agonist-induced increases in coronary and limb blood flow in hypercholesterolemic animals and humans (Osborne *et al.,* 1989). This impairment of microvascular response has physiological consequences. For example, we have observed that exercise tolerance is reduced in hypercholesterolemic Apo E-deficient mice, in part due to an impairment of exercise-induced increases in limb blood flow (Maxwell *et al.,* 1998). When mice are exercised on a treadmill in a metabolic chamber, the maximal oxygen uptake and the distance run by Apo E-deficient (E^- animals) mice are less than controls. This abnormality appears to be due in large part to a reduced ability to deliver blood to the exercising limbs (Fig. 4). Mice were placed on a treadmill, and fluorescent microspheres were injected (through a cannula inserted from the left carotid artery into the thoracic aorta) at rest or during maximal exercise. Limb blood flow during exercise was reduced in E^- animals. Furthermore, the expected increase in urinary nitrogen oxide (U_{NOx}) after exercise was absent in E^- animals (Fig. 5). ADMA levels are increased in E^- animals, and vascular NO biosynthesis is reduced (as measured in aortic segments by chemiluminescence). Administration of L-arginine (6% solution *ad libitum*) for 1 week prior to exercise enhanced distance run during exercise (by 61%) and elevated U_{NOx} postexercise by fivefold in E^- mice. In contrast, administration of the NOS inhibitor L-nitroarginine (L-NA) to normal mice reduced limb blood flow and distance run during exercise.

To summarize, NO plays a critical role in exercise-induced increases in limb blood flow. These findings are consistent with those of Böger *et al.* (1997a, 1998c) in patients with peripheral arterial disease whereby L-arginine enhances NO biosynthesis (Fig. 6) and limb blood flow.

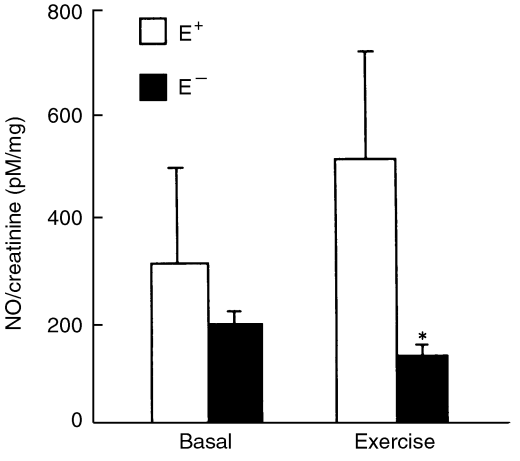


Figure 5 Exercise-induced increase in NO biosynthesis is abrogated in E^- mice (modified from Maxwell *et al.,* 1998, *Circulation* 98(4), 369–374).

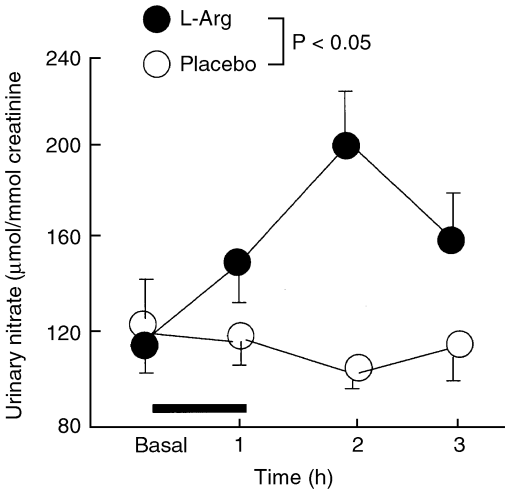


Figure 6 L-Arginine infusion increases urinary nitrate in patients with peripheral arterial disease (modified from Böger *et al.,* 1998c).

In patients with peripheral arterial disease, plasma ADMA levels are elevated two- to fivefold and are correlated to the clinical severity of the disease (Boger *et al.,* 1997a). In patients with critical limb ischemia due to atherosclerosis, an intravenous infusion of L-arginine (30 g over 30 min) increases limb blood flow (approximately twofold) and to the same extent as prostaglandin E_1 (PGE_1) (Maxwell *et al.,* 1998). This is significant because PGE_1 is demonstrably effective in relieving rest pain and healing ischemic ulcers in these individuals (European Working Group on Critical Leg Ischemia, 1991). The observed increase in limb blood flow in these patients is due to the conversion of the exogenous L-arginine to NO, as reflected by parallel increases in urinary nitrogen oxides (Fig. 6) and urinary cGMP (Böger *et al.,* 1997a).

These effects of L-arginine are associated with symptomatic relief in patients with peripheral arterial disease. In a randomized placebo controlled trial, intravenous administration of L-arginine (8 g twice daily) for 2 weeks, improved walking distance by 150%, significantly better than vehicle and the active control PGE_1 (Böger *et al.,* 1998c).

Symptomatic benefits of L-arginine are also observed in individuals with coronary artery disease (CAD) (Lerman *et al.,* 1998; Ceremuzynski *et al.,* 1997). Investigators at the Mayo Clinic reported a double-blind placebo controlled trial of L-arginine (9 g per day) in patients with symptomatic CAD (Lerman *et al.,* 1998). The coronary blood flow response to intracoronary acetylcholine (10^{-6} *M*) was markedly depressed at the start of therapy but was dramatically improved after 6 months of therapy with L-arginine (an absolute increase of 149% compared to 6% for placebo). Angina episodes were reduced by 70% in the L-arginne-treated group after 1 week, an effect that was maintained at 6 months. A similar response to oral L-arginine was observed by Ceremuzynski *et al.* (1997). They observed that in comparison to placebo, treadmill-walking time is significantly increased, as is time to 1 mm ST segment depression in CAD patients given L-arginine capsules orally (6 g p.o. daily) (Lerman *et al.,* 1998).

These data indicate that there is an impairment of the NOS pathway in atherosclerosis that is at least in part mediated by ADMA. Endothelial vasodilator dysfunction is reversed by intravenous or oral administration of L-arginine, which appears to be due to its conversion to nitric oxide. The subsequent improvements in coronary or peripheral blood flow provide symptomatic relief. Therefore, it appears that the elevation in plasma ADMA is of physiological relevance. Accordingly, an understanding of the mechanisms by which ADMA becomes elevated is of scientific merit and potential clinical relevance.

Dysregulation of DDAH: Novel Mechanism for Endothelial Dysfunction

As previously mentioned, plasma ADMA is elevated in patients with renal failure due to reduced clearance. In these individuals, dialysis clears ADMA from the plasma and improves endothelium-dependent vasodilation, as does administration of L-arginine (Hand *et al.,* 1998).

Plasma ADMA is elevated in patients with atherosclerosis, as well as in patients with risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, diabetes mellitus, tobacco exposure, and hyperhomocysteinemia. However, in these individuals the elevation in plasma ADMA levels is likely not due to reduced renal clearance. We have developed evidence that reduced degradation of ADMA may be involved in the observed increase in plasma ADMA levels in hypercholesterolemia and hyperglycemia.

Dimethylarginine dimethylaminohydrolase (DDAH) is the enzyme that degrades ADMA to citrulline and dimethylamine (MacAllister *et al.,* 1996). We have accumulated data indicating that a reduction in DDAH activity may be responsible for the elevation in ADMA observed in conditions where glucose and/or cholesterol are elevated. Specifically we have observed that when cultured endothelial cells are exposed to oxidized LDL cholesterol (ox-LDL, 0–100 μg/ml) or tumor necrosis factor α (TNF-α)(0–200 IU), they elaborate increased amounts of ADMA into the conditioned medium (Fig. 7). During this time there was no change in the expression of DDAH mRNA or protein by Northern or Western analysis, respectively. However, the increase in ADMA accumulation is temporally related to a decline in the activity of the DDAH enzyme (Fig. 8). These data indicate that the increase in ADMA observed in hypercholesterolemia may be due to a decline in its degradation by DDAH (Ito *et al.,* 1999a). In subsequent studies, we extended this observation *in vivo,* demonstrating that DDAH activity is depressed by 50% in the thoracic aorta of NZW hypercholesterolemic rabbits (Ito *et al.,* 1999a).

To summarize, DDAH activity of endothelial cells was decreased to almost 60% of the baseline values by incubation with oxidized LDL or tumor necrosis factor. Similarly,

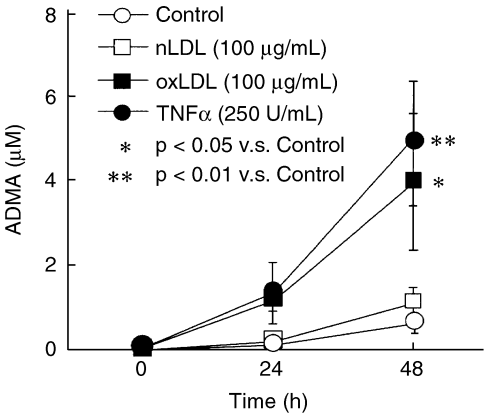


Figure 7 ADMA release by human aortic endothelial cells (HAECs) is increased by oxidized lipoprotein or cytokine stimulation (modified from Ito *et al.,* 1999a, *Circulation* 99, 3092–3095).

DDAH activity was significantly decreased in rabbits fed a high cholesterol diet, in the absence of any changes in the protein expression. These results are similar to those obtained when endothelial cells are exposed to *S*-2-amino-4-(3-methylguanidino)butanoic acid (4124W) (1 m*M*), which inhibits DDAH activity and increases the accumulation of ADMA in the conditioned medium of endothelial cells (MacAllister *et al.,* 1996).

Intriguingly, we observed similar results when endothelial cells are exposed to high glucose conditions. Accordingly, we examined another model of endothelial vasodilator dysfunction, the streptozotocin-treated rat. In these animals, hyperglycemia induced by streptozotocin was associated with an increase in plasma ADMA levels and a decline in DDAH activity in the vessel wall without a change in DDAH expression by Western analysis (Ito *et al.,* 1999b). These studies indicate that the increase in plasma ADMA observed in hypercholesterolemia or hyperglycemia may be due to reductions in DDAH activity. ADMA and DDAH are widely distributed in tissues (Kimoto *et al.,* 1995), and they may provide a mechanism for controlling NO synthesis in physiological and/or pathological states. Our results reveal that

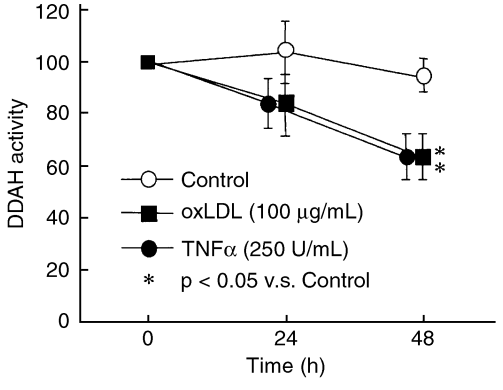


Figure 8 DDAH activity is reduced by oxidized lipoprotein or cytokine stimulation (modified from Ito *et al.,* 1999a, *Circulation* 99, 3092–3095).

lipoproteins or cytokines may increase endothelial elaboration of ADMA by reducing DDAH activity. Decreased activity of DDAH may lead to local accumulation/release of intracellular ADMA and inhibition of NO synthase in disease states including hypercholesterolemia. The increase in ADMA levels may explain the therapeutic benefit of supplemental L-arginine observed in patients with endothelial dysfunction (Lerman *et al.,* 1998).

The mechanism for the depression of DDAH activity has not been delineated. However, DDAH has critical sulfhydryl groups in its structure, and SH-blocking agents such as *p*-chloromercuribenzoate and $HgCl_2$ are known to inhibit enzyme activity (Ogawa *et al.,* 1987). It may be that oxidative stress induced by hypercholesterolemia or hyperglycemia reduces DDAH activity by oxidizing these critical sulfhydryl groups.

Hypertension and NOS

Nitric oxide plays a major role in the regulation of systemic vascular resistance. It is therefore conceivable that endothelial vasodilator dysfunction could precipitate hypertension. Indeed, an endothelial abnormality is associated with hypertension in animal models (Lüscher *et al.,* 1987). Depending on the experimental model, the reduction in endothelium-dependent relaxation is due to an attenuation of endothelium mediated NO-dependent activity or is due to the augmented elaboration of an endothelium-derived contracting factor (possibly a prostanoid). Whether endothelial dysfunction is primary in the initiation of hypertension or is merely an epiphenomenon is not clear. Treatment of the elevated blood pressure normalizes endothelium-dependent relaxation, suggesting that the endothelial abnormality is secondary in the hypertensive process (Cooke and Dzau, 1997a,b; Lüscher *et al.,* 1987; Shultz and Raij, 1989). Conversely, infusions of nitric oxide synthase antagonists produce marked increases in blood pressure in experimental animals (Ribeiro *et al.,* 1992). These inhibitors have been considered nonspecific, and the effect on blood pressure could conceivably be due to an effect on the neuronal NOS. However, more definitive data for a primary role of nitric oxide in the regulation of blood pressure was provided by Huang *et al.* (1995). They found that inactivation of the mouse endothelial nitric oxide synthase gene by homologous recombination produced mice that were significantly hypertensive.

There is extensive evidence for endothelial vasodilator dysfunction in hypertensive humans (Linder *et al.,* 1990; Panza *et al.,* 1990). The most direct evidence has come from measurements of forearm blood flow by strain-gauge plethysmography in response to intra-arterial infusions of endothelium-dependent and -independent vasodilators. In young patients with mild essential hypertension, endothelium-independent vasodilation is relatively undisturbed. In contrast, cholinergic vasodilation (presumably endothelium-dependent) is attenuated. Whether this is a primary or secondary phenomenon is not known. The impairment of en-

dothelium-dependent vasodilation is likely multifactorial and may involve abnormalities of signal transduction, NO activity, or NO biosynthesis. There is preliminary evidence that, at least in some cases, the endothelial deficit may precede the appearance of essential hypertension (Panza *et al.,* 1990). In young normotensive individuals with hypertensive parents cholinergic forearm vasodilation is impaired; in contrast, endothelium-independent vasodilation is normal.

NO and Angiogenesis

In addition to the role of NO as a potent vasodilator, data from our laboratory and others suggest that NO plays a critical role in angiogenesis. First, a number of angiogenic factors are known to stimulate the NOS pathway. Vascular endothelial growth factor (VEGF) stimulates the release of NO from cultured human umbilical venous endothelial cells (HUVECs), as well as upregulates the expression of NOS (Hood *et al.,* 1998; Van der Zee *et al.,* 1997). Vascular segments of rabbit thoracic aorta release NO in response to VEGF; preincubation with L-arginine increases basal and VEGF-stimulated NO release twofold (Murohara *et al.,* 1998a). Transforming growth factor β (TGF-β) upregulates eNOS in bovine aortic endothelial cells (BAECs) and increases NO elaboration as detected by chemiluminescence (Inoue *et al.,* 1995). In the rat, acidic or basic fibroblast growth factor (bFGF) dilates skeletal muscle microvessels and increases blood flow, an effect that is blocked by NOS inhibitors but not by indomethacin (Wu *et al.,* 1996). Similarly, bFGF relaxes porcine coronary arterioles by an NO-dependent mechanism (Tiefenbacher and Chilian, 1997). VEGF-induced vascular permeability is also mediated in part by NO (Murohara *et al.,* 1998a). Second, the angiogenic effect of a number of growth factors is mediated in part by nitric oxide. Human umbilical venous endothelial cells grown in a three-dimensional fibrin matrix form a network of capillary-like tubules under the influence of bFGF or VEGF or TGF-β. The effect of bFGF, VEGF, or TGF-β to induce endothelial tubule formation is abrogated by inhibitors of NOS (Babaei *et al.,* 1998; Papapetropoulos *et al.,* 1997a,b). Furthermore, administration of NO donor agents mimics this effect of angiogenic factors *in vitro* (Babaei *et al.,* 1998; Papapetropoulos *et al.,* 1997b).

The healing of acid-induced ulcerations of the rat stomach is dependent on angiogenesis. Administration of FGF enhances healing, an effect which is associated with increased expression of eNOS; the effect of FGF is blocked by NOS antagonists (Akiba *et al.,* 1997). Administration of L-arginine, but not D-arginine, enhances gastric blood flow and healing of ulcers, effects that are abrogated by antagonists of NOS (Brzozowski *et al.,* 1995, 1997).

Significantly, in conditions associated with reduced NO bioactivity, angiogenesis is attenuated. Vascular explants from rabbit thoracic aorta or human coronary artery will manifest capillary-like outgrowth when placed into a collagen matrix (Chen *et al.,* 1997; Chen and Henry, 1997). The growth of these capillary-like microtubes is inhibited by oxidized LDL cholesterol (Chen *et al.,* 1997; Chen and Henry, 1997), which is also known to reduce NO bioactivity (Kugiyama *et al.,* 1990; Simon *et al.,* 1990). In hypercholesterolemic rabbits, endothelium-dependent nitric oxide-mediated vasodilation is blunted, as is the angiogenic response to hind limb ischemia (Van Belle *et al.,* 1997). More definitively, the angiogenic response to hind limb ischemia is impaired in the eNOS-deficient mice, an effect that cannot be reversed by VEGF (Murohara *et al.,* 1998a).

The mechanisms by which NO mediates angiogenesis are incompletely understood. However, under certain experimental conditions NO enhances endothelial cell proliferation and migration (Morbidelli *et al.,* 1996; Ziche *et al.,* 1994, 1997). NO may enhance migration by increasing endothelial cell podokinesis (Noiri *et al.,* 1998) and/or by increasing the expression of the endothelial integrin $\alpha_v\beta_3$, which is involved in endothelial migration (J. Isner, 1999, personal communication). NO may also increase the production of urokinase-type plasminogen activator (uPA) via its upregulation of bFGF (Ziche *et al.,* 1997). The NO-induced activation of uPA is significant, since migration of endothelial cells requires dissolution of the surrounding extracellular matrix. Finally, the hemodynamic effects of this potent vasodilator may play a role in its angiogenic effects. Increased shear stress (induced by administration of prazosin) in the skeletal microcirculation is associated with increased uptake of bromodeoxyuridine by capillary endothelial cells; this indicates that endothelial cell proliferation may be stimulated by increases in blood flow (Hudlicka, 1998).

ADMA: An Endogenous Antiangiogenic Factor

Studies indicate that endothelium-derived nitric oxide plays a critical role in angiogenesis (see earlier). Accordingly, we hypothesized that ADMA, by inhibiting NOS, may be an endogenous antiangiogenic factor. We have used a disk-angiogenesis system (DAS) to test this hypothesis (Kowalski *et al.,* 1992). The disk is composed of a polyvinyl alcohol sponge covered on both surfaces by filter paper so that vessel ingrowth occurs only from the side. A slow-release pellet containing a pro or anti growth factor can be placed in the center of the disk. The disk is implanted subcutaneously and harvested 2 weeks later. The disk is sectioned and stained, and the surface area of fibrovascular growth (which is directly related to the area of vascular ingrowth) is measured. Figure 9 shows a high-power photomicrograph of the disk revealing endothelium-lined tubes. These capillary-like structures are in continuity with the systemic circulation as indicated by intraluminal leuconyl blue which was injected into the aorta prior to sacrifice (Fig. 9).

In hypercholesterolemic Apo E-deficient mice (E^-), plasma ADMA levels are elevated, and aortic NO synthesis is reduced (Maxwell *et al.,* 1998). In these animals, basal and FGF-stimulated angiogenesis in the disk is attenuated in comparison to normal animals (E^+). Dietary administration of L-arginine normalizes angiogenesis in the hypercholester-



Figure 9 High power photomicrograph of fibrovascular growth into alcohol sponge placed subcutaneously. Note the capillaries outlined by leuconyl blue dye injected systemically prior to sacrifice.

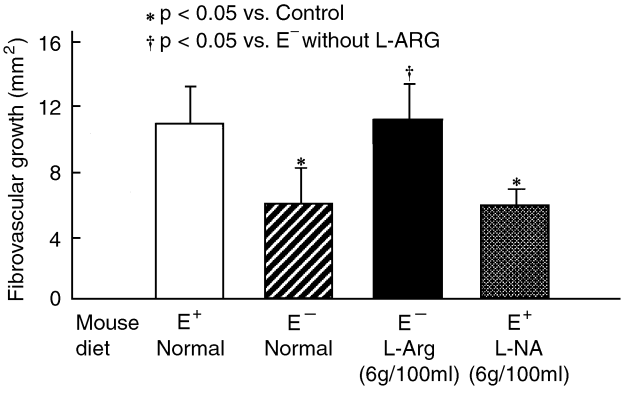


Figure 10 Angiogenesis in subcutaneously implanted disks is inhibited by hypercholesterolemia (Ho *et al.,* 1999). E− stands for Apo E-deficient hypercholesterolemic mice; E+, normal mice; L-Arg, L-arginine added to the drinking water; L-NA, nitroarginine added to the drinking water.

olemic E^- animals (Fig. 10). In contrast, oral administration of the NOS inhibitor L-nitroarginine (L-NA) inhibits angiogenesis in normal (E^+) animals. These studies indicate that NO biosynthesis is required for normal angiogenic response.

More recently, we have examined the role of ADMA in modulating angiogenesis in a murine model of hind limb ischemia. After the surgical induction of hind limb ischemia, normocholesterolemic mice exhibit a 70% reduction in exercise tolerance. Over a period of 7 weeks, the animals recover to their baseline treadmill performance. In association with this recovery, there is an increase in capillary density and an associated improvement in hind limb blood flow as measured by magnetic resonance (MR) perfusion studies. In comparison, the recovery from hind limb ischemia of hypercholesterolemic Apo E-deficient mice is blunted, as assessed by the histological and MR parameters. Oral administration of L-arginine to the animals in their drinking water restores the angiogenic response. In contrast, administration of L-nitroarginine to the normal animals impairs angiogenic response as assessed by the histological and functional measures.

NO and Atherogenesis

In addition to its effects on vascular reactivity and angiogenesis, NO inhibits platelet aggregation, leukocyte adhesion, proliferation of vascular smooth muscle, and endothelial generation of superoxide anion, all of which are key processes in atherogenesis (Bath *et al.,* 1991a,b; Clancy *et al.,* 1992; Garg *et al.,* 1989; Kubes *et al.,* 1991; Niu *et al.,* 1994; Radomski *et al.,* 1987; Stamler *et al.,* 1989). NO exerts its effects on these processes acutely through cGMP-mediated phosphorylation of signaling proteins, but also via cGMP-independent effects on transcriptional events. NO suppresses the transcription of oxidant-sensitive genes, including adhesion molecules mediating monocyte infiltration. NO may exert this effect by (1) combining directly with superoxide anion to form peroxynitrite anion; (2) inhibiting oxidative enzymes and/or terminating the autocatalytic chain of lipid peroxidation, and/or (3) enhancing the expression of mRNA for IκBα, the protein inhibitor of NF-κB (Garg *et al.,* 1989; Hogg *et al.,* 1993; Niu *et al.,* 1994; Spiecker *et al.,* 1997). The antiatherogenic effects of NO may be due in part to its inhibition of oxidative stress.

The interaction of nitric oxide and superoxide anion forms the highly reactive free radical peroxynitrite anion, which is generally deemed to enhance oxidative stress. However, it is possible that peroxynitrite anion could subsequently nitrosate sulfhydryl groups to form *S*-nitrosothiols. This class of molecules is known to induce vasodilation, inhibit platelet aggregation, and interfere with leukocyte adherence to the vessel wall (Radomski *et al.,* 1987; Stamler *et al.,* 1992).

Another mechanism by which NO may ameliorate oxidative stress is by terminating the autocatalytic chain of lipid peroxidation that is initiated by ox-LDL or intracellular generation of oxygen-derived free radicals. Indeed, exogenous NO inhibits copper-induced oxidation of LDL cholesterol, causing a lag in the formation of conjugated dienes (Hogg *et al.,* 1993). Alternatively, NO may directly suppress the generation of oxygen-derived free radicals by nitrosylating, and thereby inactivating, oxidative enzymes. This hypothesis is supported by the observation that the generation of superoxide anion by stimulated neutrophils is reduced by their exposure to exogenous NO (Niu *et al.,* 1994). This is due to the inactivation of NADPH oxidase, a multimeric enzyme with cytosolic and particulate components. The particulate component is vulnerable to nitrosylation by NO (at either its heme moiety or sulfhydryl group), which prevents its association with the cytosolic component and reconstitution of the active enzyme. A similar phenomenon may occur in endothelial cells. This would explain the observation of Niu and colleagues (1994), who reported that antagonism of endogenous NO production increases oxidative stress in HUVECs, as demonstrated with redox-sensitive fluorophores. Furthermore, Pagano and colleagues (1993) showed that exogenous NO donor agents inhibit the generation of

superoxide anion by the endothelium of rabbit thoracic aortas treated *ex vivo* with antagonists of superoxide dismutase.

It is well established that hypercholesterolemia reduces the activity and/or synthesis of endothelium-derived NO (Heistad *et al.,* 1984; McLenahan *et al.,* 1991). In parallel, the endothelium begins to generate superoxide anion (Ohara *et al.,* 1993). It is currently believed that the resulting endothelial oxidative stress triggers a transcriptional cascade that results in the activation of genes encoding molecules that regulate endothelial adhesiveness (Marui *et al.,* 1993; Weber *et al.,* 1994). Cytokines and lysophosphatidylcholine induce the expression by HUVECs of vascular cell adhesion molecule-1 (VCAM-1), an endothelial immunoglobulin implicated in monocyte adhesion and atherogenesis (Cybulsky and Gimbrone, 1991; DeCaterina *et al.,* 1995). This expression is regulated by an NF-κB-mediated transcriptional pathway that is blocked by exposure of the cells to the antioxidant pyrolidinethiocarbamate or aspirin (Marui *et al.,* 1993; Weber *et al.,* 1994).

The cytokine-induced activation of vascular cell adhesion molecule-1 and macrophage colony-stimulating factor in human saphenous vein endothelial cells is suppressed by NO donor agents (DeCaterina *et al.,* 1995; Peng *et al.,* 1995). This effect of NO appears to be due in part to stabilization and/or increased expression of IκBα, which complexes with NF-κB to inhibit its transcriptional activity (DeCaterina *et al.,* 1995).

Zeiher and colleagues (1995) observed that NO inhibits monocyte chemotactic protein 1 (MCP-1) expression in cytokine-stimulated HUVECs in a cGMP-independent fashion. Our data are also consistent with this model of an oxidant-responsive NF-κB-mediated pathway that is modulated by NO.

Because ADMA inhibits eNOS, it is capable of activating the oxidant-sensitive transcriptional pathways that result in endothelium-monocyte interaction. To demonstrate this, we exposed endothelial cells to ADMA or to the synthetic NOS inhibitor L-NMMA (both 0.1–100 μ*M*), each of which induced a concentration-dependent inhibition of NO formation that was reversed by coincubation with L-arginine (1 m*M*). The biologically inactive stereoisomer symmetric dimethylarginine (SDMA) did not inhibit NO release. ADMA or L-NMMA (10 μ*M*) increased endothelial superoxide elaboration (by 110 and 150%, respectively; $p < 0.05$). A similar elevation of superoxide release was induced by 100 mg/dl of native LDL (nLDL) (+140%). ADMA and L-NMMA also stimulated MCP-1 formation by endothelial cells. This effect was paralleled by activation of the redox-sensitive transcription factor NF-κB. Preincubation of endothelial cells with ADMA or L-NMMA increased the adhesiveness of endothelial cells for THP-1 human monocytoid cells in a concentration-dependent manner. ADMA- and L-NMMA-induced monocyte binding was diminished by L-arginine or by a neutralizing anti-MCP-1 antibody. These studies indicated that ADMA, the endogenous NO synthase antagonist, induces an endothelial oxidative stress that activates oxidant-responsive genes involved in monocyte adhesion.

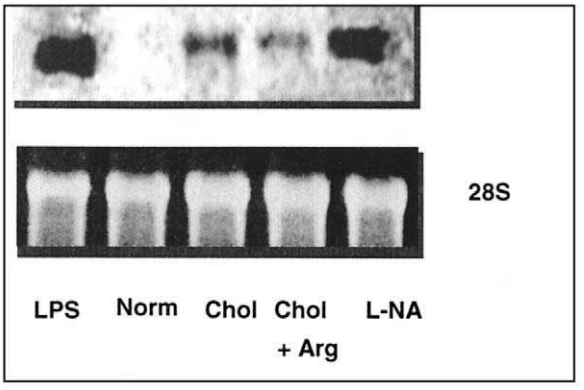


Figure 11 Northern analysis of aortic MRNA (Tsao *et al.,* 1997). Expression of monocyte chemotactic protein (MCP-1) in the rabbit thoracic aorta is increased by feeding the animals a 1% cholesterol diet (CHOL) for 2 weeks, an effect that is partially reversed by the coadministration of L-arginine (CHOL + ARG). Normocholesterolemic animals administered the NOS antagonist L-nitroarginine (L-NA) manifest a marked increase in aortic MCP-1 expression. LPS represents a positive control, namely, an aortic segment from normal animal treated *ex vivo* for 2 hours with bacterial lipopolysaccharide.

NO also regulates oxidant-sensitive gene expression *in vivo* (Fig. 11). We find that in hypercholesterolemic animals, chronic administration of supplemental L-arginine doubles plasma arginine levels and causes a sustained increase in vascular NO generation, as measured *ex vivo* by chemiluminescence (Tsao *et al.,* 1997). In contrast, administration of L-NA suppresses vascular NO generation. Vascular generation of superoxide anion is increased by 2 weeks of a high cholesterol diet, and it is reversed by coadministration of supplemental L-arginine. Hypercholesterolemia increases MCP-1 expression in the thoracic aorta of the NZW rabbit, an effect that is attenuated by administration of L-arginine (Fig. 11) (Tsao *et al.,* 1997). In contrast, inhibition of NO generation by the NOS antagonist L-NA in the rabbits markedly increases vascular expression of MCP-1. Using an *ex vivo* functional binding assay, we demonstrated that hypercholesterolemia increased endothelial adhesiveness for monocytes (Tsao *et al.,* 1994). Administration of L-arginine to the hypercholesterolemic rabbits reduced endothelial adhesiveness, whereas administration of the NOS inhibitor L-NA markedly increased endothelial adhesiveness for monocytes.

Chronic dietary manipulation of NO synthesis affects the progression of lesion formation in hypercholesterolemic animals. Administration of L-arginine to fat-fed NZW rabbits for 10 weeks restores endothelial vasodilator function and markedly inhibits progression of intimal lesions (Fig. 12) (Cooke *et al.,* 1992; Wang *et al.,* 1994). Inhibitory effects of L-arginine on atherogenesis have been confirmed by other investigators in the fat-fed NZW rabbit as well as in the LDL receptor knockout mouse (Aji *et al.,* 1997; Candipan *et al.,* 1996; Cayatte *et al.,* 1994; Davies *et al.,* 1995). In contrast, chronic administration of NOS inhibitors increases endothelium–monocyte interaction and accelerates lesion formation in the fat-fed NZW rabbit (Cayatte *et al.,* 1994; Tsao *et al.,* 1995).

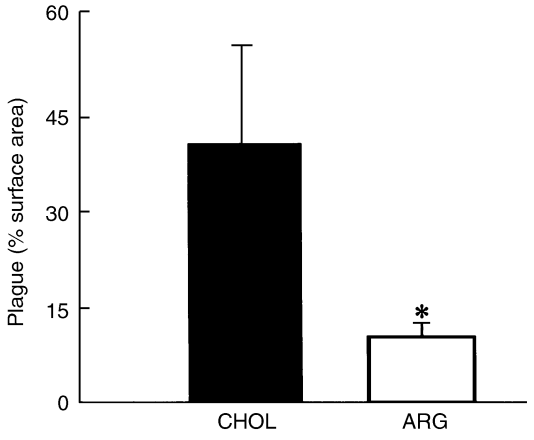


Figure 12 Oral administration of L-arginine decreases plaque area in the hypercholesterolemic rabbit (Cooke *et al.,* 1992). *, Significantly different from CHOL value.

The antiatherogenic effect of L-arginine is at least as potent as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibition in animal models (Böger *et al.,* 1997b). Böger and colleagues (1997b) fed NZW rabbits a 1.0% cholesterol diet for 16 weeks, together with vehicle, lovastatin, or supplemental L-arginine. Lovastatin reduced total cholesterol levels and inhibited lesion formation in the carotid artery by 55%. L-Arginine had no effect on the lipoprotein profile, but it enhanced endothelium-dependent, NO-mediated vasodilation, reduced vascular generation of O_2^-, and inhibited lesion formation by 68%.

Flow, NO, and Vascular Disease

The distribution of atherosclerotic lesions throughout the vascular tree is nonuniform. As early as the nineteenth century, the great pathologists Rokitansky (1952) and Virchow (1860) independently speculated that this nonuniformity was due to local alterations in hemodynamic forces impinging on the vascular tree. Indeed, at sites vulnerable to plaque formation, namely, bends, branches, and bifurcations, unidirectional laminar flow is disturbed, with areas of recirculation characterized by low and fluctuating shear stress (Asakura and Karino, 1990; Cornhill *et al.,* 1985; Glagov *et al.,* 1988; Montenegro and Eggen, 1968; Sinzinger *et al.,* 1980). In these regions of low shear stress, the vulnerability to plaque formation may be a result of enhanced monocyte binding. Adhesive interactions between leukocytes in the vessel wall are less likely to be disrupted under conditions of low shear stress. Furthermore, experimental reductions of blood flow enhance endothelial adhesiveness for monocytes (Walpola *et al.,* 1993, 1995). This may be in part because low-shear regions exhibit enhanced permeability to macromolecules, including LDL. Accumulation and modification of LDL in the vessel wall could trigger a cascade of events leading to changes in the chemotactic and adhesive properties of the endothelium (Berliner *et al.,* 1995; Schwenke and Carew, 1988; Walpola *et al.,* 1995).

Tractive forces of fluid flow also modulate the gene expression of endothelial adhesion molecules and cytokines that participate in monocyte binding. In the NZW rabbit, an adhesion molecule homologous to the human vascular adhesion molecule-1 (VCAM-1) is upregulated by hypercholesterolemia and is expressed at sites of early lesion formation (Cybulsky and Gimbrone, 1991; Li *et al.,* 1993). The expression of VCAM-1 in a murine endothelial cell line is reduced by 75% after 24 hours of exposure to laminar fluid flow (Ohtsuka *et al.,* 1993). The expression of VCAM-1 is regulated in part by oxidant-responsive transcriptional activation (Marui *et al.,* 1993). Oxidized LDL and cytokines induce the expression of VCAM-1 via a transcriptional pathway modulated by NF-κB; this NF-κB-mediated gene expression can be abrogated by antioxidants (Marui *et al.,* 1993).

The most important physiological stimulus of endothelial NO synthesis is blood flow (Cooke *et al.,* 1990, 1991b; Pohl *et al.,* 1986). We and others have demonstrated that the tractive force of fluid flow stimulates the release of endothelium-derived NO and increases the expression of NO synthase. In addition to its major role in flow-mediated vasodilation, the release of NO under conditions of flow may influence circulating blood elements.

Flow-stimulated NO has acute effects on monocyte–endothelial cell interaction, as well as more chronic effects on the expression of genes mediating monocyte adherence. Monocyte adherence to endothelial cells in culture may be inhibited by administration of NO with a short time course that implies an effect on signal transduction of adhesion pathways (Bath *et al.,* 1991b). We have also shown that the adherence of monocytoid cells to bovine aortic endothelial cells is inhibited by brief (i.e., 15-min) exposure to NO donor agents or by increases in endogenous NO in the absence of any changes in expression of VCAM-1 or intracellular adhesion molecule-1 (ICAM-1) (Tsao *et al.,* 1995).

However, chronic exposure of endothelial cells to laminar flow may have a chronic and persisting effect on their adhesiveness for monocytes. We have examined the long-term effects of laminar flow on endothelial adhesiveness for monocytes. Confluent monolayers of human aortic endothelial cells were exposed to static or fluid flow conditions for 4 hours. The medium was replaced, and cells were then incubated with native or oxidized LDL or lipopolysaccharide (LPS) plus TNF-α for an additional 4 hours. Functional binding assays using THP-1 monocytes were then performed. Superoxide production by human aortic endothelial cells was monitored by lucigenin chemiluminescence, and expression of VCAM-1 and ICAM-1 were quantified by flow cytometry. Whereas native LDL had little effect, incubation with either oxidized LDL or LPS/TNF-α significantly increased superoxide production, NF-κB activity, VCAM-1 expression, and endothelial adhesiveness for monocytes. Previous exposure to fluid flow inhibited these sequelae of exposure to cytokines or oxidized lipoprotein. The effect of

fluid flow was due in part to shear-induced release of NO, because coincubation with L-NA completely abolished these effects of flow. These studies indicated that the flow-induced release of NO suppressed endothelial generation of superoxide anion and thereby inhibited an oxidant-sensitive transcriptional pathway for endothelial adhesive molecules (Tsao *et al.,* 1996).

These effects of flow were mimicked by the NO donor agent PAPA-NONOate, whereas a cGMP analog had only a partial effect. This implies that NO exerts its effects in part by cGMP-independent pathways. Similarly, Zeiher *et al.* (1995) found that cGMP analogs did not mimic the effect of exogenous NO donor agents in inhibiting NF-κB activity and MCP-1 expression in cultured endothelial cells. This is in contrast to the *in vivo* work of Kurose and colleagues (1993), who found that the enhanced adhesion of leukocytes to vessels perfused by nitro-L-arginine methyl ester could be completely reversed by 8-Br-cGMP. This discrepancy may be due to other effects of the cGMP analog *in vivo* (e.g., vasodilation with attendant increases in flow or direct effects on monocyte adhesive proteins). However, Kuchan and Frangos (1993) have also demonstrated that the negative regulation of endothelin-1 by flow is dependent on NO and is mimicked by 8-Br-cGMP. The mechanism of action may differ from that in the present study, as the endothelin promoter does not appear to contain an NF-κB consensus sequence.

It is interesting to note that the effects of fluid flow seen in the study by Tsao *et al.* (1996) persisted even after cessation of flow. In contrast, NO production quickly falls to baseline levels after cessation of fluid flow. Kanai *et al.* (1995) used a porphyrinic microsensor to detect NO elaborated by cultured endothelial cells in response to fluid flow. With cessation of the flow stimulus, the NO concentration in the conditioned medium declined at a rate that would be predicted by oxidative degradation of the NO released into the medium during flow. This observation suggests that NO elaboration ceases instantaneously with discontinuation of the flow stimulus. However, in the study by Tsao *et al.,* the effect of flow on endothelial adhesiveness persisted for at least 4 hours. We speculate that this persistent effect may be due to the inactivation by NO of oxidative enzyme activity, which probably has a long time constant, given the extraordinary affinity of NO for heme proteins.

We found that the effects of flow to inhibit NF-κB-mediated VCAM-1 expression and endothelial adhesiveness could be accounted for by elaboration of endogenous NO. The effect of NO on NF-κB and IκB, however, may not totally explain the effects of flow on VCAM-1 expression or monocyte binding. VCAM-1 promoter activity has been demonstrated to be under the complex control of both stimulatory and inhibitory *trans*-activating factors. Moreover, deletion analysis has demonstrated that NF-κB binding activity is important for optimal stimulation of the VCAM-1 promoter but is not essential for VCAM-1 transcription. In addition, NF-κB activity does not fully explain the effect on adhesion molecule expression, since ICAM-1 expression was only minimally affected by NO. Other nuclear binding proteins may dominate in cytokine- or lipid-induced ICAM-1 expression. Furthermore, the ICAM-1 promoter contains the shear stress responsive element (SSRE) GAGACC first defined by Resnick *et al.* (1993). This SSRE may contribute to the positive regulation by shear stress of ICAM-1 that we have observed. This same SSRE is found in the promoter region of a number of genes regulated by flow, including endothelial cell NOS (Marsden *et al.,* 1993; Nishida *et al.,* 1992). Therefore, mechanisms independent of NO may contribute to the effects of flow on endothelial adhesiveness. For example, flow enhances the release of prostacyclin and increases the expression of superoxide dismutase, which might also contribute to the flow-induced inhibition of endothelial adhesion (Frangos *et al.,* 1985; Inoue *et al.,* 1996; Kubes *et al.,* 1991).

Nevertheless, the acute and chronic effects of NO on endothelial adhesiveness may play an important role in atherogenesis. The effect of habitual exercise in inhibiting atherosclerotic lesion formation (Haskell *et al.,* 1994; Richard *et al.,* 1990) may be due in part to the effect of exercise in enhancing the vascular expression of endothelial cell NOS and elaboration of NO (Sessa *et al.,* 1994). Similarly, the predisposition to lesion formation at sites of branching may be due in part to the reduced elaboration of NO at these sites (McLenahan *et al.,* 1991). This speculation is supported by our previous observations that enhancement of vascular NO activity in the hypercholesterolemic rabbit inhibits endothelial adhesiveness for monocytes and reduces lesion formation (Cooke *et al.,* 1992; Tsao *et al.,* 1994; Wang *et al.,* 1994). In contrast, chronic administration of NOS antagonists enhances endothelial adhesiveness and increases monocyte accumulation in the vessel wall (Cayette *et al.,* 1994; Naruse *et al.,* 1994). Similar effects of NO on endothelial cell–neutrophil interactions have been observed in hypercholesterolemia (Lefer and Ma, 1993). Taken together, these studies implicate NO as an endogenous antiatherogenic molecule, which exerts its effects in part via its modulation of oxidant-responsive transcriptional pathways. Derangements in the NOS pathway, induced by various risk factors, impair the regulation by NO of the oxidant responsive transcriptional pathway, leading to the expression of adhesion molecules and chemokines involved in monocyte adhesion (Fig. 13).

Regression and Progression: Dependency on NO

In 1996 we published our findings that administration of L-arginine to hypercholesterolemic rabbits with preexisting lesions can induce apparent regression of preexisting intimal lesions (Candipan *et al.,* 1996). Specifically, NZW rabbits were fed a 0.5% cholesterol diet for 10 weeks, at which time they exhibited intimal lesions occupying 30% of the surface area of the thoracic aorta. Subsequently, animals received L-arginine (2.25%) or vehicle in their drinking water while the 0.5% cholesterol diet was continued. Thoracic aortas

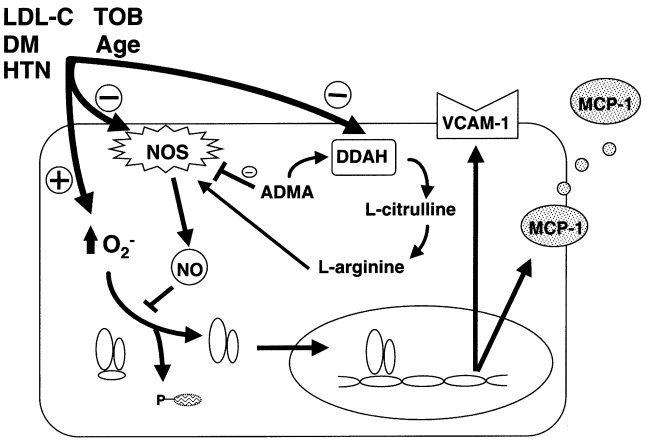


Figure 13 Cardiovascular risk factors may induce atherogenesis, in part, by modulation of the nitric oxide pathway. DM, diabetes mellitus; HTN, hypertension; TOB, tobacco; NOS, NO synthase; ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; VCAM-1, vascular cell adhesion molecule; MCP-1, monocyte chemotactic protein.

were harvested at subsequent weeks for vascular reactivity studies and histomorphometry. In those animals receiving vehicle, there was a progressive impairment of endothelium-dependent vasodilation and an increase in lesion surface area (to 60% of the thoracic aorta) over the course of weeks 10 to 23. In contrast, in the L-arginine-treated group, there was an improvement in endothelium-dependent vasodilation and a significant reduction in lesion surface area. Indeed, after 23 weeks, those L-arginine-treated animals that still manifested an improvement in endothelium-dependent NO-mediated vasodilation exhibited a lesion surface area of only 5% (Candipan *et al.,* 1996).

This study suggested to us that enhancement of vascular NO activity could induce regression of preexisting intimal lesions. We hypothesized that NO-induced regression may be mediated by apoptosis of cells in the intimal lesion. To test this hypothesis, male NZW rabbits were fed a 0.5% cholesterol diet for 10 weeks to develop intimal lesions. After 10 weeks L-arginine hydrochloride (2.5%) was added to the drinking water, and the cholesterol diet was continued for 2 weeks, at which time the aortas were harvested for histological studies. L-Arginine treatment increased the number of apoptotic cells (largely macrophages) in the intimal lesions by threefold. In subsequent studies, aortas were harvested for *ex vivo* studies. Aortic segments were incubated in cell culture medium for 4 to 24 hours with modulators of the NO synthase pathway. The tissues were then collected for histological studies, and the conditioned medium was collected for measurement of nitrogen oxides by chemiluminescence. Addition of sodium nitroprusside (10^{-5} mol/liter) to the medium caused a time-dependent increase in apoptosis of vascular cells (largely macrophages) in the intimal lesion. L-Arginine (10^{-3} mol/liter) had an identical effect on apoptosis, which was associated with an increase in nitrogen oxides released into the medium. These effects were not mimicked by D-arginine, and they were antagonized by the NO synthase inhibitor L-NA (10^{-4} mol/liter). The effect of L-arginine was not influenced by an antagonist of cGMP-dependent protein kinase, nor was the effect mimicked by the agonist of protein kinase G or 8-Br-cGMP. These studies indicate that supplemental L-arginine induces apoptosis of macrophages in intimal lesions by its metabolism to NO, which acts through a cGMP-independent pathway (Wang *et al.,* 1999).

Apoptosis occurs in vascular cells of human atherosclerotic plaques (Bennet *et al.,* 1995; Björkerud and Björkerud, 1996; Geng and Libby, 1995; Han *et al.,* 1995; Isner *et al.,* 1995; Kockx *et al.,* 1996). Factors involved in the initiation and regulation of apoptosis in atherosclerosis have not been fully elucidated, but immunohistochemical studies provide evidence for several proteins known to participate in apoptosis, including p53 and interleukin-1β-converting enzyme (Isner *et al.,* 1995; Messmer *et al.,* 1994; Messmer and Brune, 1995). Among the myriad pathways that may be involved, there is accumulating evidence to implicate L-arginine/NOS. Cytokine-mediated activation of inducible NOS (iNOS) induces apoptosis of macrophages and vascular smooth muscle cells *in vitro* (Geng *et al.,* 1996a,b; Albina *et al.,* 1993). The effect of iNOS activation *in vitro* is augmented by additional L-arginine and attenuated by antagonists of NOS. In our study, L-arginine-enhanced apoptosis was associated with increases in the level of NO_x released into the medium (Wang *et al.,* 1999). The involvement of the NOS pathway was also indicated by the observation that the effect of L-arginine was blocked by an antagonist of NOS.

Although the evidence in this study supports a role for NO in apoptosis of macrophages, it should be acknowledged that NO has been shown to have antiapoptotic effects as well. NO donor agents have been demonstrated to inhibit apoptosis of cultured endothelial cells and lymphocytes (Dimmeler *et al.,* 1997; Mannick *et al.,* 1994). The effect of NO as a modulator of apoptosis appears to be cell specific and contextual (e.g., dependent on the presence of certain cytokines, growth factors, or oxidative stress) (Geng and Libby, 1995).

It is likely that iNOS expressed by cells within the lesion is responsible for the effect of L-arginine to induce apoptosis of lesion cells and regression. Indeed, previous immunohistochemical studies have detected iNOS in the intimal macrophages and vascular smooth muscle cells of human atherosclerotic plaques (Buttery *et al.,* 1996). Under these conditions, vascular cells also produce superoxide anion (Mügge *et al.,* 1991; Ohara *et al.,* 1993; White *et al.,* 1994). In this milieu, the product of iNOS is quickly transformed into peroxynitrite anion, a highly reactive free radical (Beckman and Koppenol, 1996). Peroxynitrite anion is cytotoxic and may induce apoptosis initially by causing DNA strand fragmentation (Nguyen *et al.,* 1992). Peroxynitrite anion can also affect cell function by nitrosating tyrosine residues that are involved in the signal transduction of transmembrane receptors (Martin *et al.,* 1990). Using monoclonal antibodies directed against nitrotyrosine, evidence of peroxynitrite formation has been observed in human atherosclerotic plaques

(Beckman *et al.,* 1994; Mügge *et al.,* 1991). This is relevant to the observation of L-arginine-induced regresion, because peroxynitrite anion may be the NO species mediating macrophage apoptosis.

Previous studies have suggested that apoptosis of macrophages induced by iNOS activity is independent of cGMP (Messmer *et al.,* 1995). Consistent with this observation is our finding that the various manipulations of the cGMP pathway did not influence apoptosis in the lesions of this animal model. In contrast, in cultured vascular smooth muscle cells, NO donor agents induce apoptosis by a cGMP-dependent mechanism (Pollman *et al.,* 1996).

The activation of iNOS may have complex effects on the evolution of atherosclerotic plaques. By inducing cell death, iNOS activation may contribute to the development of the ''necrotic core'' of complex lesions. One might also speculate that iNOS may be involved in the characteristic atrophy of the media beneath atheroma or the dissolution of the fibrous cap by activated macrophages. NO or peroxynitrite anion produced by activated macrophages could induce apoptosis of vascular smooth muscle (Dimmeler *et al.,* 1997; Rajagopalan *et al.,* 1996). Furthermore, NO or peroxynitrite anion may reduce collagen formation by vascular cells and activate metalloproteinases, which degrade extracellular matrix (Kolpakov *et al.,* 1995; Rajagopalan *et al.,* 1996).

These actions of peroxynitrite anion would contribute to plaque instability and have led some investigators to explore antagonism of iNOS as a potential therapeutic avenue in atherosclerosis. However, it is likely that such a strategy would have unintended consequences. Antagonism of iNOS activity could promote platelet aggregation, leukocyte adherence, vasoconstriction, and proliferation of vascular smooth muscle cells and macrophages. It should be considered that iNOS may be in fact a countervailing force in the accretion of atherosclerotic plaque. Furthermore, by reducing proliferation and by promoting apoptosis of macrophages in the lesion, iNOS activation may lead to plaque stabilization and even regression, as suggested by our observations. It is worthy of emphasis that both macrophages and vascular smooth muscle cells contribute to the intimal lesion in the balloon-injured hypercholesterolemic rabbit, but in our observations of this animal model, apoptosis was largely observed in the central, macrophage-rich area of the lesions. Further evidence to support a role for iNOS as a beneficial force in atherogenesis comes from studies of the iNOS-deficient mouse. In these animals, transplant atherosclerosis is accelerated, indicating that NO derived from iNOS (as well as that derived from eNOS) has beneficial effects on the progression of atherosclerosis, possibly by curtailing inflammation and/or cell proliferation.

Restenosis and NOS

The long-term benefit of coronary balloon angioplasty and atherectomy is limited by the considerably high occurrence of restenosis (40 to 50%) 3 to 6 months after the procedure (Holmes *et al.,* 1984). Restenosis is in part due to myointimal hyperplasia, a process characterized by vascular smooth muscle cell migration and proliferation (Forrester *et al.,* 1991; Popma *et al.,* 1991). A decrease in total vessel area termed ''negative remodeling'' or shrinkage also contributes to the luminal encroachment (Kakuta *et al.,* 1994; Post *et al.,* 1994). Medical therapies to prevent smooth muscle cell proliferation have been uniformly unsuccessful, whereas vascular stenting to obtain optimal lumen gain and inhibition of remodeling has modestly reduced the restenosis rate (Fischman *et al.,* 1994; Serruys *et al.,* 1994, 1996). However, intimal thickening still plays a significant role in stent restenosis.

The architecture of the vessel wall is remodeled in response to changes in the balance of paracrine factors that regulate the proliferation and biosynthetic activity of vascular cells. One of the substances that participates in this vascular homeostasis is NO. In addition to relaxing smooth muscle cells, NO inhibits their proliferation (Bath *et al.,* 1991b). As mentioned previously, NO also inhibits the interaction of circulating blood elements with the vessel wall (Bath *et al.,* 1991a,b; Kubes *et al.,* 1991; Radomski *et al.,* 1987; Stamler *et al.,* 1989; Tsao and Lefer, 1990). These effects of vascular NO may explain the observation that long-term administration of L-arginine inhibits myointimal hyperplasia after balloon injury (McNamara *et al.,* 1993; Hamon *et al.,* 1994). Because the endothelium is removed at the time of the intervention and is not fully regenerated at the site of injury for several weeks in this animal model, it is not likely that endothelium-derived NO is responsible for the effect of intramural L-arginine on myointimal hyperplasia. It is more likely that the activity of inducible NO synthase expressed by vascular smooth muscle cells after injury is inhibiting their proliferation. We have directly tested the hypothesis that generation of NO by vascular smooth muscle cells *in vivo* can inhibit their migration and/or proliferation. After balloon-catheter injury, transfection of vascular smooth muscle cells with a plasmid construct containing NO synthase (but not the control vector) enhanced NO generation locally and inhibited myointimal hyperplasia (Von der Leyen *et al.,* 1995).

More recently, in a hypercholesterolemic rabbit model of balloon angioplasty, we have observed that a single intramural administration of a concentrated solution of L-arginine could affect vascular function and structure after balloon angioplasty of the iliac artery. Specifically, administration of L-arginine (800 mg/5 ml over 15 min) with the use of a drug-delivery catheter after angioplasty enhanced vascular NO generation (by fivefold as long as 1 week after the angioplasty), and it inhibited lesion formation by about 60% 4 weeks after the procedure (Schwarzacher *et al.,* 1997). This study is consistent with earlier observations that oral administration of L-arginine suppresses myointimal hyperplasia after vascular injury (McNamara *et al.,* 1993; Tarry and Makhoul, 1994).

After vascular injury and denudation of the endothelium, adjacent endothelial cells begin to proliferate and migrate

into the area of injury. The endothelial cells that resurface the denuded area initially appear morphologically distinct from endothelial cells in noninjured segments of the vessel. The regenerating endothelial cells are cuboidal rather than flat, and they are not aligned with flow. They are also functionally abnormal. Endothelium-dependent NO-mediated vasorelaxation is reduced after vascular injury (Creager *et al.,* 1990; Shimokawa *et al.,* 1987; Verbeuren *et al.,* 1986; Weidinger *et al.,* 1990). The impairment of endothelium-dependent vasodilation is associated with increased intracellular levels of ADMA (Azumi *et al.,* 1995).

ADMA: A Novel Risk Factor for Atherosclerosis?

These preclinical studies pose an intriguing question: Does the reduced activity or elaboration of vascular nitric oxide participate in the progression of atherosclerosis or restenosis in humans? It is quite possible that with a loss of vascular NO activity the processes of inflammation, adhesion, and proliferation would be favored to cause progression of vascular disease and even destabilization of atherosclerotic plaques. Some tantalizing preliminary evidence for this hypothesis is now forthcoming. In patients with coronary artery disease, endothelial vasodilator dysfunction (as assessed by coronary reactivity to acetylcholine) is predictive of vascular events (Murakami *et al.,* 1998). Data from our laboratory are also supportive of this hypothesis. Specifically, in collaboration with Imaizumi and co-workers, we have examined the relationship between plasma ADMA and atherosclerosis in humans (Miyazaki *et al.,* 1999). To identify risk factors in atherosclerosis, subjects ($n = 116$, age 52 ± 1 years, male:female ratio of 100:16) underwent a history and physical examination; determination of serum chemistries and ADMA levels; and duplex scanning of the carotid arteries to quantitate intimal–medial thickness (IMT) (Fig. 14). These individuals had no symptoms of coronary or peripheral artery disease and were taking no medications. Univariate analyses of the effects of each potential risk factor or ADMA on IMT were performed with linear regression for continuous variables (age, systolic, diastolic and mean blood pressure, total cholesterol, response to oral glucose tolerance test [total glucose], and ADMA) and with one-way analysis of variance for categorical variables (smoking and family history). The interaction between risk factors, ADMA, and IMT was then examined using multiple stepwise regression analysis. Multivariate analyses revealed that plasma levels of ADMA were positively correlated to age ($p < 0.0001$), mean arterial pressure, ($p < 0.0001$), and total glucose ($p < 0.0006$) (Fig. 15). Most intriguingly, ADMA levels were better correlated to IMT of the carotid artery ($r = 0.51$, $p < 0.0001$) than to all traditional risk factors including age, arterial blood pressure (systolic, diastolic, or mean arterial pressure), cholesterol (total, LDL, HDL, or triglycerides), plasma glucose (fasting level or AUC after an oral glucose tolerance test), tobacco use, or family history. After stepwise multiple regression analysis, IMT was significantly correlated with age and ADMA only. The correlation between ADMA and IMT was still significant even after adjusting for age ($r = 0.33$, $p = 0.0003$). This study suggests that plasma ADMA may be a novel risk factor for atherosclerosis (Miyazaki *et al.,* 1999). Intriguingly, another study revealed that tissue ADMA is elevated in human atheroma (Matsuoka *et al.,* 1998).

These studies raise the intriguing question of whether enhancement of NO synthesis in humans may have a favorable effect on the progression of vascular disease. Although there is as yet no published evidence that L-arginine therapy prevents progression and/or stabilizes lesions, there is evidence in humans that L-arginine therapy inhibits cellular processes involved in atherogenesis. Specifically, several investigators have shown that L-arginine can inhibit the adhesiveness of peripheral blood mononuclear cells and can inhibit platelet aggregation. The effects on platelet aggregation are modest, but the effect on mononuclear cell adherence is dramatic.

Previous preclinical studies had shown that NO inhibits monocyte and platelet adhesion. Endothelium-derived NO

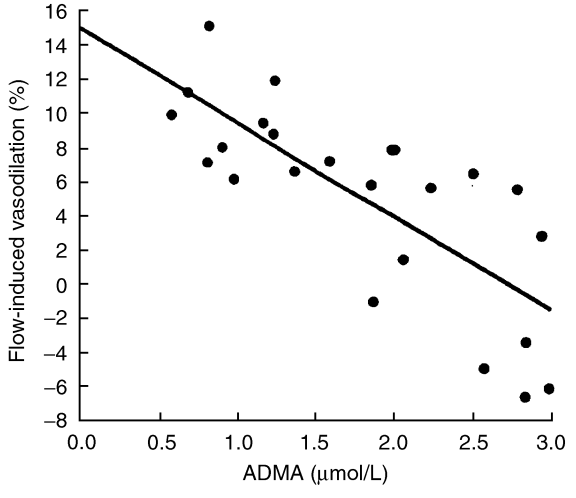


Figure 14 ADMA is negatively correlated with flow-mediated vasodilation of the human brachial artery (Böger *et al.,* 1998b).

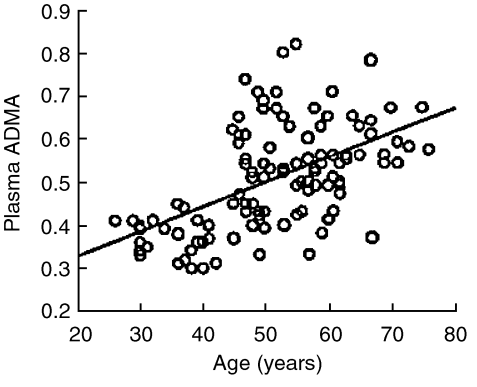


Figure 15 ADMA is positively correlated with risk factors for carotid atherosclerosis, including age (Miyazaki *et al.,* 1999).

released into the lumen increases platelet cGMP and inhibits platelet aggregation (Kowalski *et al.,* 1992; Pohl and Busse, 1989; Radomski *et al.,* 1987). Accordingly, NO modulates the reactivity of circulating leukocytes and platelets. In hypercholesterolemic (HC) humans, platelet and monocyte adhesiveness is increased. We hypothesized that this might be due in part to a deficit of endothelium-derived NO.

Accordingly, we performed a study to determine the role of ADMA and NOS inhibition in the increased mononuclear cell adhesiveness observed in human hypercholesterolemia. Plasma ADMA levels were determined by HPLC. Functional mononuclear leukocyte adhesion assays were performed in parallel, and flow cytometry was used to characterize bound monocytes and T lymphocytes. Hypercholesterolemic patients were then placed on an oral L-arginine regimen of 14 or 21 g/day and studied over 12 weeks.

Plasma ADMA levels and adhesiveness of mononuclear cells (specifically monocytes and T lymphocytes) were elevated in hypercholesterolemic patients. Adhesiveness was inversely correlated with the plasma L-arginine/ADMA ratio. Oral administration of L-arginine normalized plasma L-arginine/ADMA ratios and attenuated monocyte and T-lymphocyte adhesiveness. These studies revealed that the adhesiveness of circulating monocytes and T cells was augmented by an increase in plasma ADMA level. To determine if this was a direct effect of ADMA on mononuclear cells, or if it was mediated by a suppression of eNOS, we studied the effect of ADMA on isolated monocytes and on those cocultured with endothelial cells. Transformed human umbilical vein (ECV 304) aortic endothelial cells were incubated with varied concentrations of ADMA. Monocytoid cells were cocultured with these cells, and monocytoid cell adhesiveness assessed using a binding assay. Flow cytometry was used to quantitate adhesion molecule expression.

ADMA had no direct effect on the adhesiveness of mononuclear cells. However, monocytes became hyperadhesive when cultured in the conditioned medium from ADMA-exposed endothelial cells.

These studies revealed that in human hypercholesterolemia, the plasma L-arginine/ADMA ratio is inversely correlated with mononuclear cell adhesiveness. Restoration of the L-arginine/ADMA ratio to control levels normalizes mononuclear cell adhesiveness. The elaboration of endothelium-derived NO affects the behavior of circulating T lymphocytes and monocytes, and inhibition of endothelial cell NO production by ADMA leads to hyperadhesive T lymphocytes and monocytes (Chan *et al.,* 2000; Theilmeier *et al.,* 1997).

Monocytes and T lymphocytes are the predominant inflammatory cells found in atherosclerotic plaques. Indeed, the adhesion of mononuclear cells to the endothelium is a key initial event in atherogenesis preceding the formation of fatty streaks (Libby and Hansson, 1991; Ross, 1993; Gimbrone, 1995). In hypercholesterolemic humans, peripheral blood mononuclear cells exhibit increased adhesiveness for endothelial cells in *ex vivo* adhesion assays (Jongkind *et al.,* 1995; Theilmeier *et al.,* 1997). Differences in monocyte surface markers have been detected by flow cytometry between hypercholesterolemic and normocholesterolemic humans, although the functional significance is unclear (Rothe *et al.,* 1996). Molecular signaling mechanisms for the altered behavior of mononuclear cells are likely multifactorial and may involve inflammatory cytokines (Stemme and Hansson, 1994), low density lipoprotein (Berliner *et al.,* 1990; Couffinhal *et al.,* 1993), platelet-activating factor (Lorant *et al.,* 1996), circulating soluble adhesion molecules (Hackman *et al.,* 1996), and reduced bioactivity of endothelium-derived NO.

Nitric oxide has been shown to modulate the behavior of circulating blood elements. *In vivo,* NO inhibits leukocyte adherence in the early stages of hypercholesterolemia in the rat (Gauthier *et al.,* 1995). Furthermore, endothelium-derived NO is able to increase cGMP and reduce the ability of platelets to aggregate as they pass through the microvasculature of the rabbit heart (Pohl and Busse, 1989). In a clinical study of hypercholesterolemic and age- and gender-matched subjects, platelet aggregation to ADP was assessed. Hypercholesterolemic subjects manifested platelet hyperaggregation. In these individuals, administration of L-arginine (8.4 g daily) attenuated platelet aggregation by over 25% (Wolf *et al.,* 1997). Similar effects of treatment with oral L-arginine on platelet and monocyte reactivity (Adams *et al.,* 1995, 1997) have been reported by other investigators. These observations suggest that endothelium-derived NO, released into the lumen, plays an important role as a modulator of leukocyte and platelet function. Changes in the production or bioactivity of endothelium-derived NO can thus be expected to modulate the adhesiveness of circulating blood cells.

Using an independent method, we confirmed observations made by Jongkind *et al.* (1995) that peripheral blood monocytes from hypercholesterolemic patients are hyperadhesive in *ex vivo* adhesion assays (Fig. 16) (Theilmeier *et al.,* 1997). In addition, we found that T (but not B) lymphocytes also exhibit increased adhesiveness in hypercholesterolemia. The increased adhesiveness of circulating

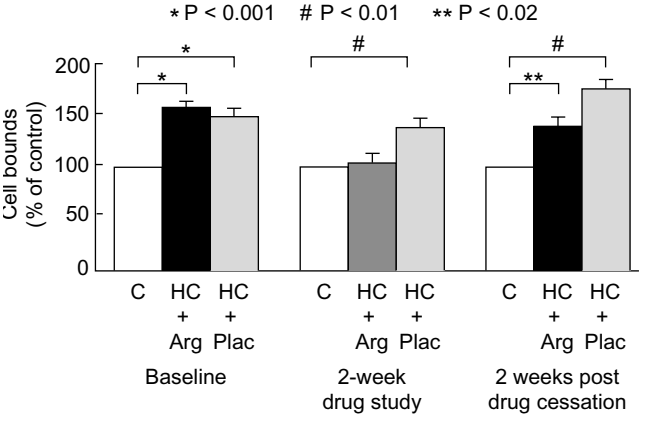


Figure 16 Oral L-arginine reduces mononuclear cell adhesion in hypercholesterolemic individuals (modified from Theilmeier *et al.,* 1997).

monocytes and T lymphocytes in hypercholesterolemic individuals is consistent with the observation that both monocytes and T cells are present in atherosclerotic plaques and adhere to the endothelium in regions predisposed to atherosclerosis before the formation of fatty streaks (Hansson *et al.,* 1991).

Pharmacokinetics, Pharmacoefficacy, and Safety of Oral L-Arginine Administration in Humans

In the reported studies of L-arginine administration and its effects on human vascular biology, no significant adverse effects have been reported. In our own studies, L-arginine supplementation (9 g/day) caused a slight rise in blood urea nitrogen from 14 to 19 μg/dl. No other changes in serum chemistries, complete blood count, or lipid profile have been observed. No side effects of L-arginine have been reported by the subjects, except for one case of recurrence of oral herpes lesions, which resolved after discontinuance of L-arginine. However, to gain additional information regarding the clinical administration of L-arginine we have performed pharmacokinetic and pharmacoefficacy studies in different populations.

To learn more about the pharmacokinetics of oral L-arginine, in one study (Tangphao *et al.,* 1999a), eight healthy males (age 43 ± 4 years) with no cardiovascular risk factors were admitted to the General Clinical Research Center at Stanford University in the morning, after an overnight fast, for a 24-hour period of an L-arginine-free diet (diet A) or a diet with a fixed amount of L-arginine intake (3.8 g; diet B), during a 2-day cross-over study. Subjects were randomly selected to begin with diet A or B, and on the second day consumed the other diet. During diet A, plasma L-arginine fell from 21.4 ± 2.0 to 11.9 ± 1.1 μg/ml. This study indicated that plasma L-arginine concentrations manifest a diurnal rhythm that is influenced by dietary intake.

A second study was performed to determine if supplemental high-dose L-arginine (14 g/day) was tolerable (Tangphao *et al.,* 1999b), if it would maintain a persistent elevation of plasma L-arginine, and if it would produce a sustained therapeutic effect. We studied 24 hypercholesterolemic (HC) patients (LDL-C 175 ± 5 mg/ml; age 45 ± 3 years) and 18 normocholesterolemic individuals (LDL-C 110 ± 6 mg/ml; age 42 ± 3 years). In HC subjects, basal plasma ADMA levels were elevated by over 60% (2.1 ± μ*M*), whereas plasma L-arginine levels (59 ± 9 μ*M*) were not different. L-Arginine was administered at 14 g/day for 12 weeks to the HC individuals, and plasma L-arginine, ADMA, serum biochemistries, and monocyte studies were performed at 4-week intervals. Oral L-arginine supplementation induced a 60% increase in plasma L-arginine levels, which was sustained throughout the 12-week study. Notably, the plasma ADMA concentration did not change during the study. Furthermore, basal levels of plasma insulin, growth hormone, and glucagon did not increase during L-arginine treatment. There were no significant alterations in serum biochemistries and hematology studies (except for a clinically insignificant increase in blood urea nitrogen), and no adverse events were reported. Mononuclear cell adhesiveness was increased by 47% in HC individuals and was highly correlated to the ADMA/arginine ratio ($r = 0.62$, $p < 0.0005$). Arginine treatment normalized mononuclear cell adhesiveness, an effect that was maintained for the entire 12-week study period. Together, these studies suggest that high-dose L-arginine is safe in this population of patients, demonstrably increases plasma arginine, and has effects on platelet and monocyte behavior that may be clinically beneficial.

An L-Arginine-Enriched Medical Food for Vascular Disease

As described earlier, there is compelling evidence for the safety and potential benefit of L-arginine in cardiovascular disease. However, the dose of L-arginine required to increase plasma L-arginine levels and to enhance the synthesis of endothelium-derived nitric oxide is in the range of 6–9 g per day. Administered orally in the usual 500 mg tablets, this represents 12–18 capsules daily. The compliance with this many pills would not be high, and in this form, L-arginine therapy is impractical. Another approach would be to increase the intake of dietary L-arginine. The average American diet contains 5.4 g of L-arginine (Visek, 1986). This average intake must be supplemented by 6 to 9 g L-arginine daily to obtain measurable increases in plasma L-arginine and (in hypercholesterolemic individuals) to enhance endothelium-dependent vasodilation and indices of NO biosynthesis (Theilmeier *et al.,* 1997; Böger *et al.,* 1998b). One could increase L-arginine in the diet by selectively using arginine-rich foods. Some foods rich in L-arginine (e.g., red meat) contain saturated fat, which adversely influences endothelial function, and these would not be a good source of L-arginine. Preferably, one should use foods rich in L-arginine (e.g., fish, nuts, legumes, soy protein) that contain other agents that can enhance endothelial function (e.g., eicosapentanoic acid, vitamin E, phytoestrogens) or have other beneficial effects on vascular structure.

A complementary approach is to provide an enriched source of dietary L-arginine. To do so, we developed an L-arginine enriched nutrient bar for the dietary treatment of vascular disease. Because endothelial vasodilator dysfunction is multifactorial and depends on the underlying disorder, it seemed most practical that the bar contain other agents that have been shown to enhance endothelial function. Therefore, in addition to the L-arginine (3.3 g), the bar contains vitamins C (250 mg), E (200 IU), B_6 (2 mg), B_{12} (5 μg), folate (200 μg), and niacin (25 mg) in a soy protein base.

To determine if this medical food could enhance endothelial vasodilator function, we performed a study in 43 patients with hypercholesterolemia (LDL-C > 160 mg/dl). To assess endothelial function we measured flow-mediated vasodilation of the brachial artery as assessed by high-resolution ultrasound before and after 1 week of treatment with the nutrient bar (two per day) or a placebo control. The placebo

bar contained no vitamins, contained no added L-arginine, and consisted of a whey protein base (whey protein is arginine-poor, with less than 1% arginine residues, whereas soy protein is arginine-rich, at 7% arginine). This study revealed that the L-arginine-enriched bar, but not the placebo bar, normalized flow-mediated vasodilation in middle-aged hypercholesterolemic individuals.

On the basis of these data and the evidence that L-arginine could enhance vascular NO biosynthesis and improve limb blood flow in animal models and in patients with peripheral arterial disease (Bode-Böger *et al.,* 1996a), we studied the effect of the medical food in patients with symptomatic arterial occlusive disease of the extremities. Accordingly, 41 patients with peripheral artery disease underwent treadmill testing (Gardner protocol) and quality-of-life questionnaires (SF-36) before and 2 weeks after treatment with zero, one, or two arginine-enriched nutrient bars daily in a placebo-controlled randomized trial. Patients were studied after an overnight fast (i.e., trough level).

Over the course of 2 weeks of treatment there was no change in lipid profile or serum chemistries, and the bar was well tolerated. Pain-free walking distance improved 66% (over 100 m) in the two bars per day group, and absolute walking distance improved by over 60 m (Fig. 17). Quality of life measures improved significantly in the two bars per day group, but were unchanged in the other two groups. The beneficial effects of the nutrient bar were likely due to the L-arginine content, because a portion of the subjects in the placebo group received a placebo bar containing all the other vitamins of the active bar. However, the soy protein base of the nutrient bar may also have contributed (soy protein has antioxidant effects and phytoestrogenic properties that may influence the vasculature). The effect of the nutrient bar is equivalent or superior to available medical therapy for symptoms of peripheral artery disease. Whereas this small clinical trial is encouraging, it needs to be confirmed by a larger multicenter study.

Contraindications to Therapeutic Use of L-Arginine

Although to date the administration of L-arginine to patients with cardiovascular disease has not been associated with any significant adverse effects, there are theoretical concerns that should be kept in mind when considering the use of L-arginine in certain individuals. Because nitric oxide plays a major role in the hypotension associated with septic shock, the use of L-arginine in patients with a severe infection is not advisable (Kilbourn *et al.,* 1993). Another subset of patients that may be adversely affected by L-arginine administration are those with active autoimmune disorders, where the NOS pathway (and peroxynitrite generation) may contribute to the pathogenesis of these disorders (Nussbaum, 1994; Sakurai *et al.,* 1995; Ueki *et al.,* 1996). Because of the role of NO in angiogenesis, the use of L-arginine may be contraindicated in conditions associated with pathological angiogenesis. Vascular endothelial growth factor may contribute to the pathogenesis of diabetic retinopathy (Aiello *et al.,* 1997). Because the angiogenic effect of VEGF may be in part mediated by NO (Murohawa *et al.,* 1998b), L-arginine should be used with caution in patients with diabetic retinopathy. Finally, the effect of L-arginine in individuals with an active malignancy is unknown. Preclinical studies are mixed, with some demonstrating a beneficial immunomodulatory effect of L-arginine, whereas other studies show an adverse effect of L-arginine on the progression of disease, possibly mediated by an angiogenic effect of L-arginine. In animal models of breast and colon cancer, the majority of studies show beneficial immunomodulatory effects of L-arginine, associated with a reduction in tumor growth and metastasis, and increased survival (Pryme, 1978; Reynolds *et al.,* 1988), although there are some studies that are contradictory (Yeatman *et al.,* 1991). A majority of preclinical and clinical studies indicate a positive immunomodulatory effect of L-arginine administration in other disease states, with increased number and activity of cytotoxic T cells (Barbul *et al.,* 1980, 1981, 1990; Nussbaum, 1994).

L-Arginine (30 g p.o. daily for 3 days) has been given to women with breast cancer. Subsequently, biopsies revealed increased leucine incorporation and PCNA staining in tumor tissue (Park *et al.,* 1992); however, neither tumor growth nor metastasis was examined. Indeed, in a follow-up study of 96 patients with breast cancer, L-arginine (30 g/day for three days) did not increase sensitivity to chemotherapy, an anticipated response if L-arginine stimulated tumor growth (Brittenden *et al.,* 1994a; Heys *et al.,* 1998). Indeed, these investigators found a better histopathological response in the L-arginine treated group, which may be attributed to improved immune function and/or increased tumor cell apoptosis (Brittenden *et al.,* 1994a). In subsequent studies, L-arginine has been shown to have beneficial immunomodulatory effects in women with breast cancer, with an increase in killer T activity (Brittenden *et al.,* 1994b,c; Heys *et al.,* 1998; Park *et al.,* 1992; Pittari *et al.,* 1993). A majority of preclinical and clinical studies indicate a positive immunomodulatory effect of L-arginine administration in other disease states, with increased numbers and activity of cytotoxic T cells (Barbul *et al.,* 1981, 1990; Bode-Böger *et al.,* 1996a; Jongkind *et al.,* 1980).

L-Arginine is widely used as secretagogue in endocrine function studies (30 g i.v.) without adverse effect (Ghigo *et*

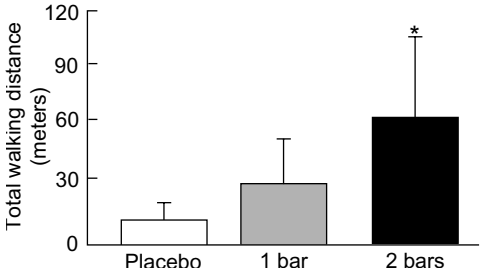


Figure 17 Increase in total walking distances in patients with claudication after 2 weeks of treatment with an L-arginine-enriched nutrient bar (one or two bars per day) or a placebo bar (Maxwell *et al.,* 2000).

al., 1996). L-Arginine is given chronically (21 g p.o. daily for life) to individuals with the inborn error of metabolism resulting in citrullinemia (Visek 1986). L-Arginine has been given (4–8 g p.o. daily) to elderly individuals and to children of short stature in an effort to increase growth hormone levels (so as to increase muscle mass or enhance growth, respectively, in these groups) (Ghigo *et al.,* 1994; Bellone *et al.,* 1993). These doses of L-arginine were well tolerated for up to 6 months in children (Pittari *et al.,* 1993). To summarize, there is extensive experience with the use of L-arginine in a wide variety of cardiovascular and noncardiovascular disorders. In patients with cardiovascular disease, the preponderance of the data indicates that administration of L-arginine enhances endothelial function, particularly in those individuals with atherosclerosis or risk factors for atherosclerosis, particularly those with hypercholesterolemia. Enhancement of endothelial NO synthesis has real clinical benefit, with improvements in limb and coronary blood flow and relief of symptoms in patients with peripheral or coronary artery disease, the rivals more traditional pharmacotherapies. Although the use of L-arginine seems quite safe in most patients, caution should be exercised in those individuals with conditions characterized by pathological angiogenesis or in those patients with severe infectious, inflammatory, or neoplastic disorders, because of the uncertainties with respect to the use of L-arginine in these circumstances.

Conclusion

To conclude, endothelium-derived nitric oxide is a potent modulator of the tone and structure of a blood vessel and of its interactions with circulating blood elements. NO also plays a key role in angiogenesis. Endothelium-derived nitric oxide suppresses many of the key processes involved in atherosclerosis and restenosis, including monocyte adherence, platelet aggregation, vascular smooth muscle proliferation, and generation of oxygen-derived free radicals. NO has acute effects mediated by cGMP as well as more chronic effects on gene expression mediated by its suppression of oxidant-sensitive transcriptional pathways. Accordingly, derangements of the NO synthase pathway lead to vascular disorders characterized by excessive or paradoxical vasoconstriction, inflammation, and cell proliferation. Furthermore, the deficit in NO synthesis or activity may impair the angiogenic response to vascular occlusive disease. Aberrancy of the NO synthase pathway is multifactorial, and it is dependent on underlying disease processes, environmental influences, and the genetic substrate. A greater understanding of the underlying causes of endothelial dysfunction will lead to new therapeutic strategies for atherosclerosis, restenosis, ischemic syndromes, and other vascular disorders.

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References

Adams, M. R., Forsyth, C. J., Jessup, W., Robinson, J., and Celermajer, D. S. (1995). Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. *J. Am. Coll. Cardiol.* **26,** 1054–1061.

Adams, M. R., McCredie, R., Jessup, W., Robinson, J., Sullivan, D., and Celermajer, D. S. (1997). Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. *Atherosclerosis* **129,** 261–269.

Aiello, L. P. (1997). Vascular endothelial growth factor and the eye: Biochemical mechanisms of action and implications for novel therapies. *Ophthalmic Res.* **29,** 354–362.

Aji, W., Ravalli, S., Szabolcs, M., *et al.* (1997). L-Arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. *Circulation* **95,** 430–437.

Akiba, Y., Nakamura, M., Oda, M., Kimura, H., Miura, S., Tsuchiya, M., and Ishii, H. (1997). Basic fibroblast growth factor increases constitutive nitric oxide synthase during healing of rat gastric ulcers. *J. Clin. Gastroenterol.* **25**(Suppl. 1), S122–S128.

Albina, J. E., Shinjun, C., Mateo, R. B., and Reichner, J. S. (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150,** 5080–5085.

Andrews, T. C., Raby, K., Barry, J., Naimi, C. L., Allred, E., Ganz, P., and Selwyn, A. P. (1997). Effect of cholesterol reduction on myocardial ischemia in patients with coronary disease. *Circulation* **95,** 324–328.

Arnal, J. F., Munzel, T., Venema, R. C., James, N. L., Bai, C. L., Mitch, W. E., and Harrison, D. G. (1995). Interactions between L-arginine and L-glutamine change endothelial NO production. An effect independent of NO synthase substrate availability. *J. Clin. Invest.* **95,** 2565–2572.

Asakura, T., and Karino, T. (1990). Flow patterns and spatial distribution of atherosclerotic lesions in human coronary arteries. *Circ. Res.* **66,** 1045–1066.

Azumi, H., Sato, J., Hamasaki, H., Sugimoto, A., Isotani, E., and Obayashi, S. (1995). Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *Br. J. Pharmacol.* **115,** 1001–1004.

Babaei, S., Teichert-Kuliszewska, K., Monge, J. C., Mohamed, F., Bendeck, M. P., and Stewart, D. J. (1998). Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. *Circ. Res.* **82,** 1007–1015.

Barbul, A., Wasserkrug, H. L., Seifter, E., Rettura, G., Levenson, S. M., and Efron, G. (1980). Immunostimulatory effects of arginine in normal and injured rats. *J. Surg. Res.* **29,** 228–235.

Barbul, A., Sisto, D. A., Wasserkrug, H. L., and Efron, G. (1981). Arginine stimulates lymphocyte immune response in healthy human beings. *Surgery* **90,** 244–251.

Barbul, A., Lazarou, S. A., Efron, D. T., Wasserkrug, H. L., and Efron, G. (1990). Arginine enhances wound healing and lymphocyte immune responses in humans. *Surgery* **108,** 331–336.

Bath, P. M. W., Gladwin, A. M., and Martin, J. F. (1991a). Human monocyte characteristics are altered in hypercholesterolemia. *Arteriosclerosis* **90,** 175–181.

Bath, P. M. W., Hassall, D. G., Gladwin, A.-M., *et al.* (1991b). Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler. Thromb.* **11,** 254–260.

Beckman, J. S., Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunochemistry. *Biol. Chem. Hoppe-Seyler.* **375,** 81–88.

Bellone, J., Bartolotta, E., Cardinale, G., Arvat, E., Cherubini, V., Aimaretti, G., Maccario, M., Mucci, M., Camanni, F., and Ghigo, E. (1993). Low dose orally administered arginine is able to enhance both basal and growth hormone-releasing hormone-induced growth hormone secretion in normal short children. *J. Endocrinol. Invest.* **16,** 521–525.

Bennet, M. R., Evan, G. I., and Schwarz, S. M. (1995). Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J. Clin. Invest.* **95,** 2266–2274.

Berliner, J. A., Territo, M. C., Sevanian, A., Ramin, S., Kim, J. A., Bamshad, B., Esterson, M., and Fogelman, A. M. (1990). Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* **85,** 1260–1266.

Berliner, J. A., Navab, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A., Watson, A. D., and Lusis, A. J. (1995). Atherosclerosis: Basic mechanisms: oxidation, inflammation, and genetics. *Circulation.* **91,** 2488–2496.

Björkerud, S., and Björkerud, B. (1996). Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T-cells), and may contribute to the accumulation of gruel and plaque instability. *Am. J. Pathol.* **149,** 367–380.

Bode-Böger, S. M., Böger, R. H., Alfke, H., Heinzel, D., Tsikas, D., Creutzig, A., Alexander, K., and Frölich, J. C. (1996a). L-Arginine induces nitric oxide-dependent vasodilation in patients with critical limb ischemia: A randomized, controlled study. *Circulation* **93,** 85–90.

Bode-Böger, S. M., Böger, R. H., Kienke, S., Junker, W., and Frölich, J. C. (1996b). Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. *Biochem. Biophys. Res. Commun.* **219,** 598–603.

Böger, R. H., Bode-Böger, S. M., Mügge, A., Kienke, S., Brandes, R., Dwenger, A., and Frölich, J. C. (1995). Supplementation of hypercholesterolaemic rabbits with L-arginine reduces the vascular release of superoxide anions and restores NO production. *Atherosclerosis* **117,** 273–284.

Böger, R. H., Bode-Böger, S. M., Thiele, W., *et al.* (1997a). Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* **95,** 2068–2074.

Böger, R. H., Bode-Böger, S. M., Brandes, R. P., *et al.* (1997b). Dietary L-arginine reduces the progression of atherosclerosis in cholesterol-fed rabbits: Comparison with lovastatin. *Circulation* **96,** 1282–1290.

Böger, R. H., Bode-Böger, S. M., Heistad, D., and Lentz, S. R. (1998a). Elevated plasma concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, in monkeys with hyperhomocysteinemia. Abstracts from the 71st Scientific Sessions, American Heart Association, Dallas, Texas, Nov. 8–11. *Circulation* **98**(Suppl.), 1733.

Böger, R., Bode-Böger, S., Szuba, A., Tsao, P. S., Chan, J., Tangphao, O., Blaschke, T., and Cooke, J. P. (1998b). Asymmetric dimethylarginine (ADMA): A novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation* **98,** 1842–1847.

Böger, R. H., Bode-Böger, S. M., Thiele, W., Creutzig, A., Alexander, K., and Frolich, J. C. (1998c). Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. *J. Am. Coll. Cardiol.* **32,** 1336–1344.

Bossaller, C., Yamamoto, H., Lichtlen, P. R., and Henry, P. D. (1987). Impaired cholinergic vasodilation in the cholesterol-fed rabbit in vivo. *Basic Res. Cardiol.* **82,** 396–404.

Brittenden, J., Park, K. G., Heys, S. D., Ross, C., Ashby, J., Ah-See, A., and Eremin, O. (1994a). L-Arginine stimulates host defenses in patients with breast cancer. *Surgery* **115,** 205–212.

Brittenden, J., Heys, S. D., Miller, I., Sarkar, T. K., Hutcheon, A. W., Needham, G., Gilbert, F., McKean, M., Ah-See, A. K., and Eremin, O. (1994b). Dietary supplementation with L-arginine in patients with breast cancer (>4 cm) receiving multimodality treatment: Report of a feasibility study. *Br. J. Cancer* **69,** 918–921.

Brittenden, J., Heys, S. D., Ross, J., Park, K. G., and Eremin, O. (1994c). Natural cytotoxicity in breast cancer patients receiving neoadjuvant chemotherapy: Effects of L-arginine supplementation. *Eur. J. Surg. Oncol.* **20,** 467–472.

Brzozowski, T., Konturek, S. J., Drozdowicz, D., Dembinski, A., and Stachura, J. (1995). Healing of chronic gastric ulcerations by L-arginine. Role of nitric oxide, prostaglandins, gastrin and polyamines. *Digestion* **56,** 463–471.

Brzozowski, T., Konturek, S. J., Sliwowski, Z., Drozdowicz, D., Zaczek, M., and Kedra, D. (1997). Role of L-arginine, a substrate for nitric oxide-synthase, in gastroprotection and ulcer healing. *J. Gastroenterol.* **32,** 442–452.

Buttery, L. D., Springall, D. R., Chester, A. H., Evans, T. J., Standfield, E. N., Parums, D. V., Yacoub, M. H., and Polak, J. M. (1996). Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Lab. Invest.* **75,** 77–85.

Candipan, R. C., Wang, B., Buitrago, R., Tsao, P. S., and Cooke, J. P. (1996). Regression or progression: Dependency on vascular nitric oxide. *Arterioscler. Thromb. Vasc. Biol.* **16,** 44–50.

Cayatte, A. J., Palacino, J. J., Horten, K., Cohen, R. A. (1994). Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler. Throm.* **14,** 753–759.

Celermajer, D. S., Sorensen, K. E., Gooch, V. M., Speigelhalter, D. J., Miller, O. W., Sullivan, I. D., Lloyd, J. K., and Deanfield, J. E. (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* **340,** 1111–1115.

Celermajer, D. S., Sorensen, K. E., Bull, C., Robinson, J., and Deanfield, J. E. (1994). Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects related to coronary risk factors and their interaction. *J. Am. Coll. Cardiol.* **24,** 1468–1474.

Ceremuzynski, L., Chamiec, T., and Herbaczynska-Cedro, K. (1997). Effect of supplemental oral L-arginine on exercise capacity in patients with stable angina pectoris. *Am. J. Cardiol.* **80,** 331–333.

Chan, J., Böger, R. H., Bode-Böger, S. M., Tangphao, O., Tsao, P., Blaschke, T., and Cooke, J. P. (2000). Asymmetric dimethylarginine increases mononuclear cell adhesiveness in hypercholesterolemic humans. *Arterioscler. Thromb. Vasc. Biol.* **20,** 1040–1046.

Chen, C. H., and Henry, P. D. (1997). Atherosclerosis as a microvascular disease: Impaired angiogenesis mediated by suppressed basic fibroblast growth factor expression. *Proc. Assoc. Am. Physicians* **109,** 351–361 *Circulation* **95,** 324–328.

Chen, C. H., Cartwright, J., Jr., Li, Z., Lou, S., Nguyen, N. H., Gotto, A. M., Jr., and Henry, P. D. (1997). Inhibitory effects of hypercholesterolemia and ox-LDL on angiogenesis-like endothelial growth in rabbit aortic explants. Essential role of fibroblast growth factor. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1303–1312.

Clancy, R. M., Leszczynska, P., Piziak, J., and Abramson, S. B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on NADPH oxidase. *J. Clin. Invest.* **90,** 1116–1121.

Clarkson, P., Adams, M. R., Powe, A. J., Donald, A. E., McCredie, R., Robinson, J., McCarthy, S. N., Keech, A., Celermajer, D. S., and Deanfield, J. E. (1996). Oral L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults. *J. Clin. Invest.* **97,** 1989–1994.

Cohen, R. A., Zitnay, K. M., Haudenschild, C. C., and Cunningham, L. D. (1988). Loss of selective endothelial cell vasoactive functions in pig coronary arteries caused by hypercholesterolemia. *Circ. Res.* **63,** 903–910.

Cooke, J. P., and Dzau, V. J. (1997a). Nitric oxide synthase: Role in the genesis of vascular disease. *Annu. Rev. Med.* **48,** 489–509.

Cooke, J. P., and Dzau, V. J. (1997b). Derangements of the nitric oxide synthase pathway, L-arginine, and cardiovascular diseases (editorial). *Circulation* **95,** 379–382.

Cooke, J. P., Rossitch, E., Andon, N., Loscalzo, J., and Dzau, V. J. (1991b). Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J. Clin. Invest.* **88,** 1663–1671.

Cooke, J. P., Stamler, J. S., Andon, N., Davies, P. R., and Loscalzo, J. (1990). Flow stimulates endothelial cells to release a nitrovasodilator

that is potentiated by reduced thiol. *Am. J. Physiol.* (*Heart Circ. Physiol.*) **28,** H804–H812.

Cooke, J. P., Andon, N. A., Girerd, X. J., Hirsch, A. T., and Creager, M. A. (1991a). Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta. *Circulation* **83,** 1057–1062.

Cooke, J. P., Singer, A. H., Tsao, P., *et al.* (1992). Anti-atherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J. Clin Invest.* **90,** 1168–1172.

Cornhill, J. F., Barrett, W. A., Herderick, E. E., Mahley, R. W., and Fry, D. F. (1985). Topographic study of sudanophilic lesions in cholesterol-fed minipigs by image analysis. *Atherosclerosis* **5,** 415–426.

Cosentino, F., and Katusic, Z. S. (1995). Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. *Circulation* **91,** 139–144.

Couffinhal, T., Duplaa, C., Labat, L., Moreau, C., Bietz, I., and Bonnet, J. (1993). Effect of low density lipoprotein on monocyte adhesiveness to endothelial cells in vitro. *Atherosclerosis* **99,** 35–45.

Creager, M. A., Cooke, J. P., Mendelsohn, M. E., Gallagher, S. J., Coleman, S. M., Loscalzo, J., and Dzau, V. J. (1990). Impaired vasodilatation of forearm resistance vessels in hypercholesterolemic humans. *J. Clin. Invest.* **86,** 228–234.

Creager, M. A., Gallagher, S. J., Girerd, X. J., Coleman, S. M., Dzau, V. J., and Cooke, J. P. (1992). L-Arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J. Clin. Invest.* **90,** 1248–1253.

Cybulsky, M. I., and Gimbrone, M. A., Jr. (1991). Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **251,** 788–791.

Davies, M. G., Dalen, H., Kim, J. H., Barber, L., Svendsen, E., and Hagen, P. O. (1995). Control of accelerated vein graft atheroma with the nitric oxide precursor: L-Arginine. *J. Surg. Res.* **59,** 35–42.

DeCaterina, R., Libby, P., Peng, H.-B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., Jr., Shin, W. S., and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. *J. Clin. Invest.* **96,** 60–68.

Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A. M. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1β-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.* **185,** 601–607.

Drexler, H., Zeiher, A. M., Meinzer, K., and Just, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* **338,** 1546–1550.

Drexler, H., Fischell, T. A., Pinto, F. J., Chenzbraun, A., Cooke, J. P., and Alderman, E. A. (1994). Effect of L-arginine on coronary endothelial function in cardiac transplant recipients: Relationship to vessel wall morphology. *Circulation* **89,** 1615–1623.

Dubois-Rande, J. L., Zelinsky, R., Chabrier, P. E., Castaigne, A., Geschwind, H. (1992). Adnot, S. L-Arginine improves endothelium-dependent relaxation of conductance and resistance coronary arteries in coronary artery disease. *J. Cardiovasc. Pharmacol.* **20**(Suppl. 12), S211–S213.

European Working Group on Critical Leg Ischemia (1991). Second European Consensus Document on chronic critical leg ischemia. *Circulation* **84**(Suppl. 4), IV1–IV26.

Faraci, F. M., Brian, J. E., and Heistad, D. D. (1995). Response of cerebral blood vessels to an endogenous inhibitor of nitric oxide synthase. *Am. J. Physiol.* **269,** H1522–H1527.

Fischman, D. L., Leon, M. B., Baim, D., Schatz, R. A., Savage, M. P., Penn, I., Detre, K., Veltri, L., Ricci, D., Nobuyoshi, M., Cleman, M., Heuser, R., Almond, D., Teirstein, P. S., Fish, R. D., Colombo, A., Brinker, J., Moses, J., Shaknovich, A., Hirshfeld, J., Bailey, S., Ellis, S., Rake, R., and Goldberg, S., for the Stent Restenosis Study Investigators. (1994). A randomized comparison of coronary stent placement and balloon angioplasty in the treatment of coronary artery disease. *N. Engl. J. Med.* **331,** 496–501.

Forrester, J. S., Fishbein, M., Helfant, R., and Fagin, J. (1991). A paradigm for restenosis based on cell biology: Clues for the development of new preventive therapies. *J. Am. Coll. Cardiol.* **17,** 758–769.

Förstermann, U., Warmuth, G., Dudel, C., and Alheid, U. (1989). Formation and functional importance of endothelium-derived relaxing factor (EDRF) and prostaglandins in the microcirculation. *Z. Kardiol.* **78**(Suppl. 6), 85–91.

Frangos, J. A., Eskin, S. G., Mcintire, L. V., and Ives, C. L. (1985). Flow effects prostacyclin production of cultured human endothelial cells. *Science* **227,** 1477–1479.

Garg, U. C., Hassid, A., *et al.* (1989). Nitric oxide-generating vasodilators and 8-bromocyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83,** 1774–1777.

Gauthier, T. W., Scalia, R., Murohara, T., Guo, J. P., and Lefer, A. M. (1995). Nitric oxide protects against leukocyte–endothelial interaction of early hypercholesterolemia. *Arterioscler. Thromb. Vasc. Biol.* **15,** 1652–1659.

Geng, Y. J., and Libby, P. (1995). Evidence for apoptosis in advanced human atheroma, colocalization with interleukin-1β-converting enzyme. *Am. J. Pathol.* **147,** 251–266.

Geng, Y. J., Wu, Q., Muszynski, M., Hansson, G. K., and Libby, P. (1996b). Apoptosis of vascular smooth muscle cells induced by in vitro stimulation with interferon-γ, tumor necrosis factor-α, and interleukin-1β. *Arterioscler. Thromb. Vasc. Biol.* **16,** 19–27.

Ghigo, E., Ceda, G. P., Valcavi, R., Goffi, S., Zini, M., Mucci, M., Valenti, G., Cocchi, D., Muller, E. E., and Camanni, F. (1994). Low doses of either intravenously or orally administered arginine are able to enhance growth hormone response to growth hormone releasing hormone in elderly subjects. *J. Endocrinol. Invest.* **17,** 113–117.

Ghigo, E., Bellone, J., Aimaretti, G., Bellone, S., Loche, S., Cappa, M., Bartolotta, E., Dammacco, F., and Camanni, F. (1996). Reliability of provocative tests to assess growth hormone secretory status. Study in 472 normally growing children. *J. Clin. Endocrinol. Metab.* **81,** 3323–3327.

Gimbrone, M. A., Jr. (1995). Vascular endothelium: An integrator of pathophysiologic stimuli in atherosclerosis. *Am. J. Cardiol.* **75,** 67B–70B.

Girerd, X. J., Hirsch, A. T., Cooke, J. P., Dzau, V. J., and Creager, M. A. (1990). L-Arginine augments endothelium-dependent vasodilation in cholesterol-fed rabbits. *Circ. Res.* **67,** 1301–1308.

Glagov, S., Zarins, C., Giddens, D. P., and Ku, D. N. (1988). Hemodynamics and atherosclerosis: Insights and perspectives gained from studies of human arteries. *Arch. Pathol. Lab. Med.* **112,** 1018–1031.

Goode, G. K., and Heagerty, A. M. (1995). In vitro responses of human peripheral small arteries in hypercholesterolemia and effects of therapy. *Circulation* **91,** 2898–2903.

Hackman, A., Abe, Y., Insull, W., Jr., Pownall, H., Smith, L., Dunn, K., Gotto, A. M., Jr., and Ballantyne, C. M. (1996). Levels of soluble cell adhesion molecules in patients with dyslipidemia. *Circulation* **93,** 1334–1338.

Hamon, M., Vallet, B., Bauters, C., Wernert, N., McFadden, E. P., Lablanche, J., Dupuis, B., and Bertrand, M. E. (1994). Long-term oral administration of L-arginine reduces intimal thickening and enhances neoendothelium-dependent acetylcholine-induced relaxation after arterial injury. *Circulation* **90,** 1357–1362.

Han, D. K. M., Haudenschild, C. C., Hong, M. K., Tinkle, B. T., Leon, M. B., and Liau, G. (1995). Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. *Am. J. Pathol.* **147,** 267–277.

Hand, M. F., Haynes, W. G., and Webb, D. J. (1998). Hemodialysis and L-arginine, but not D-arginine, correct renal failure-associated endothelial dysfunction. *Kidney Int.* **53,** 1068–1077.

Hansson, G. K., Seifert, P. S., Olsson, G., and Bondjers, G. (1991). Immunohistochemical detection of macrophages and T lymphocytes in atherosclerotic lesions of cholesterol-fed rabbits. *Arterioscler. Thromb.* **11,** 745–750.

Haskell, W. L., Alderman, E. L., Fair, J. M., Maron, D. J., Mackey, S. F., Superko, H. R., Williams, P. T., Johnstone, I. M., Champagne, M. A., Krauss, R. M., and Farquhar, J. W. (1994). Effects of intensive multiple risk factor reduction on coronary atherosclerosis and clinical cardiac events in men and women with coronary artery disease: The Stan-

ford Coronary Risk Intervention Project (SCRIP). *Circulation* **89,** 975–990.

Heistad, D. D., Armstrong, M. L. I., Marcus, M. L., Piegors, D. J., and Mark, A. L. (1984). Augmented responses to vasoconstrictor stimuli in hypercholesterolemic and atherosclerotic monkeys. *Circ. Res.* **43,** 711–718.

Heys, S., Ogston, K., Miller, I., Hutcheon, A., Walker, L., Sarker, T., Dewar, J., Ah-See, A., and Eremin, O. (1998). Potentiation of the response to chemotherapy in patients with breast cancer by dietary supplementation with L-arginine: Results of a randomised controlled trial. *Int. J. Oncol.* **12,** 221–225.

Ho, H. K., Jang, J. J., Adimoolam, S., and Cooke, J. P. (1999). Dietary L-arginine reverses the inhibitory effects of asymmetric dimethylarginine on angiogenesis in hypercholesterolemia. *J. Am. Coll. Cardiol. Suppl. A* **33,** 1A.

Hogg, N., Kalyanaramer, B., Joseph, J., Struck, A., and Parthasarathy, S. (1993). Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett.* **334,** 170–174.

Holmes, D. R., Vliestra, R. E., Smith, H. C., Vetrovec, G. W., Kent, K. M., Cowley, M. J., Faxon, D. P., Gruentzig, A., Kelsey, S. F., Detre, K. M., van Raden, M. J., and Mock, M. B. (1984). Restenosis after percutaneous transluminal coronary angioplasty (PTCA): A report from the PTCA registry of the National Heart, Lung, and Blood Institute. *Am. J. Cardiol.* **53,** 77C–81C.

Hood, J. D., Meininger, C. J., Ziche, M., and Granger, H. J. (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am. J. Physiol.* **274**(Part 2), H1054–H1058.

Huang, P. L., Hyang, Z., Mashimo, H., Block, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **377,** 239–242.

Hudlicka, O. (1998). Is physiological angiogenesis in skeletal muscle regulated by changes in microcirculation? *Microcirculation* **5,** 7–23.

Hutchison, S. J., Reitz, M. S., Sudhir, K., Sievers, R. E., Zhu, B. Q., Sun, Y. P., Chou, T. M., Deedwania, P. C., Chatterjee, K., Glantz, S. A., and Parmley, W. W. (1997). Chronic dietary L-arginine prevents endothelial dysfunction secondary to environmental tobacco smoke in normocholesterolemic rabbits. *Hypertension* **29,** 1186–1191.

Inoue, N., Venema, R. C., Sayegh, H. S., Ohara, Y., Murphy, T. J., and Harrison, D. G. (1995). Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. *Arterioscler. Thromb. Vasc. Biol.* **15,** 1255–1261.

Inoue, N., Ramasamy, S., Fukai, T., Nerem, R. M., and Harrison, D. G. (1996). Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. *Circ. Res.* **79,** 32–37.

Isner, J. M., Kearney, M., Bortman, S., and Passeri, J. (1995). Apoptosis in human atherosclerosis and restenosis. *Circulation* **91,** 2703–2711.

Ito, A., Tsao, P. S., Adimoolam, S., Kimoto, M., Ogawa, T., and Cooke, J. P. (1999a). A novel mechanism for endothelial dysfunction: Dysregulation of dimethylarginine dimethylaminohydrolase. *Circulation* **99,** 3092–3095.

Ito, A., Asagami, T., Tsao, P. S., Adimoolam, S., Kimoto, M., Tsuji, H., Ogawa, T., Reaven, G., and Cooke, J. P. (1999b). ''Dysregulation of dimethylarginine dimethylaminohydrolase: A mechanism for endothelial dysfunction in diabetes mellitus'' (Published abstract). Western Regional Meeting, American Federation for Medical Research, Jan. 27–30, 1999, Carmel, California.

Joannides, R., Haefeli, W. E., Linder, L., Richard, V., Bakkali, E. H., Thuillez, C., and Luscher, T. F. (1995). Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* **1,** 91, 1314–1319.

Jongkind, J. F., Verkerk, A., and Hoogebrugge, N. (1995). Monocytes from patients with combined hypercholesterolemia-hypertriglyceridemia and isolated hypercholesterolemia show an increased adhesion to endothelial cells in vitro: III. Influence of intrinsic and extrinsic factors on monocyte binding. *Metabolism* **44,** 374–378.

Kakuta, T., Currier, J. W., Haudenschild, C. C., Ryan, T. J., and Faxon, D. P. (1994). Differences in compensatory vessel enlargement, not neointimal formation, account for restenosis following angioplasty in the hypercholesterolemic rabbit model. *Circulation* **89,** 2809–2815.

Kanai, A. J., Strauss, H. C., Truskey, G. A., Crews, A. L., Grunfeld, S., and Malinski, T. (1995). Shear stress induces ATP-independent transient nitric oxide release from vascular endothelial cells, measured directly with a porphyrinic microsensor. *Circ. Res.* **77,** 284–293.

Kikuta, K., Sawamura, T., Miwa, S., Hashimoto, N., and Masaki, T. (1998). High-affinity arginine transport of bovine aortic endothelial cells is impaired by lysophosphatidylcholine. *Circ. Res.* **83,** 1088–1096.

Kilbourn, R. G., Griffith, O. W., and Gross, S. S. (1993). Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.* **329,** 1427–1428.

Kimoto, M., Whitley, G. S., Tsuji, H., and Ogawa, T. (1995). Detection of N^G, N^G-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. *J. Biochem.* (*Tokyo*) **117,** 237–238.

Kockx, M. M., De Meyer, G. R. Y., Murhing, J., Bult, H., Bultnick, J., and Herman, A. G. (1996). Distribution of cell replication and apoptosis in atherosclerotic plaques of cholesterol-fed rabbits. *Atherosclerosis* **120,** 115–124.

Kolpakov, V., Gordon, D., and Kulik, T. J. (1995). Nitric oxide-generating compounds inhibit total protein and collagen synthesis in cultured vascular smooth muscle cells. *Circ. Res.* **76,** 305–309.

Kowalski, J., Kwan, H. H., Prionas, S. D., Allison, A. C., and Fajardo, L. F. (1992). Characterization and applications of the disc angiogenesis system. *Exp. Mol. Pathol.* **56,** 1–19. (Review.)

Kubes, P., Suzuki, M., *et al.* (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4651–4685.

Kuchan, M. I., and Frangos, J. A. (1993). Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *Am. J. Physiol.* **264,** H150–H156.

Kugiyama, K., Kerns, S. A., Morrisett, J. D., Roberts, R., and Henry, P. D. (1990). Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* **344,** 160–162.

Kurose, I., Kubes, P., Wolf, R., Anderson, D. C., Paulson, J., Miyasaka, M., and Granger, D. N. (1993). Inhibition of nitric oxide production: Mechanisms of vascular albumin leakage. *Circ. Res.* **73,** 164–171.

Lefer, A. M., and Ma, X. L. (1993). Decreased basal nitric oxide release in hypercholesterolemia increases neutrophil adherence to rabbit coronary artery endothelium. *Arterioscler. Thromb.* **13,** 771–776.

Lerman, A., Holmes, D. R., Jr., Bell, M. R., Garratt, K. N., Nishimura, R. A., and Burnett, J. C., Jr. (1995). Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation* **92,** 2426–2431.

Lerman, A., Burnett, J. C., Jr., Higano, S. T., McKinley, L. J., and Holmes, D. R., Jr. (1998). Long-term L-arginine supplementation improves small-vessel coronary endothelial function in humans. *Circulation* **97,** 2123–2128.

Li, H., Cybulsky, M. I., Gimbrone, M. A., Jr., and Libby, P. (1993). An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler. Thromb.* **13,** 197–204.

Libby, P., and Hansson, G. K. (1991). Involvement of the immune system in human atherosclerosis: Current knowledge and unanswered questions. *Lab. Invest.* **64,** 5–15.

Linder, L., Kiowski, W., Buhler, F. R., and Luscher, T. F. (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo: Blunted response in essential hypertension. *Circulation* **81,** 1762–1767.

Lorant, D. E., Zinu rierman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Platelet activating factor mediates procoagulant activity on the surface of endothelial cells by promoting leukocyte adhesion. *Semin. Cell Biol.* **6,** 295–303.

Ludmer, P. L., Selwyn, A. P., Shook, T. L., Wayne, R. R., Mudge, G. H., Alexander, R. W., and Ganz, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N. Engl. J. Med.* **315,** 1046–1051.

Lüscher, T. F., Vanhoutte, P. M., and Raij, L. (1987). Antihypertensive treatment normalizes decreased endothelium-dependent relaxations

in rats with salt-induced hypertension. *Hypertension* **9**(Suppl. III), 193–197.

Maxwell, A. J., Anderson, B. E., and Cooke, J. P. (2000). Nutritional therapy for peripheral arterial disease: A double-blind, placebo-controlled, randomized trial of HeartBar. *Vasc. Med.* **5,** 11–19.

McDonald, K. K., Zharikov, S., Block, E. R., and Kilberg, M. S. (1997). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the ''arginine paradox.'' *J. Biol. Chem.* **272,** 31213–31216.

McLenahan, J. M., William, J. K., Fish, R. D., Ganz, P., and Selwyn, A. P. (1991). Loss of flow-mediated endothelium-dependent dilation occurs early in the development of atherosclerosis. *Circulation* **84,** 1273–1278.

McNamara, D. B., Bedor, B., Aurora, H., Tena, L., Ignarro, L. J., Kadowitz, P. J., and Akers, D. L. (1993) L-Arginine inhibits balloon catheter-induced intimal hyperplasia. *Biochem. Biophys. Res. Commun.* **193,** 291–296.

MacAllister, R. J., Parry, H., Kimoto, M., *et al.* (1996). Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. *Br. J. Pharmacol.* **119,** 1533–1540.

Mannick, J. B., Asano, K., Izumi, K., Kieff, E., and Stamler, J. S. (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* **79,** 1137–1146.

Marsden, P. A., Heng, H. H. Q., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L., and Schappert, K. T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase. *J. Biol. Chem.* **268,** 17478–17488.

Martin, B. L., Wu, D., Jakes, S., and Graves, D. J. (1990). Chemical influences on the specificity of tyrosine phosphorylation. *J. Biol. Chem.* **265,** 7108–7111.

Marui, N., Offerman, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W., and Medford, R. M. (1993). Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* **92,** 1866–1874.

Matsuoka, H., Itoh, S., Kimoto, M., Kohno, K., Tamai, O., Wada, Y., Yasukawa, H., Iwami, G., Okuda, S., and Imaizumi, T. (1997). Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* **29**(Part 2), 242–247.

Matsuoka, H., Miyazaki, H., *et al.* (1998). Endogenous nitric oxide synthase inhibitor is elevated in human atheromatous lesion. Abstracts from the 71st Scientific Sessions, American Heart Association, Dallas, Texas, Nov. 8–11, 1998. Supplement to *Circulation* **98,** I–116.

Maxwell, A. J., Schauble, E., Bernstein, D., and Cooke, J. P. (1998). Limb blood flow during exercise is dependent upon nitric oxide. *Circulation* **98,** 369–374.

Mehta, S., Stewart, D. J., and Levy, R. D. (1996). The hypotensive effect of L-arginine is associated with increased expired nitric oxide in humans. *Chest* **109,** 1550–1555.

Messmer, U. K., and Brüne, B. (1995). Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem. J.* **319,** 299–305.

Messmer, U. K., Ankarcrona, M., Nicotera, P., and Brüne, B. (1994). p53 expression in nitric oxide-induced apoptosis. *FASEB Lett.* **3,** 23–26.

Messmer, U. K., Lapetina, E. G., and Brüne, B. (1995). Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds. *Mol. Pharmacol.* **47,** 757–765.

Minor, R. L., Myers, P. R., Guerra, R., Jr., Bates, J. N., and Harrison, D. G. (1990). Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J. Clin. Invest.* **86,** 2109–2116.

Miyazaki, H., Matsuoka, H., Cooke, J. P., Usui, M., Ueda, S., Okuda, S., and Imaizumi, T. (1999). Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation* **99,** 1141–1146.

Montenegro, M. R., and Eggen, D. A. (1968). Topography of atherosclerosis in the coronary arteries. *Lab. Invest.* **18,** 586–593.

Morbidelli, L., Chang, C. H., Douglas, J. G., Granger, H. J., Ledda, F., and Ziche, M. (1996). Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. *Am. J. Physiol.* **270**(Part 2), H411–H415.

Mügge, A., Elwell, J. H., Peterson, T. E., Hofmeyer, T. G., Heistad, D. D., and Harrison, D. G. (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ. Res.* **69,** 1293–1300.

Murakami, T., Mizuno, S., and Kaku, B. N. B. (1998). Clinical morbidities in subjects with Doppeler-evaluated endothelial dysfunction of coronary artery. *J. Am. Coll. Cardiol. Suppl. A* 31, 419A. (Abstract 1165–64).

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J. F., Fishman, M. C., Huang, P. L., and Isner, J. M. (1998a). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101,** 2567–2578.

Murohara, T., Horowitz, J. R., Silver, M., Tsurumi, Y., Chen, D., Sullivan, A., and Isner, J. M. (1998b). Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation* **97,** 99–107.

Nabel, E. G., Selwyn, A. P., and Ganz, P. (1990). Large coronary arteries in humans are responsive to changing blood flow: An endothelium-dependent mechanism that fails in patients with atherosclerosis. *J. Am. Coll. Cardiol.* **16,** 349–356.

Naruse, K., Shimizu, K., Muramatsu, M., Toki, Y., Miyazaki, Y., Okumura, K., Hashimoto, H., Ito, T. (1994). Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta: PGH_2 does not contribute to impaired endothelium-dependent relaxation. *Arterioscler. Thromb.* **14,** 746–752.

Nguyen, T., Brunson, D., Crepsi, C. L., Penman, B. W., Wishnok, J. S., and Tannenbaum, S. R. (1992). DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 3030–3034.

Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W., and Murphy, T. J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.* **90,** 2090–2096.

Niu, X.-F., Smith, C. W., and Kubes, P. (1994). Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ. Res.* **74,** 1133–1140.

Noiri, E., Lee, E., Testa, J., Quigley, J., Colflesh, D., Keese, C. R., Giaever, I., and Goligorsky, M. S. (1998). Podokinesis in endothelial cell migration: Role of nitric oxide. *Am. J. Physiol.* **274**(Part 1), C236–C244.

Nussbaum, M.S. (1994). Arginine stimulates wound healing and immune function in elderly human beings. *JPEN J. Parenter, Enteral. Nutr.* **18,** 194.

Ogawa, T., Kimoto, M., and Sasaoka, K. (1987). Occurrence of a new enzyme catalyzing the direct conversion of N^G, N^G-dimethyl-L-arginine to L-citrulline in rats. *Biochem. Biophys. Res. Commun.* **148,** 671–677.

Ohara, Y., Peterson, T. E., and Harrison, D. G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* **91,** 2546–2551.

Ohtsuka, A., Ando, J., Korenaga, R., Kamiya, A., Toyama-Sorimachi, N., and Miyasaka, M. (1993). The effect of flow on the expression of vascular adhesion molecule-1 by cultured mouse endothelial cells. *Biochem. Biophys. Res. Commun.* **193,** 303–310.

Osborne, J. A., Siegman, M. J., Sedar, A. W., Mooers, S. U., and Lefer, A. M. (1989). Lack of endothelium-dependent relaxation in coronary resistance arteries of cholesterol-fed rabbits. *Am. J. Physiol.* **256**(Part 1), C591–C597.

Pagano, P. J., Tornheim, K., and Cohen, R. A. (1993). Superoxide anion production by rabbit thoracic aorta: Effect of endothelium-derived nitric oxide. *Am. J. Physiol.* **265,** H707–H712.

Panza, J., Quyyumi, A. A., Brush, J. E., Jr., and Epstein, S. E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N. Engl. J. Med.* **323,** 22–27.

Papapetropoulos, A., Desai, K. M., Rudic, R. D., Mayer, B., Zhang, R., Ruiz-Torres, M. P., Garcia-Cardena, G., Madri, J. A., and Sessa, W. C.

(1997a). Nitric oxide synthase inhibitors attenuate transforming-growth-factor-beta 1-stimulated capillary organization in vitro. *Am. J. Pathol.* **150,** 1835–1844.

Papapetropoulos, A., Garcia-Cardena, G., Madri, J. A., and Sessa, W. C. (1997b). Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100,** 3131–3139.

Park, K. G., Heys, S. D., Blessing, K., Kelly, P., McNurlan, M. A., Eremin, O., and Garlick, P. J. (1992). Stimulation of human breast cancers by dietary L-arginine. *Clin Sci.* **82,** 413–417.

Peng, H.-B., Rajavashisth, T. B., Libby, P., and Liao, J. K. (1995). Nitric oxide inhibits macrophage-colony stimulating factor gene transcription in vascular endothelial cells. *J. Biol. Chem.* **270,** 17050–17055.

Pittari, A. M., Becherucci, P., La Cauza, F., and Seminara, S. (1993). [Therapy with arginine chlorohydrate in children with short constitutional stature.] *Minerva Pediatr.* **45,** 61–65.

Pohl, U., and Busse, R. (1989). EDRF increases cyclic GMP in platelets during passage through the coronary vascular bed. *Circ. Res.* **65,** 1798–1803.

Pohl, U., Holtz, J., Busse, R., and Bassenge, E. (1986). Crucial role of endothelium in the vasodilator response to increase flow in vivo. *Hypertension* **8,** 37–44.

Pollman, M. J., Yamada, T., Horiuchi, M., and Gibbons, G. H. (1996). Vasoactive substances regulate vascular smooth muscle cell apoptosis: Countervailing influences of nitric oxide and angiotensin II. *Circ. Res.* **79,** 748–756.

Pollock, J. S., Försterman, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H., Nakane, M., and Murad, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10480–10484.

Popma, J. J., Califf, M. C., and Topol, E. J. (1991). Clinical trials of restenosis after coronary angioplasty. *Circulation* **84,** 1426–1436.

Post, M., Borst, C., and Kuntz, R. E. (1994). The relative importance of arterial remodeling compared with intimal hyperplasia in lumen narrowing after balloon angioplasty: A study in the normal rabbit and in the hypercholesterolemic Yucatan micropig. *Circulation* **89,** 2816–2821.

Pritchard, K. A., Jr., Groszek, L., Smalley, D. M., Sessa, W. C., Wu, M., Villalon, P., Wolin, M. S., and Stemerman, M. B. (1995). Native low-density lipoprotein increases endothelial cell-nitric oxide synthase generation of superoxide anion. *Circ. Res.* **77,** 510–518.

Pryme, I. F. (1978). The effects of orally administered L-arginine HCl on the development of myeloma tumors in BALB/c mice following the injection of single cell suspensions, cell aggregates or tumor fragments; and on the growth of two ascites tumour cell lines. *Cancer Lett.* **5,** 19–23.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987). The anti-aggregating properties of vascular endothelium interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.* **92,** 629–636.

Rajagopalan, S., Meng, X. P., Ramasamy, S., Harrison, D. G., and Galis, Z. S. (1996). Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro: Implications for atherosclerotic plaque stability. *J. Clin. Invest.* **98,** 2572–2579.

Randall, M. D., Ujiie, H., and Griffith, T. M. (1994). L-Arginine reverses the impairment of nitric oxide-dependent collateral perfusion in dietary-induced hypercholesterolaemia in the rabbit. *Clin. Sci.* **87,** 53–59.

Resnick, N., Collins, T., Atkinson, W., Bonthron, D. T., Dewey, C. F., Jr., and Gimbrone, M. A., Jr. (1993). Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 4591–4595.

Reynolds, J. V., Thom, A. K., Zhang, S. M., Ziegler, M. M., Naji, A., and Daly, J. M. (1988). Arginine, protein malnutrition, and cancer. *J. Surg. Res.* **45,** 513–522.

Ribeiro, M. O., Antunes, E., de Nucci, G., Lovisolo, S. M., and Zatz, R. (1992). Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. *Hypertension* **20,** 298–303.

Richard, M. J., Davis, L. D., and Jacobson, N. L. (1990). The domestic goat: A useful model to determine effects of diet and exercise on cholesterol accumulation in the body. *Comp. Biochem. Physiol.* **95,** 275–280.

Rokitansky, C. (1952). The pathological anatomy of the organs of respiration and circulation. *In* "Manual of Pathological Anatomy" (G. E. Day, ed.), Vol. 4. Sydenham Society, London.

Ross, R. (1993). The pathogenesis of atherosclerosis—A perspective for the 1990s. *Nature* **362,** 801–809.

Rossitch, E., Jr., Alexander III, E., Black, P. M., and Cooke, J. P. (1991). L-Arginine normalizes endothelial function in cerebral vessels from hypercholesterolemic rabbits. *J. Clin. Invest.* **87,** 1295–1299.

Rothe, G., Gabriell H., Kovacs, E., Klucken, J., Stohr, J., Kindermann, W., and Schmitz, G. (1996). Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia. *Arterioscler. Thromb. Vasc. Biol.* **16,** 1437–1447.

Sakurai, H., Kohsaka, H., Liu, M. F., Higashiyama, H., Hirata, Y., Kanno, K., Saito, I., and Miyasaka, N. (1995). Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *J. Clin. Invest.* **96,** 2357–2363.

Schuschke, D. A., Miller, F. N., Lominadze, D. G., and Feldhoff, R. C. (1994). L-Arginine restores cholesterol-attenuated microvascular responses in the rat cremaster. *Int. J. Microcirc. Clin. Exp.* **14,** 204–211.

Schwarzacher, S. P., Lim, T. T., Wang, B. Y., Kernoff, R. S., Niebauer, J., Cooke, J. P., and Yeung, A. C. (1997). Local intramural delivery of L-arginine enhances nitric oxide generation and inhibits lesion formation after balloon angioplasty. *Circulation* **95,** 1863–1869.

Schwenke, D. E., and Carew, T. E. (1988). Quantification in vivo of increased LDL content and rate of LDL degradation in normal rabbit aorta occurring at sites susceptible to early atherosclerotic lesions. *Circ. Res.* **62,** 699–710.

Serruys, P. W., de Jaegere, P., Kiemeneij, F., Macaya, C., Rutsch, W., Heyndrickx, G., Emanuelsson, H., Marco, J., Legrand, V., Materne, P., Belardi, J., Sigwart, U., Colombo, A., Goy, J. J., van der Heuvel, P., Delcan, J., and Morel, M., for the Benestent Study Group (1994). A comparison of balloon expandable stent implantation with balloon angioplasty in patients with coronary artery disease. *N. Engl. J. Med.* **331,** 489–495.

Serruys, P. W., Emanuelsson, H., van der Giessen, W., Lunn, A. C., Kiemeneij, F., Macaya, C., Rutsch, W., Heyndrickx, G., Suryapranata, H., Legrand, V., Goy, J. J., Materne, P., Bonnier, H., Morice, M. C., Fajadet, J., Belardi, J., Colombo, A., Garcia, E., Ruygrok, P., de Jaegere, P., and Morel, M., on behalf of the Benestent-II Study Group. (1996). Heparin-coated Palmaz-Schatz stents in human coronary arteries: Early outcome of the Benestent-II pilot study. *Circulation* **93,** 412–422.

Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J., and Hintze, T. H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* **74,** 349–353.

Shimokawa, H., Aarhus, L. L., and Vanhoutte, P. M. (1987). Porcine coronary arteries with regenerated endothelium have a reduced endothelium-dependent responsiveness to aggregating platelets and serotonin. *Circ. Res.* **61,** 256–270.

Shultz, P. J., and Raij, L. (1989). Effects of antihypertensive agents on endothelium-dependent and endothelium-independent relaxations. *Br. J. Clin. Pharmacol.* **28**(Suppl. 2), 151S–157S.

Simon, B. C., Cunningham, L. D., and Cohen, R. A. (1990). Oxidized low density lipoproteins cause contraction and inhibit endothelium-dependent relaxation in the pig coronary artery. *J. Clin. Invest.* **86,** 75–79.

Sinzinger, H., Siberbauer, K., and Auerswald, W. (1980). Quantitative investigation of sudanophilic lesions around the aortic ostia of human fetuses, newborns and children. *Blood Vessels* **17,** 44–52.

Spiecker, M., Peng, H.-B., and Liao, J. K. (1997). Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves

the induction and nuclear translocation of IKBα. *J. Biol. Chem.* **272,** 30969–30974.

Stamler, J. S., Mendelsohn, M. E., Amarante, P., Smick, D., Andon, N., Davies, P. F., Cooke, J. P., and Loscalzo, J. (1989). *N*-acetylcysteine potentiates platelet inhibition by endothelium-derived relaxing factor. *Circ. Res.* **65,** 789–795.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stemme, S., and Hansson, G. K. (1994). Immune mechanisms in atherogenesis. *Ann. Med.* **26,** 141–146.

Stroes, E. S., Koomans, H. A., de Bruin, T. W., and Rabelink, T. J. (1995). Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* **346,** 467–471.

Tangphao, O., Chalon, S., Coulston, A. M., Moreno, H., Jr., Chan, J. R., Cooke, J. P., Hoffman, B. B., and Blaschke, T. F. (1999a). L-Arginine and nitric oxide-related compounds in plasma: Comparison of normal and arginine-free diets in a 24-h crossover study. *Vasc. Med.* **4,** 27–32.

Tangphao, O., Chalon, S., Moreno, H., Hoffman, B. B., and Blaschke, T. F. (1999b). Pharmacokinetics of L-arginine during chronic administration to hypercholesterolemic patients. *Clin. Sci.* **96,** 199–207.

Tarry, W. C., and Makhoul, R. G. (1994). L-Arginine improves endothelium-dependent vasorelaxation and reduces intimal hyperplasia after balloon angioplasty. *Arterioscler. Thromb.* **14,** 938–943.

Tesfamariam, B., and Cohen, R. A. (1992). Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am. J. Physiol.* **263,** H321–H326.

Theilmeier, G., Chan, J. R., Zalpour, C., Anderson, B., Wang, B.-Y., Wolf, A., McEvoy, L. M., Butcher, E. C., Tsao, P. S., and Cooke, J. P. (1997). Adhesiveness of mononuclear cells in hypercholesterolemic humans is normalized by dietary L-arginine. *Arterioscler. Thromb. Vasc. Biol.* **17,** 3557–3564.

Tiefenbacher, C. P., and Chilian, W. M. (1997). Basic fibroblast growth factor and heparin influence coronary arteriolar tone by causing endotheliium-dependent dilation. *Cardiovasc. Res.* **34,** 411–417.

Treasure, C. B., Klein, J. L., Weintraub, W. S., Talley, J. D., Stillabower, M. E., Kosinski, A. S., Zhang, J., Boccuzzi, S. J., Cedarholm, J. C., and Alexander, R. W. (1995). Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. *N. Engl. J. Med.* **332,** 481–487.

Tsao, P. S., and Lefer, A. M. (1990). Time course and mechanism of endothelial dysfunction in isolated ischemic and hypoxic rat hearts. *Am. J. Physiol.* **259,** C738–C745.

Tsao, P., McEvoy, L. M., Drexler, H., *et al.* (1994). Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation* **89,** 2176–2182.

Tsao, P. S., Lewis, N. P., Alpert, S., and Cooke, J. P. (1995). Exposure to shear stress alters endothelial adhesiveness: Role of nitric oxide. *Circulation* **92,** 3513–3519.

Tsao, P. S., Buitrago, R., Chan, J. R., and Cooke, J. P. (1996). Fluid flow inhibits endothelial adhesiveness: Nitric oxide and transcriptional regulation of VCAM-1. *Circulation* **94,** 1682–1689.

Tsao, P. S., Wang, B. Y., Buitrago, R., *et al.* (1997). Nitric oxide regulates monocyte chemotactic protein-1. *Circulation* **96,** 934–940.

Ueki, Y., Miyake, S., Tominaga, Y., and Eguchi, K. (1996). Increased nitric oxide levels in patients with rheumatoid arthritis. *J. Rheumatol.* **23,** 230–236.

Usui, M., Matsuoka, H., Miyazaki, H., Ueda, S., Okuda, S., and Imaizumi, T. (1998). Increased endogenous nitric oxide synthase inhibitor in patients with congestive heart failure. *Life Sci.* **62,** 2425–2430.

Vallance, P., Leone, A., Calver, A., Collier, J., and Moncada, S. (1992). Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *J. Cardiovasc. Pharmacol.* **20**(Suppl. 12), S60–S62.

Van Belle, E., Rivard, A., Chen, D., Silver, M., Bunting, S., Ferrara, N., Symes, J. F., Bauters, C., and Isner, J. M. (1997). Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines. *Circulation* **96,** 2667–2674.

van der Zee, R., Murohara, T., Luo, Z., Zollmann, F., Passeri, J., Lekutat, C., and Isner, J. M. (1997). Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* **95,** 1030–1037.

Verbeuren, T. J., Jordaens, F. H., Zonnekeyn, L. L., Van Hove, C. E., Coene, M. C., and Herman, A. G. (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ. Res.* **58,** 552–564.

Virchow, R. (Clance, F., transl.). (1860). ''Cellular Pathology as Based upon Physiological and Pathological Histology.'' Churchill, London.

Visek, W. J. (1986). Arginine needs, physiological state and usual diets. A reevaluation. *J. Nutr.* **116,** 36–46.

von der Leyen, H. E., Gibbons, G. H., Morishita, R., Lewis, N. P., Zhang, L., Nakajima, M., Kaneda, Y., Cooke, J. P., and Dzau, V. J. (1995). Gene therapy inhibiting neointimal vascular lesion: In vivo transfer of endothelial cell nitric oxide synthase gene. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1137–1141.

Walpola, P. L., Gottlieb, A. I., and Langille, B. L. (1993). Monocyte adhesion and changes in endothelial cell number, morphology, and F-actin distribution elicited by low shear stress in vivo. *Am. J. Pathol.* **142,** 1392–1400.

Walpola, P. L., Gottlieb, A. I., Cybulsky, M. I., and Langille, B. L. (1995). Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. *Arterioscler. Thromb. Vasc. Biol.* **15,** 2–10.

Wang, B., Singer, A., Tsao, P., Drexler, H., Kosek, J., and Cooke, J. P. (1994). Dietary arginine prevents atherogenesis in the coronary artery of the hypercholesterolemic rabbit. *J. Am. Coll. Cardiol.* **23,** 452–458.

Wang, B. Y., Hoai-Ky, V. H., Lin, P. S., Schwarzacher, S. P., Pollman, M. J., Gibbons, G. H., Tsao, P. S., and Cooke, J. P. (1999). Regression of atherosclerosis: Role of nitric oxide and apoptosis. *Circulation* **99,** 1236–1241.

Weber, C., Erl, W., Pietsch, A., Strobel, M., Ziegler-Heitbrock, H. W. L., and Weber, P. C. (1994). Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-β mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. *Arterioscler. Thromb.* **14,** 1665–1673.

Weidinger, F. F., McLenachan, J. M., Cybulsky, M., Gordon, J. M., Rennke, H. A., Hollenberg, N. K., Ganz, P., and Cooke, J. P. (1990). Persistent dysfunction of regenerated endothelium following balloon angioplasty of rabbit iliac artery. *Circulation* **81,** 1667–1679.

White, C. R., Brock, T. A., Chang, L. Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A., and Tarpey, M. M. (1994). Superoxide and peroxynitrite in atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1044–1048.

Wolf, A., Zalpour, C., Theilmeier, G., Wang, B.-Y., Ma, A., Anderson, B., Tsao, P. S., and Cooke, J. P. (1997). Dietary L-arginine supplementation normalizes platelet aggregation in hypercholesterolemic humans. *J. Am. Coll. Cardiol.* **29,** 479–485.

Wu, H. M., Yuan, Y., McCarthy, M., and Granger, H. J. (1996). Acidic and basic FGFs dilate arterioles of skeletal muscle through a NO-dependent mechanism. *Am. J. Physiol.* **271**(Part 2), H1087–H1093.

Yeatman, T. J., Risley, G. L., and Brunson, M. E. (1991). Depletion of dietary arginine inhibits growth of metastatic tumor. *Arch. Surg.* **126,** 1376–1381; discussion 1381–1382.

Yu, X. J., Li, X. J., and Xiong, Y. (1994). Increase of an endogenous inhibitor of nitric oxide synthesis in serum of high cholesterol fed rabbits. *Life Sci.* **54,** 753–758.

Zeiher, A. M., Drexler, H., Wolschlager, H., and Just, H. (1991). Modulation of coronary vasomotor tone in humans. *Circulation* **83,** 391–401.

Zeiher, A. M., Fisslthaler, B., Schray-Utz, B., and Busse, R. (1995). Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ. Res.* **76,** 980–986.

Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., Geppetti, P., and Ledda, F. (1994). Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J. Clin. Invest.* **94,** 2036–2044.

Ziche, M., Parenti, A., Ledda, F., Dell'Era, P., Granger, H. J., Maggi, C. A., and Presta, M. (1997). Nitric oxide promotes proliferation and plasminogen activator production by coronary venular endothelium through endogenous bFGF. *Circ. Res.* **80,** 845–852.

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CHAPTER 49

Cytotoxic Role of Nitric Oxide in Diabetes

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INSULIN-DEPENDENT DIABETES MELLITUS IS CHARACTERIZED BY SELECTIVE DESTRUCTION OF INSULIN-SECRETING β CELLS FOUND IN PANCREATIC ISLETS. CYTOKINES, DERIVED FROM ACTIVATED MACROPHAGES AND LYMPHOCYTES, ARE BELIEVED TO PARTICIPATE IN β-CELL DAMAGE BY STIMULATING INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) EXPRESSION AND NITRIC OXIDE PRODUCTION IN ISLETS. IN ANIMAL MODELS OF AUTOIMMUNE DIABETES, iNOS INHIBITORS HAVE BEEN SHOWN TO ATTENUATE OR DELAY THE ONSET OF DIABETES. IN THIS CHAPTER, THE MECHANISMS BY WHICH CYTOKINES STIMULATE NITRIC OXIDE SYNTHASE EXPRESSION AND THE RAT, MOUSE, AND HUMAN ISLET CELLULAR SOURCES OF THIS ENZYME ARE EXAMINED. ALSO, THE MECHANISMS BY WHICH NITRIC OXIDE IMPAIRS β-CELL FUNCTION AND STIMULATES β-CELL DEATH ARE DISCUSSED. IN ADDITION, STUDIES DEMONSTRATING A ROLE FOR NITRIC OXIDE IN THE DEVELOPMENT OF DIABETES IN ANIMAL MODELS ARE EVALUATED. VIRAL INFECTION HAS BEEN IMPLICATED AS ONE ENVIRONMENTAL AGENT THAT MAY STIMULATE DIABETES IN GENETICALLY SUSCEPTIBLE INDIVIDUALS. IN THIS CHAPTER, EVIDENCE TO SUPPORT A ROLE FOR NITRIC OXIDE IN VIRAL-INDUCED DIABETES IS EXAMINED. LAST, THE ROLE OF NITRIC OXIDE IN MEDIATING β-CELL ''BURN-OUT'' IN ANIMAL MODELS OF TYPE II OR NON-INSULIN-DEPENDENT DIABETES IS DISCUSSED.

Introduction

Diabetes, defined as an excess of sugar in the blood, is caused by the inability of insulin to stimulate glucose disposal or by the absence or reduced production of insulin by pancreatic β cells. In the United States, 16 million people have been diagnosed with diabetes. This disease is the leading cause of kidney failure, amputations, and blindness, and it is also a major risk factor for cardiovascular disease and stroke (Gaster and Hirsch, 1998; Marre, 1999; Swidan and Montgomery, 1998). In the United States, over $100 billion is spent annually in health care costs related to diabetes (Pathak and Burke, 1998). This figure is expected to increase, as currently there is no cure for diabetes. Treatments include agents that either stimulate insulin action or increase insulin production by β cells, and insulin therapy.

There are several types of diabetes. The most common form is type 2 diabetes (non-insulin-dependent diabetes mellitus or NIDDM), a disease that primarily affects adults. Type 2 diabetes was originally termed adult onset because this disease usually occurs in individuals over the age of 40. However, the prevalence of type 2 diabetes is increasing in children, and this increase appears to reflect a similar increase in the number of obese children (Caprio and Tamborlane, 1999; Trissler, 1999). Type 2 diabetes is characterized by defects in insulin secretion by β cells or defects in insulin action. Defects in insulin action usually occur in middle-aged individuals and are associated with obesity (Lebovitz,

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1999). In the United States, over 55% of the population is overweight (body mass index over 25 kg/m^2), and 80–90% of type 2 diabetics are overweight (Jovanovic and Gondos, 1999; Must *et al.,* 1999).

Type 1 or insulin-dependent diabetes mellitus (IDDM) is the second most common form of diabetes. Type 1 diabetes is caused by an immune-mediated attack directed against insulin-producing β cells found in pancreatic islets of Langerhans (Gepts, 1965). The destruction of β cells results in insulin deficiency that is treated by daily injections of insulin. Type 1 diabetes is typically diagnosed in childhood, but it can occur at any age. Autoimmune destruction of β cells is associated with both genetic risk factors and environmental factors (Bain *et al.,* 1997; Yoon, 1990). The genetic risk factors that most strongly predispose individuals to autoimmune diabetes include the major histocompatibility complex (MHC, or HLA, human leukocyte antigen) found on chromosome 6 (Todd, 1999). Over 80% of type 1 diabetics have an allele in HLA DR3 or DR4; however, many individuals with these alleles do not develop autoimmune diabetes (Todd, 1999). Support for the involvement of environmental factors in the development of autoimmune diabetes comes from the observations that the concordance rate for diabetes in identical twins is less than 40% (Medici *et al.,* 1999; Olmos *et al.,* 1988). Environmental factors that may contribute to autoimmune diabetes include infectious agents, dietary factors, and environmental toxins.

Although it is clear that environmental and genetic risk factors contribute to the development of autoimmune diabetes, the underlining biochemical and immunological mechanisms by which β cells are destroyed are poorly defined. Studies suggest that nitric oxide (NO) may participate in the development of diabetes by functioning as an effector molecule that mediates β-cell damage. In this chapter, evidence to support a role for nitric oxide in mediating β-cell damage during the development of autoimmune diabetes will be reviewed. In addition, the involvement of nitric oxide in mediating β-cell destruction during the development of type II diabetes will also be discussed. Although nitric oxide has been implicated in the regulation of glucose uptake by muscle (Balon, 1998) and in complications of diabetes (Baynes and Thorpe, 1999; Ido *et al.,* 1997), these topics have been covered in other reviews and will not be elaborated in this chapter.

Overview of Autoimmune Diabetes

Autoimmune diabetes is characterized by selective destruction of insulin-secreting β cells found in pancreatic islets of Langerhans (Gepts, 1965). β cells are destroyed during an inflammatory reaction in and around islets, termed insulitis. A number of inflammatory cell types comprise this destructive insulitic reaction. These include CD4$^+$ and CD8$^+$ T lymphocytes, macrophages, and a limited number of β lymphocytes (Bach, 1994). In animal models of diabetes, it has been shown that insulitis is preceded by peri-insulitis, which comprises a mononuclear cellular infiltrate that localizes around the islet periphery (Campbell and Harrison, 1990; Kolb, 1997). T cells are believed to play a primary role in mediating β-cell destruction during the development of autoimmune diabetes. It is possible to transfer diabetes to irradiated nondiabetic animals [both nonobese diabetic mice (NOD) and BioBreeding rats (BB)] using splenocytes obtained from diabetic animals (Like *et al.,* 1985; Wicker *et al.,* 1986), or by transferring purified T cells and T-cell clones established from lesions from diabetic animals (Bergman and Haskins, 1997). Antibody depletion of CD4$^+$ and CD8$^+$ T lymphocytes prevents the development of diabetes in the NOD mouse, further supporting a role for both subsets of T cells in disease progression (Hayward *et al.,* 1993; Wang *et al.,* 1996). The progression of diabetes is dependent on the T-cell subtype. CD4$^+$ Th_1 T cells, which produce interleukin-2 (IL-2) and γ-interferon (IFN-γ), are associated with the progression to diabetes. In contrast, CD4$^+$ Th_2 T cells, which produce IL-4 and IL-10, appear to block disease progression (Heurtier and Boitard, 1997; Lafaille, 1998; Rabinovitch, 1998). It is interesting to note that IL-4 and IL-10 have been shown to downregulate cytokine-stimulated inducible nitric oxide synthase (iNOS) expression by macrophages (Erwig *et al.,* 1998; Vouldoukis *et al.,* 1997).

Macrophage participation in disease progression is also essential. Macrophage depletion by silica treatment (Oschilewski *et al.,* 1985; Wright and Lacy, 1989) or by feeding a diet deficient in essential fatty acids (Lefkowith *et al.,* 1987; Schreiner *et al.,* 1988) prevents the development of autoimmune diabetes in a number of animal models (Benhamou *et al.,* 1995; Lefkowith *et al.,* 1990; Wright *et al.,* 1988). Macrophages may participate in the development of autoimmune diabetes by (1) producing inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF), which may directly damage β cells; (2) producing toxic free radicals (nitrogen and oxygen), which may also mediate β-cell damage; or (3) functioning as antigen presenting cells, thereby stimulating T-cell-dependent destruction of β cells. Figure 1 provides a schematic overview of the potential roles of each of these cell types and inflammatory mediators in the progression of autoimmune diabetes.

Nitric Oxide Mediates Cytokine-Induced Inhibition of Insulin Secretion By β Cells

In vitro studies provided the initial evidence that cytokines modulate β-cell function and mediate islet damage. Mandrup-Poulsen *et al.* (1985) first showed that treatment of isolated rat islets with conditioned medium derived from activated human mononuclear cells results in an inhibition of glucose-stimulated insulin secretion. The active component of this activated culture medium was determined to be the cytokine IL-1 (Bendtzen *et al.,* 1986). IL-1-induced inhibition of insulin secretion is both time and concentration dependent, and it requires mRNA transcription and *de novo*

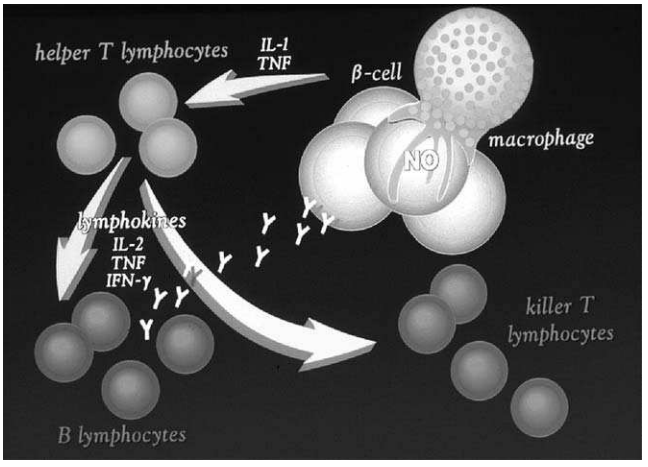


Figure 1 Schematic diagram of the cellular components of islet inflammation. The development of autoimmune diabetes is characterized by T-lymphocyte, macrophage, and monocyte infiltration into islets. Cytokines produced by these inflammatory cells may participate in the regulation of islet inflammation and may also directly stimulate β-cell expression of iNOS and production of nitric oxide. Nitric oxide produced by β cells and macrophages may directly damage β cells, stimulating the release of autoantigens and further T-lymphocyte-dependent β-cell destruction. In this chapter, evidence supporting a role for inflammatory cytokines (such as IL-1 and IFN-γ) and nitric oxide in mediating β-cell damage during the development of diabetes will be reviewed. See color insert.

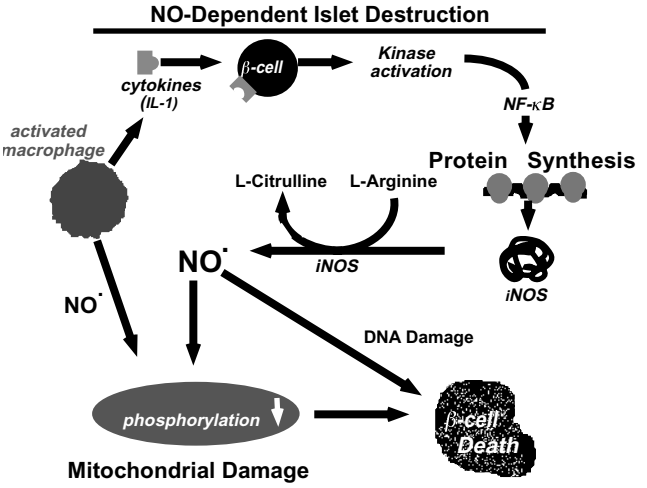


Figure 2 Mechanisms of IL-1-induced β-cell damage. This figure provides a schematic diagram of the mechanisms by which IL-1 stimulates inducible NOS (iNOS) expression and nitric oxide production by β cells, and how nitric oxide mediates β-cell damage. IL-1 binding to surface receptors activates a series of signaling pathways that result in the induction of iNOS mRNA transcription. Nuclear factor κB (NF-κB) appears to play a primary role in the regulation of iNOS expression in response to IL-1; however, other transcriptional activators may also participate. Nitric oxide, produced by β cells, stimulates β-cell damage by inhibiting islet oxidative metabolism and by inducing DNA strand breaks, leading to impairment in glucose-stimulated insulin secretion and β-cell death.

protein synthesis (Comens *et al.,* 1987; Hughes *et al.,* 1990; McDaniel *et al.,* 1988; Sandler *et al.,* 1991). The inhibitory effects of IL-1 on insulin secretion are associated with an inhibition in the oxidative metabolism of glucose to CO_2 (Eizirik *et al.,* 1989; Hughes *et al.,* 1990; Sandler *et al.,* 1991). Importantly, insulin secretion by β cells absolutely requires the oxidation of glucose to CO_2, resulting in the generation of increased levels of ATP within β cells. ATP is required for closure of the ATP-inhibited K^+ channels, leading to β-cell depolarization and Ca^{2+} influx (Misler *et al.,* 1992), events required for glucose-stimulated insulin secretion.

We first hypothesized that nitric oxide may mediate the inhibitory effects of IL-1 on insulin secretion by isolated rat islets (Corbett and McDaniel, 1992). This hypothesis was based on the observations that one of the cellular targets of nitric oxide are enzymes containing iron–sulfur centers, specifically the electron transport chain complexes I and II and the Krebs cycle enzyme aconitase (Drapier and Hibbs, 1996), and that treatment of rat islets with IL-1 results in the inhibition of islet oxidative metabolism (Sandler *et al.,* 1991). In three concurrent studies, we and others showed that treatment of rat islets with IL-1 or IL-1 plus TNF results in the production of nitrite (oxidative metabolite of nitric oxide) and that inhibitors of nitric oxide synthase (NOS) prevent cytokine-induced inhibition of insulin secretion (Corbett *et al.,* 1991; Southern *et al.,* 1990; N. Welsh *et al.,* 1991). Consistent with mitochondrial iron–sulfur centers as targets for nitric oxide-mediated damage, IL-1 was shown to induce the formation of electron paramagnetic resonance (EPR)-detectable iron–nitrosyl complexes by rat islets, to inhibit islet aconitase activity, to inhibit the oxidation of glucose to CO_2, and to reduce cellular ATP levels in rat islets (Corbett *et al.,* 1995; Cunningham and Green, 1994; Mandrup-Poulsen, 1996). Importantly, the nitric oxide synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA), nitro-L-arginine methylester (L-NAME), and aminoguanidine (AG) prevent all of these effects of IL-1 on islet function (Corbett *et al.,* 1992a, b; Southern *et al.,* 1990; N. Welsh *et al.,* 1991). In addition, studies have shown that islets isolated from iNOS-deficient mice are not adversely affected by *in vitro* treatment with IL-1 or a combination of IL-1 and IFN-γ (Flodstrom *et al.,* 1999). These studies demonstrate that nitric oxide mediates the damaging actions of cytokines on islet secretory function. A working model for the mechanism by which IL-1 inhibits glucose-stimulated insulin secretion by rat islets is shown in Fig. 2.

Does Nitric Oxide Mediate Cytokine-Induced Inhibition of Human Islet Function?

Controversy continues to exist as to whether cytokine-mediated human islet damage is dependent on the production of nitric oxide. Individually, neither IL-1, IFN-γ, nor TNF inhibit glucose-stimulated insulin secretion, nor do they stimulate iNOS expression or nitric oxide production by human islets (Corbett *et al.,* 1993a; Eizirik *et al.,* 1994a). However, in combination, IL-1, TNF, and IFN-γ stimulate iNOS expression, stimulate nitrite production, and inhibit glucose-

stimulated insulin secretion by human islets (Corbett *et al.,* 1993a; Eizirik *et al.,* 1994a). The controversy is directed at whether nitric oxide mediates the inhibitory actions of IL-1 plus TNF plus IFN-γ on human islet function or if cytokine-mediated inhibition of human islet function is independent of nitric oxide production. We first showed that treatment of human islets for 24 hours with IL-1 plus TNF plus IFN-γ results in an ~70% inhibition of insulin secretion that is significantly attenuated by L-NMMA (Corbett *et al.,* 1993a). Eizirik and co-workers showed that a 6-day incubation of human islets with the same combination of cytokines results in an inhibition of insulin secretion that is not prevented by AG, although AG did attenuate cytokine-induced nitric oxide production (Eizirik *et al.,* 1994a). Whereas the findings presented in these two studies appear to be opposed, two important issues may explain the divergent results concerning the role of nitric oxide in mediating cytokine-induced inhibition of insulin secretion by human islets. First, in the Eizirik study, human islets were cultured for 6 days with cytokines (Eizirik *et al.,* 1994a), whereas in our study human islets were cultured for only 24 hours (Corbett *et al.,* 1993a). Second, in the Eizirik study, AG in the absence of cytokines inhibited insulin secretion by human islets to levels similar to the levels inhibited by the combination of cytokines (Eizirik *et al.,* 1994a). In this study, AG was used at a concentration of 5 m*M,* a level that has been shown to inhibit insulin secretion by rodent islets (Holstad *et al.,* 1997). It is possible that the divergent results obtained in these two studies reflect differences in human islet culture; however, the results in the Eizirk study appear equivocal because AG in the absence of cytokines inhibited insulin secretion to levels similar in magnitude to the inhibitory actions of the cytokine combination (Eizirik *et al.,* 1994a). In support of a role for nitric oxide in the inhibition of insulin secretion, Eizirik and co-workers (Eizirik *et al.,* 1994b) have shown that nitric oxide donor compounds inhibit glucose-stimulated insulin secretion by isolated human islets.

Although the role of nitric oxide in cytokine-induced inhibition of human islet function remains controversial, nitric oxide has been shown to impair human islet oxidative metabolism. We have shown that IL-1 plus TNF plus IFN-γ stimulates the formation of iron–nitrosyl complexes in human islets (Corbett *et al.,* 1993a). In rodent islets, the formation of iron–nitrosyl complexes occurs under conditions in which oxidative metabolism is impaired (Corbett *et al.,* 1991, 1992b). Consistent with an impairment of oxidative metabolism, treatment of human islets with IL-1 plus IFN-γ (minimal combination of cytokines required to stimulate iNOS expression and nitrite formation by human islets) results in an ~60% inhibition of mitochondrial aconitase activity, an effect that is prevented by the iNOS inhibitor AG (Scarim *et al.,* 1997). Since glucose oxidation to CO_2 is required for insulin secretion, and since nitric oxide inhibits human islet aconitase activity (Scarim *et al.,* 1997), we believe that nitric oxide participates in cytokine-mediated human islet damage. Importantly, other factors yet to be identified also appear to participate in cytokine-mediated human islet damage, as inhibition of iNOS activity does not completely prevent the inhibitory actions of these cytokines on human islet function. This is in contrast to the effects of cytokines on rodent islets, where the inhibition of iNOS activity completely prevents cytokine-induced inhibition of insulin secretion.

Cytokine Requirements for iNOS Expression by Rodent and Human Islets

A wide array of agents (such as cytokines, bacterial and viral products, and proinflammatory molecules) have been shown to stimulate iNOS expression; however, the response to these agents is cell type and species specific (Nathan, 1992). In most cell types, iNOS expression requires a combination of two signals. For example, mouse macrophages require a combination of both TNF and lipopolysaccharide (LPS), LPS and IFN-γ, or IFN-γ and TNF for iNOS induction (Nathan, 1992). In contrast, IL-1 alone is capable of stimulating iNOS expression by rat islets (Corbett *et al.,* 1996; Mandrup-Poulsen, 1996). Similarly to isolated rat islets, insulinoma cell lines such as RINm5F, NIT, HIT, and INS also express iNOS in response to IL-1 (Corbett *et al.,* 1992b; Eizirik *et al.,* 1992; Stephens *et al.,* 1997). Importantly, the individual effects of IL-1 on iNOS expression are potentiated by IFN-γ. IFN-γ reduces the concentration of IL-1 required to stimulate maximal iNOS expression by 10-fold, such that IL-1 at concentrations as low as 0.01 pg/ml (or 0.57 p*M*) are capable of stimulating iNOS expression by rat islets (Heitmeier *et al.,* 1997). TNF appears to potentiate the level of nitric oxide produced by rat islets by ~10% (Cetkovic-Cvrlje and Eizirik, 1994; Southern *et al.,* 1990); however, TNF does not augment iNOS mRNA accumulation or protein expression, nor does TNF increase the sensitivity of rat islets to IL-1 (M. R. Heitmeier and J. A. Corbett, 2000, unpublished observation).

Islets isolated from most mouse strains require a combination of IL-1 and IFN-γ for iNOS expression (M. Welsh *et al.,* 1995; N. Welsh and Sandler, 1992). IL-1 plus IFN-γ-induced iNOS expression and nitrite formation is increased by ~10% in the presence of TNF (Thomas *et al.,* 1999). Individually, neither IFN-γ nor TNF stimulate iNOS expression by mouse islets (M. R. Heitmeier and J. A. Corbett, unpublished observation). In some mouse strains, IL-1 alone will stimulate iNOS expression by isolated islets; however, for maximal iNOS expression IFN-γ is required in addition to IL-1 (M. Welsh *et al.,* 1995; N. Welsh and Sandler, 1992). Similarly to mouse islets, human islets also require a combination of IL-1 and IFN-γ for iNOS expression (Corbett *et al.,* 1996; Eizirik *et al.,* 1994a). In addition, IL-1 plus IFN-γ-induced iNOS expression by human islets is enhanced slightly by TNF. Alone, neither IL-1, TNF, nor IFN-γ, nor the combinations of IL-1 plus TNF or TNF plus IFN-γ stimulate iNOS expression by human islets (Corbett *et al.,* 1996; Eizirik *et al.,* 1994a).

Table I Expression of iNOS in Islets[a]

Islets	Cytokines	Source of iNOS	Functional effects
Rat	IL-1	β cell	Mitochondrial function ↓
	dsRNA + IFN-γ	β cell, macrophage	Secretion ↓
	TNF + LPS	Macrophage, β cell[b]	Islet damage ↓
Human	IL-1 + IFN-γ	β cell	Mitochondrial function ↓
	TNF + LPS + IFN-γ	Macrophage, β cell[b]	Secretion ↓

[a] This table provides a list of agents that stimulate iNOS expression by islets, the cellular source of iNOS, and the effects of iNOS expression on β-cell function.

[b] β-Cell iNOS expression via intra-islet IL-1 release by macrophages.

Proinflammatory agents such as double-stranded RNA (dsRNA) and LPS also stimulate iNOS expression by rat and human islets when incubated in the presence of IFN-γ or TNF. dsRNA (in the form of polyinosinic:polycytidylic acid, polyIC) in combination with IFN-γ stimulates high levels of iNOS expression and nitrite production by both rat (Heitmeier *et al.,* 1999a) and human islets (M. R. Heitmeier and J. A. Corbett, 2000, unpublished observation). The significance of iNOS expression in response to dsRNA will be described in detail in the section concerning mechanisms of viral-induced autoimmune diabetes. Also, the combinations of TNF plus LPS and TNF plus LPS plus IFN-γ stimulate iNOS expression by rat and human islets, respectively (Arnush *et al.,* 1998a; Corbett and McDaniel, 1995). Importantly, iNOS expression in response to TNF plus LPS in rat islets and TNF plus LPS plus IFN-γ in human islets is dependent on the local release of IL-1 in islets following resident macrophage activation. A summary of the agents that stimulate iNOS expression by rat and human islets is shown in Table I.

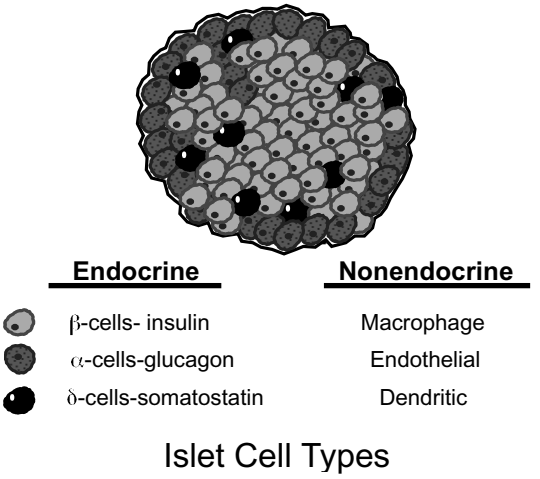


Figure 3 Islet cellular composition. Islets are composed of endocrine β cells (insulin-producing, ~70%), α cells (glucagon, ~25%), and a small percentage of somatostatin-secreting δ cells and polypeptide-secreting cells. Islets also contain a limited number of endothelial cells, macrophages, dedritic cells, and fibroblasts.

The Cellular Composition of Islets

Islets are composed of both endocrine and nonendocrine cells (Fig. 3). Insulin-secreting β cells are found in the central core of the islet and comprise ~70% of all islet cells. Glucagon-secreting α cells and somatostatin-secreting δ cells are found on the outer edge of the islet, or the islet mantel. Islets are highly vascularized, with blood flow penetrating from the mantle to the central core, where it then breaks into a number of fenestrated capillaries that flow past β cells to the islet mantle (Bonner-Weir, 1988; Bonner-Weir and Orci, 1982). Blood flow in islets represents ~20% of all blood that flows into the pancreas, a disproportionately large volume in that islets represent 1–2% of the pancreas volume (Bonner-Weir and Orci, 1982). Islets also contain a number of additional nonendocrine cell types including macrophages, dendritic cells, and fibroblasts (Setum *et al.,* 1991). Of these cell types, experimental evidence supports an effector role for endothelial cells and intraislet macrophages in mediating β-cell damage during the development of autoimmune diabetes.

Cellular Source of iNOS in Islets

β Cells

Central to understanding the mechanisms by which nitric oxide impairs β-cell function is the identification of the islet cellular sources of iNOS. This is a particularly important issue in that β cells are selectively destroyed during the development of autoimmune diabetes, whereas other islet cell types remain unharmed (Bach, 1994). As a potential mechanism to explain β-cell-selective destruction in autoimmune diabetes, we examined whether cytokine-stimulated iNOS expression is selective for β cells. In these studies, primary β cells were purified from rat islets by fluorescence activated cell sorting (FACS). Islet cell purification by FACS results in an ~95% pure population of β cells and an ~85% pure population of α cells (Pipeleers *et al.,* 1985). Treatment of FACS-purified β cells for 24 hours with IL-1 results in iNOS expression and nitric oxide production, an inhibition of glu-

cose oxidation, and an inhibition of glucose-stimulated insulin secretion (Corbett *et al.,* 1992b; Eizirik *et al.,* 1992; Strandell *et al.,* 1995). L-NMMA and AG prevent IL-1-induced nitrite formation and also the inhibitory actions of this cytokine on glucose oxidation and insulin secretion (Corbett *et al.,* 1992b). Importantly, IL-1 fails to inhibit glucose oxidation or stimulate iNOS expression by primary α cells purified by FACS (Corbett *et al.,* 1992b). Immunohistochemistry was used to confirm that β cells are the sole islet cellular source of iNOS in response to IL-1. As shown in Fig. 4, IL-1 stimulates high levels of iNOS expression (red fluorescence) in insulin-containing cells (green fluorescence) as evidenced by the intense yellow fluorescence on double exposure (Corbett and McDaniel, 1995). Importantly, not all insulin-containing cells express iNOS, and iNOS expression was not detected in non-insulin-containing cells. These findings show that the β cell is the sole islet cellular source of iNOS in response to IL-1, and β-cell production of nitric oxide results in impairment of β-cell function.

Human islets are similar to rat islets in that β cells are the major cellular source of iNOS in response to IL-1 plus IFN-γ. In human islets treated for 40 hours with IL-1 plus IFN-γ, iNOS and insulin immunoreactivity colocalize (Arnush *et al.,* 1998a). However, iNOS expression is also observed in a limited population of islet cells that do not contain insulin. These findings suggest either that a second population of islet cells other than β cells express iNOS or that the iNOS positive cells which do not contain insulin are actually β cells that have been depleted of insulin stores. Whereas human islet insulin content is reduced during culture, it is likely that this second population of iNOS-positive cells in human islets is islet ductal cells. Pavlovic *et al.* (1999a) have shown that IL-1 plus IFN-γ stimulates iNOS mRNA accumulation and nitrite production by a partially purified population of ductal cells isolated from human pancreata. In addition, iNOS expression localizes with cytokeratin-19, a ductal cell-specific marker (Pavlovic *et al.,* 1999a). These findings indicate that both β cells and ductal cells are sources of iNOS in human islets treated with IL-1 plus IFN-γ.

Endothelial Cells

Islets are highly vascularized in that 8–10 β cells surround an islet capillary, which is composed of a single layer of endothelial cells. It is possible to obtain cultures of pure islet endothelial cells from isolated rat islets by outgrowth onto a collagen matrix. The phenotype of islet endothelial cells differs from exocrine and aortic endothelium in that islet endothelial cells express high levels of the OX2 antigen (specific for thymocyte and brain endothelium), whereas the exocrine and aortic endothelial cells stain weakly for this antigen (Suschek *et al.,* 1994). Treatment of rat islet endothelial cells with a combination of IL-1 plus TNF plus IFN-γ stimulates nitric oxide production, as evidenced by increased nitrite production and the formation of EPR-detectable iron–nitrosyl complexes in these cells (Suschek *et al.,* 1994). Importantly, coculture of activated islet endothelial cells (following cytokine treatment) with islet cells at a target to effector ratio of 1:1 results in islet cell death as determined by trypan blue exclusion, DNA damage by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining, and morphological damage by electron microscopy (Steiner *et al.,* 1997). Under these conditions, islet cell death is dependent on endothelial cell production of nitric oxide. L-NMMA prevents activated endothelial cell-mediated islet cell death, and resident non-

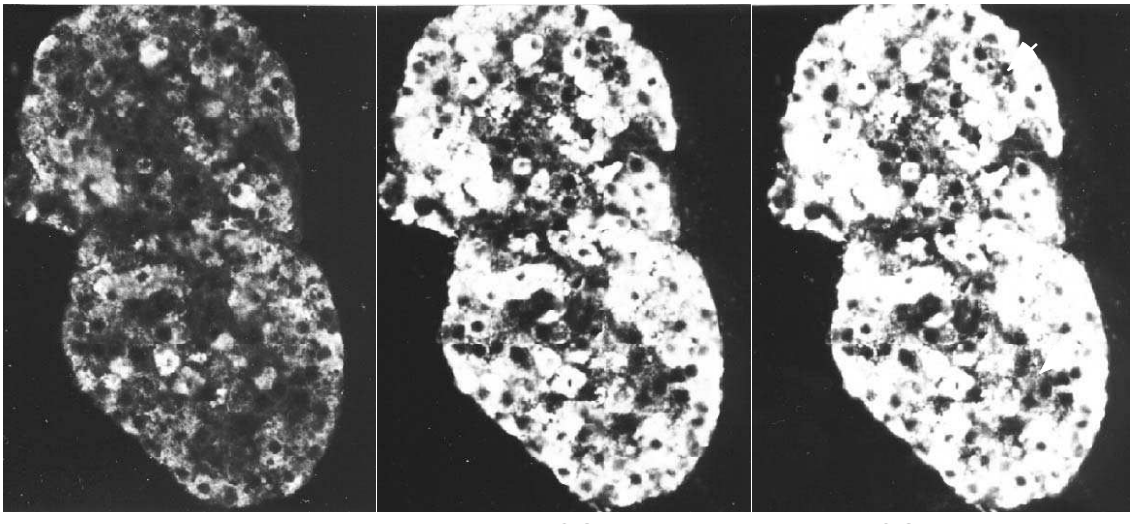


Figure 4 Immunohistochemical analysis of iNOS expression in rat islets in response to IL-1. Rat islets were treated for 40 hours with IL-1, fixed, and used to prepare thin sections. Insulin (green) and iNOS (red) expression was evaluated by immunofluorescence. IL-1 stimulates iNOS expression in insulin-containing cells (yellow). Arrows indicate a limited number of β cells that fail to express iNOS in response to IL-1. These data demonstrate that β cells are the sole islet cellular source of iNOS in response to IL-1 stimulation. Reproduced from the *J. Exp. Med.* **181,** 559–568 (Corbett and McDaniel, 1995) by copyright permission of The Rockefeller University Press. See color insert.

activated islet endothelial cells fail to kill islet cells (Steiner *et al.,* 1997). These findings indicate that islet endothelial cells are a potential source of nitric oxide that may damage β cells during the development of autoimmune diabetes.

Islet endothelial cells are also unique in that endothelial NOS (eNOS) enzymatic activity is dependent on glucose. In a concentration-dependent manner, glucose stimulates islet eNOS activity and nitric oxide production (Suschek *et al.,* 1994). This concentration dependence is similar to the concentration-dependent effects of glucose on insulin secretion. In contrast, aortic endothelial cell eNOS activity is not dependent on the ambient glucose concentration (Suschek *et al.,* 1994). The physiological relevance of the glucose-dependent eNOS activation in islet endothelial cells is unclear. Evidence suggests that nitric oxide may modulate blood flow in islets (Jansson and Andersson, 1997) and may also participate in the regulation of the initial phase of glucose-stimulated insulin secretion (Spinas *et al.,* 1998). However, eNOS regulation of islet blood flow is complex in that islet capillaries do not contain an underlining layer of smooth muscle cells or pericytes that appear to regulate blood flow in capillaries or in capillary beds (Hickey and Kubes, 1997; Kubes, 1995).

Functional defects in islet endothelial eNOS activity also correlate with the probability of diabetes development in the BB rat. In this model of diabetes, diabetes-prone (dp) BB rats naturally develop diabetes, whereas diabetes-resistant (dr) BB rats do not spontaneously develop disease (Kastern *et al.,* 1990). Suschek *et al.* (1999) have shown that islet endothelial cells isolated from dpBB and drBB rats fail to produce nitric oxide in a glucose-dependent manner. In addition, the level of nitric oxide produced by dpBB rat islet endothelial cells is three- to fourfold lower than the levels produced by endothelial cells isolated from drBB and Wistar rat islets. Although islet endothelial cell eNOS activity is impaired, the eNOS activity of aortic endothelial cells isolated from dpBB and drBB rats does not display similar defects (Suschek *et al.,* 1999). These novel findings are significant in that early functional and morphological changes in the islet vasculature resulting in an increased permeability have been suggested to facilitate extravasation of inflammatory cells into islets. Because nitric oxide inhibits leukocyte adhesion (Hickey and Kubes, 1997), a reduced capacity of islet endothelial cells to produce nitric oxide, as observed in the BB rat, may enhance both inflammatory cell adhesion and infiltration of these cells into islets. Consistent with this interpretation, pharmacological reductions in vascular permeability have been shown to attenuate diabetes development induced by multiple injections of low doses of streptozotocin in mice (Schwab *et al.,* 1986). These important observations suggest that islet endothelial cell nitric oxide production may play a primary role in the regulation of inflammatory cell adhesion and infiltration into islets during diabetes development.

MACROPHAGES

Macrophages are known to kill islet cells in a nitric oxide-dependent manner. Kroncke *et al.* (1991a) first showed that incubation of activated macrophages with islet cells at a target to effector ratio of 2:1 results in islet-cell destruction in a nitric oxide-dependent manner. Islet-cell destruction appears to be due to macrophage production of nitric oxide and not cytokine-induced iNOS expression by islet cells, in that antibody neutralization of the macrophage-derived cytokines IL-1 or TNF does not prevent activated macrophage-mediated islet-cell damage (Kroncke *et al.,* 1991a, b). These studies provided the first evidence that islet cells are sensitive to nitric oxide produced by activated macrophages. Importantly, macrophages are one of the first cell types to infiltrate islets during the progression of autoimmune diabetes (Hanenberg *et al.,* 1989; Signore *et al.,* 1989; Yoon *et al.,* 1998), suggesting that increased production of nitric oxide by these inflammatory macrophages may represent an effective mechanism for mediating β-cell destruction.

Although macrophages infiltrate islets during the progression of autoimmune diabetes, islets also contain a limited number of resident macrophages, ~10–15 per islet (or ~0.5% of islet cells) (Arnush *et al.,* 1998b; Lacy and Finke, 1991; Setum *et al.,* 1991). This resident population of macrophages has been proposed to participate in the initial events leading to β-cell damage and the development of autoimmunity directed against β cells (Lacy, 1994). Multiple lines of evidence support this hypothesis. It has been shown that incubation of isolated mouse islets with high concentrations of IFN-γ for 5 days results in islet degeneration. Depletion of MHC class II-expressing cells (including macrophages) by a 7-day culture at 24°C prevents IFN-γ-mediated islet degeneration (Lacy and Finke, 1991). Second, autoimmune diabetes induced by multiple injections of low doses of the β-cell toxin streptozotocin in CD-1 mice is attenuated by feeding these mice a diet deficient in essential fatty acids (Wright *et al.,* 1988). Third, this diet deficient in essential fatty acids also attenuates the natural progression of diabetes in dpBB rats (Lefkowith *et al.,* 1990). Importantly, the fatty acid-deficient diet has been shown to deplete tissue macrophages (Lefkowith *et al.,* 1987), suggesting that the protective effects of this diet on the development of autoimmune diabetes may be associated with the depletion of resident macrophages (Wright *et al.,* 1988; Lacy, 1994).

Other studies have examined the biochemical mechanisms by which resident islet macrophages mediate β-cell damage. Treatment of rat islets for 24 hours with TNF plus LPS, classic conditions used to activate macrophages, results in the formation of nitrite and an inhibition of insulin secretion, effects that are prevented by L-NMMA and AG (Arnush *et al.,* 1998b; Corbett and McDaniel, 1995). Importantly, TNF plus LPS fails to inhibit glucose-stimulated insulin secretion or stimulate nitrite formation by primary β cells purified by FACS, indicating that the inhibitory actions of TNF plus LPS on rat islet function are not mediated by a direct action of these agents on β cells (Corbett and McDaniel, 1995). Because activated macrophages release IL-1 in addition to producing nitric oxide, the potential role of IL-1 in mediating the inhibitory actions of TNF plus LPS on insulin secretion by rat islets was examined using the interleukin 1 receptor antagonist protein (IRAP). IRAP is a member of the

IL-1 family of cytokines that prevents IL-1 signaling by competing with IL-1 for receptor binding (Arend, 1993; Arend *et al.,* 1998). As shown in Fig. 5, IRAP prevents TNF plus LPS-induced nitrite formation and the inhibitory actions of these agents on glucose-induced insulin secretion. These findings suggest that TNF plus LPS mediates β-cell damage by stimulating the local release of IL-1 in islets and that IL-1 attains sufficient levels in islets to stimulate β-cell damage in a nitric oxide-dependent fashion. Although TNF plus LPS fails to stimulate iNOS expression by FACS-purified β cells, β cells are the primary islet cellular source of iNOS in response to TNF plus LPS. As shown in Fig. 6, TNF plus LPS stimulates iNOS expression (red fluorescence) primarily in insulin-containing cells (green fluorescence) as evidenced by the yellow fluorescence on the double exposure. Consistent with the requirement for IL-1, IRAP significantly reduces the number of islet cells that express iNOS in response to TNF plus LPS, and the few remaining iNOS-positive cells do not contain insulin (Corbett and McDaniel, 1995).

Immunological and biochemical evidence was used to identify macrophages as the islet cellular source of IL-1 in response to TNF plus LPS. As shown in Fig. 5, depletion of tissue macrophages by culturing islets for 7 days at 24°C (Lacy and Finke, 1991) prevents TNF plus LPS-induced iNOS expression, nitrite production, and inhibition of insulin secretion (Arnush *et al.,* 1998b). Also, macrophage depletion prevents TNF plus LPS-induced IL-1α and IL-1β mRNA accumulation in rat islets (Arnush *et al.,* 1998b). The isoform of IL-1 that is released in islets and that mediates rat islet damage in response to TNF plus LPS appears to be

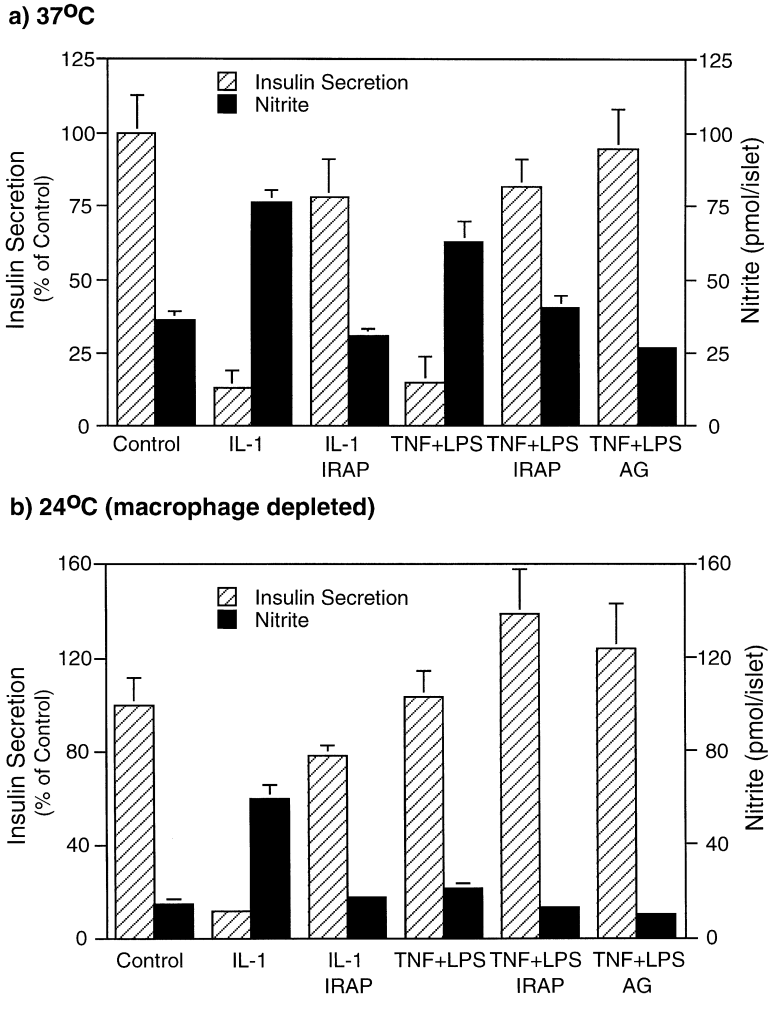


Figure 5 Activated resident macrophages mediate β-cell damage by the local release of IL-1. Treatment of freshly isolated rat islets (a) with TNF plus LPS results in the inhibition of glucose-stimulated insulin secretion and nitric oxide formation, effects that are prevented by IRAP. Depletion of resident macrophages (b) prevents the inhibitory actions of TNF plus LPS on glucose-stimulated insulin secretion by rat islets and prevents the stimulatory effects on nitrite production. These findings show that activation of macrophages results in an inhibition of β-cell function that is mediated by the local release of IL-1 in islets. Reproduced with permission from *J. Immunol.* **160,** 2684–2691 (Arnush *et al.,* 1998b), copyright 1998 by The American Association of Immunologists.

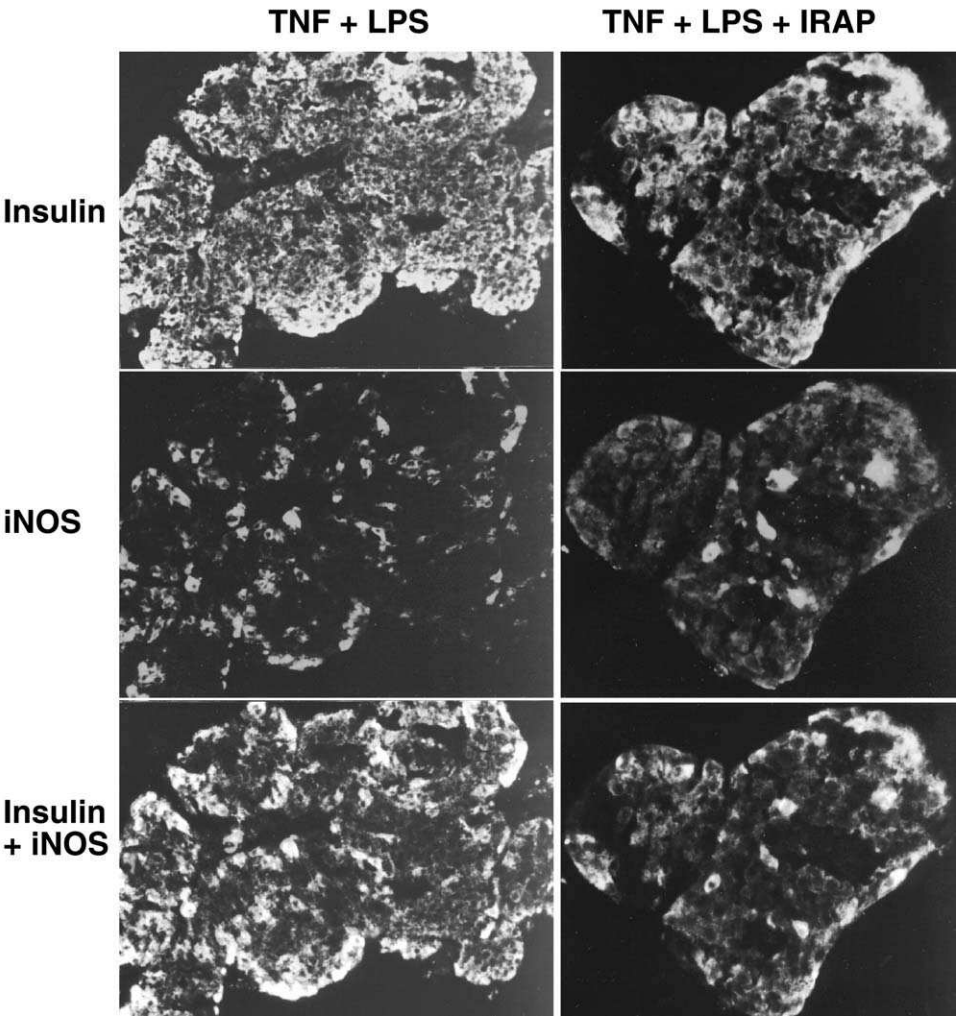


Figure 6 Immunohistochemical analysis of iNOS expression in rat islets treated with TNF plus LPS. Rat islets were treated for 40 hours with TNF plus LPS or TNF plus LPS plus IRAP, fixed, and used in preparation of thin sections. Insulin (green) and iNOS (red) expression was evaluated by immunofluorescence. TNF plus LPS stimulates iNOS expression by ~50% of islet cells, and this expression localizes with insulin-containing cells (yellow on double exposure). IRAP prevents β-cell expression of iNOS, but it does not inhibit iNOS expression by a subpopulation of islet macrophages. Reproduced from the *J. Exp. Med.* **181,** 559–568 (Corbett and McDaniel, 1995) by copyright permission of The Rockefeller University Press. See color insert.

IL-1β, as antibody neutralization of IL-1β prevents TNF plus LPS-induced nitrite production by rat islets (Arnush *et al.,* 1998b). Confirmation that macrophages are the islet cellular source of IL-1β in response to TNF plus LPS was obtained by immunohistochemical localization of IL-1β with the macrophage surface marker ED1 (Arnush *et al.,* 1998b).

If tissue macrophages participate in the development of insulin-dependent diabetes mellitus, then activation of this cell population in human islets should impair β-cell function. The combination of TNF plus LPS plus IFN-γ stimulates iNOS expression, stimulates nitrite formation, and inhibits glucose-stimulated insulin secretion by human islets (Arnush *et al.,* 1998a). IRAP attenuates the inhibitory actions of TNF plus LPS plus IFN-γ on glucose-stimulated insulin secretion, and it prevents the stimulatory actions of this combination of cytokines and endotoxin on iNOS expression and nitrite production (Arnush *et al.,* 1998a). These findings are consistent with studies in rat islets and show that IL-1 released locally in human islets impairs human islet function. AG also attenuates the inhibitory actions of TNF plus LPS plus IFN-γ on insulin secretion and completely prevents nitric oxide production, indicating that nitric oxide mediates, in part, the impairment in β-cell function under these conditions (Arnush *et al.,* 1998a). Macrophages appear to be the cellular source of IL-1 in human islets treated with TNF plus LPS plus IFN-γ. Depletion of MHC class II-positive cells by culturing for 7 days at 24°C prevents TNF plus LPS plus IFN-γ-induced iNOS and IL-1 expression and nitrite formation by human islets. Also, immunohistochemical localization of IL-1β with the human macrophage marker CD68 in human islet cells treated with TNF plus LPS plus IFN-γ provides direct evidence that macrophages are the

human islet cellular source of IL-1β (Arnush *et al.,* 1998a). In contrast to studies performed in rat islets, tissue macrophage activation in human islets also requires IFN-γ in addition to TNF plus LPS. Presently, it is not clear if IFN-γ is required to activate tissue macrophages to release IL-1 or to stimulate iNOS expression by β cells (human β cells require both IL-1 and IFN-γ for iNOS expression), or if IFN-γ participates in both events.

The studies just outlined provide biochemical and immunological evidence to support a novel mechanism by which resident islet macrophages mediate β-cell damage (Fig. 7) (McDaniel *et al.,* 1996). Activation of resident islet macrophages results in the expression and release of IL-1 in the microenvironment of islets. IL-1 attains a concentration in this microenvironment that is sufficient to stimulate iNOS expression by β cells (IFN-γ is also required for iNOS expression by human β cells). Under these conditions, β-cell production of nitric oxide mediates the inhibitory actions of activated macrophages on β-cell function. Importantly, these findings address two important and controversial issues. First, these findings provide biochemical evidence to support the hypothesis that tissue macrophages may play a critical role in precipitating events that trigger the initial destruction of β cells during the development of autoimmune diabetes (Lacy, 1994). Second, these findings address a controversy concerning human macrophage production of nitric oxide. It is clear that human macrophages express iNOS (Albina and Reichner, 1998; MacMicking *et al.,* 1997); however, the level of nitric oxide produced by human macrophages is much lower than the level produced by rodent macrophages (Albina, 1995). The importance of nitric oxide in disease progression has been questioned because of this reduced capacity of human macrophages to produce nitric oxide (Albina, 1995). The studies outlined earlier suggest that nitric oxide may participate in the development of human disease; however, the source of nitric oxide may not be macrophages, but target cells, activated by cytokines released locally by tissue macrophages. In type I diabetes mellitus, the findings described earlier suggest that human macrophages may regulate target β-cell expression of iNOS and production of nitric oxide by releasing IL-1 locally in islets, and that β-cell production of nitric oxide results in an impairment in β-cell function.

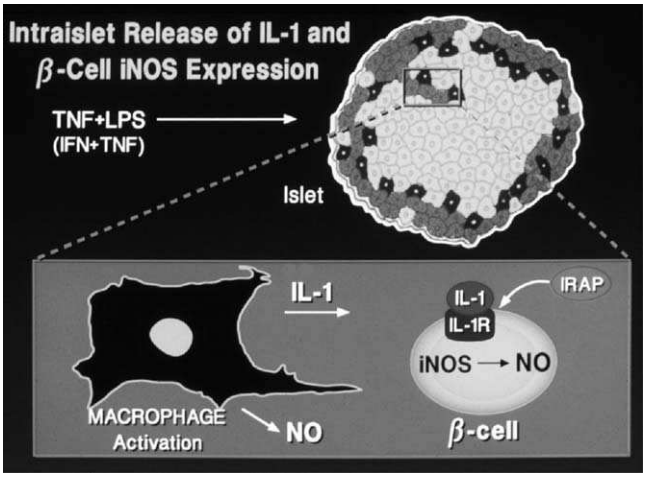


Figure 7 Mechanism by which resident macrophages mediate β-cell damage. Activation of islet macrophages results in the expression of iNOS and IL-1. IL-1 produced by macrophages accumulates in islets to levels sufficient to stimulate iNOS expression by β cells. Production of nitric oxide by β cells mediates the inhibitory actions of activated macrophages on β-cell function. Production of nitric oxide by resident macrophages (following treatment of rat islets with TNF plus LPS plus IRAP) does not mediate β-cell damage. Reproduced with permission from *Proc. Soc. Exp. Biol. Med.* (McDaniel *et al.,* 1996) by the Society for Experimental Biology and Medicine. See color insert.

Transcriptional Regulation of iNOS Expression by β Cells

A number of transcriptional regulators have been implicated in the regulation of iNOS expression, and this regulation appears to be both species and cell type specific (Förstermann and Kleinert, 1995; Taylor *et al.,* 1998; Wang and Marsden, 1995). Of these regulators, nuclear factor κB (NF-κB) appears to be essential for cytokine-stimulated iNOS expression by β cells (Eizirik *et al.,* 1996a). NF-κB is a ubiquitously expressed protein composed primarily of p50 and p65 subunits that is found sequestered in the cytosol of unstimulated cells by its inhibitor protein, IκB (Baeuerle and Henkel, 1994; Ghosh, 1999). On stimulation, IκB is phosphorylated on specific serine residues, ubiquinated, and targeted for degradation by the 26S proteasome (Baeuerle and Henkel, 1994; Ghosh, 1999). Free of IκB, NF-κB translocates to the nucleus to activate gene transcription. The rat, mouse, and human iNOS promoters contain consensus sequences for two NF-κB binding sites, one proximal and one distal to the TATA start site, and both appear to be required for maximal iNOS promoter activity in mouse macrophages (Beck and Sterzel, 1996; Chu *et al.,* 1995; Eberhardt *et al.,* 1996; Xie *et al.,* 1993). In rat insulinoma cells and rat islets, IL-1 stimulates the degradation of IκB and stimulates nuclear localization and activation of NF-κB (Darville and Eizirik, 1998; Flodstrom *et al.,* 1996; Kwon *et al.,* 1995; Saldeen and Welsh, 1994; Scarim *et al.,* 1998). Inhibitors of NF-κB, diethyl dithiocarbamate (DETC) and pyrrolidinedithiocarbamate (PDTC), prevent IL-1-induced iNOS expression by RINm5F cells and rat islets (Flodstrom *et al.,* 1996; Kwon *et al.,* 1995; Saldeen and Welsh, 1994). These findings suggest that NF-κB is required for IL-1-induced iNOS expression by β cells. Using a reporter construct containing 1.5 kb of the rat iNOS promoter, mutations in the two NF-κB response elements prevent IL-1-induced reporter activity in transfected RINm5F cells and primary β cells (Darville and Eizirik, 1998). These findings confirm that NF-κB plays a primary role in the regulation of iNOS expression by rat β cells in response to IL-1.

In addition to NF-κB, the rat, mouse, and human iNOS promoter contains IFN response elements, IFN-γ response elements (γ-IRE), IFN-stimulated response elements (ISRE), and γ-activated sequence sites (GAS) (Beck and Sterzel, 1996; Chu *et al.,* 1995; Eberhardt *et al.,* 1996; Xie *et al.,*

1993). IFN-γ is required, in addition to IL-1, for iNOS expression by NOD mouse and human islets (Corbett *et al.,* 1993a; Eizirik *et al.,* 1994a; Heitmeier *et al.,* 1999b; Thomas *et al.,* 1999). IFN-γ also primes for IL-1-induced iNOS expression by NOD mouse and rat islets, and it potentiates IL-1-induced iNOS expression by RIN cells and rat and human islets (Heitmeier *et al.,* 1997, 1999b). These findings suggest that IFN-γ may also play an essential role in cytokine-stimulated iNOS expression by β cells. IFN-γ activates a family of transcription factors known as the signal transducers and activators of transcription (STATs). In response to IFN-γ, STAT1 is recruited to the IFN-γ receptor, where it is phosphorylated on specific tyrosine residues by Janus kinases, JAK1 and JAK2. Thus activated, STAT1 homodimers translocate to the nucleus and activate new gene transcription by binding to consensus GAS sites (Darnell, 1998; Schindler, 1999). IFN-γ stimulates STAT1 phosphorylation and nuclear translocation in rat islets and primary rat β cells (Heitmeier *et al.,* 1999b). Also, the priming actions of IFN-γ on IL-1-induced iNOS expression by β cells are associated with prolonged nuclear localization and DNA binding activity of STAT1 (Heitmeier *et al.,* 1999b). Mutational analysis of the GAS response elements in the rat iNOS promoter significantly reduces IL-1 plus IFN-γ-stimulated iNOS promoter activity in RINm5F cells and primary β cells (Darville and Eizirik, 1998), supporting a primary role for STAT1 in mediating the priming and potentiating actions of IFN-γ on IL-1-induced iNOS expression by rat islets.

A second IFN-γ activated transcription factor, IRF-1, has been proposed to participate in iNOS expression by β cells. IRF-1 binds to ISRE sites to activate new gene transcription (Harada *et al.,* 1998; Mamane *et al.,* 1999), and it appears to be required for IFN-γ-induced iNOS expression by murine macrophages. Macrophages isolated from IRF-1-deficient mice fail to express iNOS (Kamijo *et al.,* 1994; Martin *et al.,* 1994). It has been shown that IFN-γ induces IRF-1 expression and activation in rat islets and RINm5F cells, and IRF-1 expression precedes IL-1 plus IFN-γ-induced iNOS mRNA accumulation (Akabane *et al.,* 1995; Flodstrom and Eizirik, 1997). Mutational analysis of the distal ISRE binding elements in the iNOS promoter reduces IL-1 plus IFN-γ-stimulated promoter activity by ~30% in RINm5F cells, without negatively affecting IL-1-stimulated promoter activity (Darville and Eizirik, 1998) or IL-1 plus IFN-γ-stimulated nitrite production. These findings suggest that IRF-1 may participate in IL-1 plus IFN-γ-induced iNOS expression by rat β cells. However, β cells isolated from IRF-1-deficient mice express iNOS and produce nitric oxide in response to IL-1 plus IFN-γ at levels similar to those of wild-type mice, suggesting that IRF-1 is not required for iNOS expression by β cells under these conditions (Pavlovic *et al.,* 1999b).

Cellular Signaling Mechanisms Associated with iNOS Expression by Islets

The cellular mechanisms by which cytokines stimulate iNOS expression by β cells are poorly understood. In a number of cell types, IL-1 has been shown to activate the mitogen activated protein (MAP) kinase signaling pathway, specifically p38, c-Jun NH_2-terminal kinase (JNK), and extracellular signal-related kinases (ERK1/2) (Bleich *et al.,* 1997; Larsen *et al.,* 1998; N. Welsh, 1996). In RINm5F and HIT cells, and in rat islets, IL-1 stimulates the phosphorylation of JNK and the phosphorylation and activation of p38 and ERK1/2 (Bleich *et al.,* 1997; Larsen *et al.,* 1998; N. Welsh, 1996). Pharmacological inhibitors of p38 (SB 203580) and ERK1/2 (MEK inhibitor PD 098059) attenuate and, in combination, prevent IL-1-induced iNOS expression by RINm5F cells and IL-1-induced nitrite formation by rat islets (Larsen *et al.,* 1998). In addition, inhibition of p38 also attenuates the inhibitory effects of IL-1 on media insulin accumulation from rat islets following a 24-hour culture (Larsen *et al.,* 1998). Although these studies support a role for MAP kinase activation by IL-1 in islets, additional studies will be required to determine if this signaling cascade is required for IL-1-induced iNOS expression and the damaging actions of IL-1 on β-cell function.

Does Nitric Oxide Impair β-Cell Function by Autocrine or Paracrine Mechanisms?

Nitric oxide has been shown to modulate cellular physiology by functioning as a paracrine effector molecule. The regulation of vascular tone, neuronal transmission, and macrophage-mediated immune defense relies on the ability of nitric oxide to diffuse from cells producing this free radical to target cells to mediate its action (Kerwin *et al.,* 1995; Lancaster, 1997). The question of whether nitric oxide impairs β-cell function by autocrine or paracrine mechanisms has generated interest because of the findings that activated macrophages (and endothelial cells) can kill islet cells when incubated at an effector to target ratio of 2:1 (Kroncke *et al.,* 1991a). However, in intact islets, β-cell damage only occurs under conditions in which β cells produce nitric oxide (Arnush *et al.,* 1998b; Corbett and McDaniel, 1995). For example, rat β cells are the sole islet cellular source of nitric oxide in response to IL-1, and under these conditions β-cell function is impaired. In contrast, TNF plus LPS stimulates resident islet macrophage iNOS expression and nitric oxide production; however, in the presence of IRAP (or under conditions in which the actions of locally released IL-1 are inhibited) macrophage nitric oxide production does not impair β-cell function (Arnush *et al.,* 1998b; Corbett and McDaniel, 1995). These findings can be interpreted in a number of ways. First, these findings suggest that nitric oxide may impair β-cell function in an autocrine manner, such that β cells are only sensitive to nitric oxide when they are the source of the free radical. Second, nitric oxide impairs β-cell function by paracrine mechanisms regardless of whether the source of nitric oxide is the β cell or macrophage, and the nitric oxide-mediated inhibition of β-cell function is primarily related to the levels of nitric oxide produced in islets. Third, it is possible that both mechanisms contribute to β-cell damage mediated by nitric oxide.

Islets are composed such that the majority of β cells are found in the central core, with 8–10 β cells forming a rosette-type structure surrounding an islet capillary (Bonner-Weir, 1988; Bonner-Weir and Orci, 1982). The mantle of the islet comprises primarily α cells (Bonner-Weir, 1988; Bonner-Weir and Orci, 1982). In this structure, β cells are found in close proximity, such that nitric oxide produced by one β cell may directly influence the function of adjacent β cells. The hypothesis that nitric oxide produced by β cells functions by paracrine interactions within islets and that the level of islet damage is a function of the amount of nitric oxide produced is supported by the following observations: (1) β-cell production of nitric oxide in intact islets results in a complete inhibition of insulin secretion following a 24-hour incubation (Corbett *et al.,* 1991; N. Welsh *et al.,* 1991), whereas nitric oxide produced by primary β cells purified by FACS results in only ~65% inhibition of insulin secretion (Corbett *et al.,* 1992b); and (2) in response to 1 unit/ml IL-1, FACS-purified β cells produce ~35% more nitric oxide than β cells found in intact islets [assuming that islets contain ~70% β cells, that on average islets produce ~35 pmol of nitrite per islet, and that FACS-purified β cells produce ~80 pmol of nitrite per islet equivalence, (Corbett and McDaniel, 1995)]. Although FACS-purified β cells produce more nitric oxide than intact islets, nitric oxide is less effective at inhibiting insulin secretion from purified β cells. In intact islets, β cells are in close contact with each other in the central core of the islet, whereas FACS-purified β cells are free floating in culture. If nitric oxide functions as a paracrine mediator, it would be predicted that the damaging actions of this free radical on β-cell function would be greater in intact islets than purified β cells (Fig. 8), because the diffusion distance that nitric oxide would travel between purified β cells in intact islets is much less than the diffusion distance between β cells free floating in culture. Conversely, if nitric oxide functions as an autocrine mediator of β-cell damage, then IL-1 should inhibit β-cell function by similar levels in both intact islets and purified β cells, because nitric oxide-mediated damage would not be dependent on diffusion of this free radical from cell to cell. These findings support a paracrine action of nitric oxide in mediating cytokine-induced inhibition of β-cell function, although these studies do not rule out the possibility that nitric oxide also functions as an autocrine inhibitor of β-cell function.

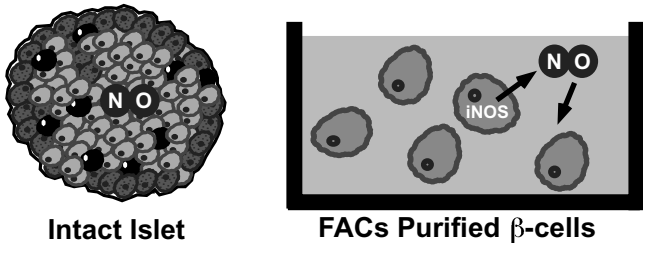


Figure 8 Does nitric oxide inhibit insulin secretion by autocrine or paracrine mechanisms? The β cells in intact islets are in close proximity, such that the diffusion distance nitric oxide would have to travel to impair the function of adjacent β cells is minimal. However, the diffusion distance that nitric oxide would have to travel to impair the function of an adjacent β cell is much larger in culture because the cells are free floating.

Mechanisms of Islet-Cell Death in Response to Nitric Oxide

Using isolated rodent islets, cytokines such as IL-1 and IL-1 in combination with TNF and IFN-γ have been shown to stimulate islet degeneration and islet-cell death (McDaniel *et al.,* 1996; Rabinovitch and Suarez-Pinzon, 1998; N. Welsh *et al.,* 1994). Islet degeneration is characterized by cell sloughing, loss of islet integrity, and morphological disaggregation (Corbett and McDaniel, 1994; Lacy and Finke, 1991). We have shown that IL-1 and IL-1 plus IFN-γ-stimulated islet degeneration is prevented by L-NMMA and AG, providing evidence that nitric oxide mediates islet morphological damage (Corbett and McDaniel, 1994; Heitmeier *et al.,* 1997; Lacy and Finke, 1991). Cytokine-stimulated islet-cell lysis also appears to be mediated, at least in part, by nitric oxide. Using electron microscopic analysis to evaluate islet-cell death, Kolb and co-workers have shown that islet-cell death mediated by IL-1 and activated macrophages (incubated with islet cells at an effector to target ratio of 2:1) is prevented by the inhibition of iNOS (Kroncke *et al.,* 1991a, b). Nitric oxide donor compounds also stimulate islet-cell death (Cunningham and Green, 1994; Delaney and Eizirik, 1996; Delaney *et al.,* 1996; Hadjivassiliou *et al.,* 1998). Although nitric oxide (supplied exogenously using nitric oxide donor compounds) kills islet cells, cell death is not selective, as both α and β cells are destroyed (Kroncke *et al.,* 1991a). Functionally, islet-cell death is characterized by an inhibition of islet oxidative metabolism and DNA damage. We and others have shown that the exogenous addition of nitric oxide as well as nitric oxide produced following cytokine stimulation of islets results in the inhibition of glucose oxidation to CO_2, and aconitase activity (Mandrup-Poulsen, 1996; McDaniel *et al.,* 1996; Rabinovitch and Suarez-Pinzon, 1998; Sandler *et al.,* 1991). Nitric oxide also has been shown to induce islet-cell DNA strand breaks as assessed by TUNEL staining, agarose gel electrophoresis, and the comet assay (Delaney and Eizirik, 1996; Delaney *et al.,* 1997; Dunger *et al.,* 1996; Hadjivassiliou *et al.,* 1998; Rabinovitch *et al.,* 1994a). These studies clearly show that cytokine-mediated islet-cell death and morphological damage are mediated by nitric oxide (Mauricio and Mandrup-Poulsen, 1998).

Central to the issue of islet-cell death in response to cytokines and nitric oxide is whether islet cells die by apoptotic or necrotic mechanisms. Apoptosis, defined as programmed cell death, is characterized by both morphological and biochemical changes, which include cell shrinking, membrane blebbing, nuclear condensation, and apoptotic body formation (Hart *et al.,* 1996; McConkey, 1998; Samali *et al.,* 1996). The net outcome of this energy-dependent mechanism of cell death is the packaging of cellular contents into

membrane-bound vesicles that are phagocytized in the absence of an inflammatory response (Nicotera *et al.,* 1999). In contrast, the loss of cell membrane continuity, membrane rupture, and leakage of cellular contents characterize necrosis (McConkey, 1998). In many cases necrosis results in acute inflammation (McConkey, 1998). The inflammatory cytokine IL-1 has been shown to induce DNA fragmentation in RINm5F cells in a NO-dependent manner, and IL-1, TNF, nitric oxide donor compounds, peroxynitrite, and oxygen radicals have been shown to stimulate DNA fragmentation in isolated rodent islets (Delaney *et al.,* 1993, 1997; Dunger *et al.,* 1996; Eizirik *et al.,* 1996b; Fehsel *et al.,* 1993; Rabinovitch *et al.,* 1994a). Apoptosis is an energy-requiring event, and nitric oxide (either from donor compounds or cytokine-induced NO production) is a potent inhibitor of mitochondrial oxidation, resulting in a reduction in cellular levels of ATP. Therefore, although nitric oxide-mediated islet-cell death displays a number of apoptotic features, mainly DNA damage, in the absence of functional mitochondria and energy-rich ATP, it is difficult to conclude that cytokine-induced islet-cell death is strictly by apoptosis. Apoptotic cell death is also associated with the activation of a family of aspartic proteases, termed caspases, that execute virtually all forms of apoptosis (Rathmell and Thompson, 1999). Although islets have been shown to express members of the caspase family of proteases (Ingelsson *et al.,* 1998; Rathmell and Thompson, 1999; Stephens *et al.,* 1997), caspase activation in islet cells in response to either nitric oxide or cytokines has yet to be established. In fact, studies have shown that nitric oxide inhibits caspase activation (Dimmeler *et al.,* 1997; Kim *et al.,* 1997; Li and Billiar, 1999). This evidence suggests that nitric oxide, either produced following cytokine stimulation or supplied exogenously to islet cells, does not stimulate islet-cell death by apoptosis, but most likely by necrosis.

The mechanism by which cytokines mediate human islet damage is more complex, as a number of studies have shown that cytokine-induced DNA damage occurs by mechanisms that are independent of nitric oxide production (Delaney *et al.,* 1997; Rabinovitch *et al.,* 1994b). It has been shown that N^G-nitro-L-arginine and L-NMMA prevent cytokine-induced nitric oxide production by human islets but fail to prevent IL-1 plus TNF plus IFN-γ-induced DNA strand breaks following prolonged exposure of 6 and 9 days (Delaney *et al.,* 1997). In addition, this study also used propidium iodide and Hoeschst 33342 staining to provide additional evidence for cytokine-mediated islet-cell apoptosis by mechanisms that are independent of human islet production of nitric oxide (Delaney *et al.,* 1997). Also, prolonged exposure of human islets for 3–4 days with IL-1 plus TNF plus IFN-γ stimulates human islet DNA damage independent of nitric oxide production (Rabinovitch *et al.,* 1994b). These studies suggest that prolonged exposure of human islets to cytokines results in DNA damage that appears to be nitric oxide independent. Cytokines have also been shown to stimulate increases in intracellular levels of Ca^{2+} in islet cells (Borg and Eizirik, 1990; McDaniel *et al.,* 1988), and increased intracellular levels of Ca^{2+} in β cells have been shown to stimulate β-cell apoptosis (Bai *et al.,* 1999; Zhou *et al.,* 1998). Also, serum deprivation induces insulinoma cell apoptosis (Mizuno *et al.,* 1998). Importantly, as islets utilize growth factors and nutrients in the culture medium during prolonged cytokine exposure (4–9 days), this could result in apoptosis similar to the effects of serum deprivation in insulinoma cells. Thus, in human islets, cytokine-induced nitric oxide-independent apoptosis may be associated with either increased intracellular levels of Ca^{2+}, loss of growth factors due to extended culture required for cytokine-induced human islet-cell apoptosis, or a combination of both pathways.

The mechanism by which DNA strand breaks result in islet-cell death appears to be associated with the activation of poly(ADP-ribose) polymerase (PARP). Okamoto and co-workers first proposed in 1981 that PARP may be responsible for β-cell death induced by DNA damage (Yamamoto *et al.,* 1981). PARP catalyzes the NAD^+-dependent addition of ADP-ribose to nuclear proteins following activation. In the Okamoto model, DNA strand breaks stimulate PARP activation, and the subsequent depletion of NAD^+ results in the depletion of ATP in an effort to replenish NAD^+ levels. Kolb and co-workers have shown in isolated islets that DNA damage induced by nitric oxide (both cytokine-induced endogenous production or NO supplied exogenously by donor compounds), oxygen radicals, and the β-cell toxin streptozotocin stimulates islet PARP activity (Heller *et al.,* 1995; Radons *et al.,* 1994). Inhibition of PARP using nicotinamide or 3-aminobenzamide prevents islet-cell lysis stimulated by nitric oxide. Importantly, three independent studies have shown that PARP-deficient mice are resistant to the development of streptozotocin-induced diabetes (Burkart *et al.,* 1999; Masutani *et al.,* 1999; Pieper *et al.,* 1999). Streptozotocin is a selective β-cell toxin that induces DNA damage possibly by releasing nitric oxide during decomposition (Kwon *et al.,* 1994; Turk *et al.,* 1993). Importantly, the studies just outlined provide direct evidence to support the Okamoto model of β-cell damage during the development of autoimmune diabetes, and they provide mechanistic information on how nitric oxide-induced DNA damage may mediate cell lysis.

Recovery from Nitric Oxide-Mediated Damage

Although the studies outlined earlier show that cytokines stimulate β-cell damage in a nitric oxide-dependent manner, islets also possess the ability to recover from nitric oxide-mediated damage. The inhibitory actions of a 15-hour incubation of rat islets with IL-1 were shown to require a 4-day incubation in the absence of this cytokine to recover normal glucose-stimulated secretory function (Comens *et al.,* 1987; Helqvist *et al.,* 1991). Importantly, the time required for islets to completely recover from the inhibitory actions of IL-1 can be reduced from 4 days to 8 hours by simply inhibiting iNOS activity. In these experiments, the addition of L-NMMA or AG to islets treated for 18 hours with IL-1 and

continued culture in the presence of both IL-1 and L-NMMA or AG result in a time-dependent recovery of insulin secretion that is complete in 8 hours (Corbett and McDaniel, 1994). The recovery of insulin secretion correlates with a similar time-dependent recovery of aconitase activity that is also complete in 8 hours (Corbett and McDaniel, 1994). The ability to recover from cytokine-mediated damage is not restricted to rat islets, as L-NMMA stimulates the recovery of human islet aconitase activity inhibited by an 18-hour incubation with IL-1 plus IFN-γ (Scarim *et al.,* 1997). These findings provide evidence that by simply inhibiting iNOS activity, islets can recover from the damaging actions of IL-1 (and IL-1 plus IFN-γ for human islets) on insulin secretion and mitochondrial oxidative metabolism.

Although islets have the ability to recover from cytokine-mediated damage, prolonged exposure of islets to this cytokine results in irreversible damage. Islets treated for periods shorter than 36 hours maintain the ability to recover from IL-1-induced damage; however, exposure of islets to IL-1 for periods longer than 36 hours results in irreversible inhibition of insulin secretion and aconitase activity (Scarim *et al.,* 1997). Consistent with the irreversible inhibition of aconitase activity and insulin secretion, islets incubated for 36 hours or longer are committed to degeneration. These studies show that islets have the ability to recover from cytokine-mediated damage; however, if β cells produce nitric oxide for periods greater than 36 hours, islets are committed to destruction.

The ability of islets to recover from cytokine-mediated damage appears to be an active process that requires new mRNA transcription. The transcriptional inhibitor actinomycin D prevents L-NMMA-induced recovery from IL-1-mediated inhibition of insulin secretion and aconitase activity by rat islets (Corbett and McDaniel, 1994; Scarim *et al.,* 1998). The stimulus for the recovery process appears to be nitric oxide. Incubation of rat islets with the nitric oxide donor compound sodium (*Z*)-1-(*N,N*-diethylamino)diazen-1-ium 1,2-diolate (DEA-NO) for 1 hour stimulates ~65% inhibition of islet mitochondrial aconitase activity (Scarim *et al.,* 1998). Removal of DEA-NO by washing, followed by a 5-hour incubation in the absence of DEA-NO, results in a complete recovery of aconitase activity. Actinomycin D, when added during the 5-hour recovery period, completely prevents islet recovery from DEA-NO-stimulated inhibition of aconitase activity (Scarim *et al.,* 1998). These studies suggest that nitric oxide stimulates the expression of factors that participate in β-cell recovery from cytokine-induced damage.

One pathway that appears to participate in islet recovery from cytokine-mediated damage is the heat-shock response. Treatment of rat islets with IL-1 stimulates heat-shock protein (hsp) 70 expression in a nitric oxide-dependent manner (Anderson *et al.,* 1995; Hao *et al.,* 1999; Scarim *et al.,* 1998; Strandell *et al.,* 1995; N. Welsh *et al.,* 1995). Importantly, islet overexpression of hsp 70 either by liposomal delivery, by heat shock, or by transient transfection of hsp 70 prevents IL-1-induced islet and RINm5F cell damage (Bellmann *et al.,* 1995, 1996; Margulis *et al.,* 1991; Rothe and Kolb, 1999). It is unclear how hsp 70 expression stimulates recovery of islets from cytokine- and nitric oxide-mediated damage; however, induction of the heat-shock response in islets attenuates cytokine signaling. IL-1- and IL-1 plus IFN-γ-induced iNOS expression by rat and human islets, respectively, requires the activation of NF-κB (Kwon *et al.,* 1995; Saldeen and Welsh, 1994). Following heat shock, IL-1 and IL-1 plus IFN-γ fail to stimulate NF-κB nuclear localization and IκB degradation (Scarim *et al.,* 1998). In addition, IL-1-induced phosphorylation of JNK is attenuated in RINm5F cells and rat islets that express hsp 70 (Maggi *et al.,* 2000). These findings indicate that nitric oxide stimulates the expression of the heat-shock response genes in islets, and that expression of heat-shock response factors in islets attenuates IL-1 signaling.

IL-1 also appears to be capable of stimulating the expression proteins that may protect islets from the damaging actions of IL-1. A20 is a zinc finger protein that has been shown to prevent TNF-induced apoptosis and proinflammatory responses in endothelial cells (Cooper *et al.,* 1996; Ferran *et al.,* 1998; Fries *et al.,* 1996; Jaattela *et al.,* 1996). More recently, IL-1 has been shown to stimulate the rapid expression of A20 in rat and human islets, and overexpression of A20 in rat islets prevents IL-1 plus IFN-γ-induced iNOS expression and nitric oxide-mediated islet damage (Grey *et al.,* 1999). Similar to heat shock, the protective actions of A20 appear to be associated with the inhibition of IL-1-induced NF-κB activation; however, the mechanisms by which A20 prevent cytokine signaling in islets are unknown.

Manganese superoxide dismutase (MnSOD) also appears to participate in islet defense and recovery from cytokine-mediated damage. Cytokines such as IL-1, TNF, and IFN-γ, alone and in combination, stimulate the expression of MnSOD by rat insulinoma cell lines and rat and human islets (Borg *et al.,* 1992; Strandell *et al.,* 1995; N. Welsh *et al.,* 1995). Islet MnSOD expression appears to be directly regulated by cytokines, as the NF-κB inhibitor PDTC prevents IL-1-induced iNOS expression, but fails to reduce IL-1-stimulated expression of MnSOD by RINm5F cells (Bigdeli *et al.,* 1994). Similar to the effects of heat shock and A20 on cytokine-mediated β-cell damage in islets, insulinoma cell lines overexpressing MnSOD are resistant to IL-1-mediated cell death (Hohmeier *et al.,* 1998). The protective actions of MnSOD are also associated with a reduction in IL-1-stimulated iNOS mRNA accumulation and nitrite production. Although the protective effects of heat shock and A20 are associated with an inhibition of IL-1 signaling in islets, it is not clear how overexpression of MnSOD prevents cytokine-induced iNOS expression or insulinoma cell damage.

Role of Nitric Oxide in the Development of Diabetes in Animal Models

Two animal models of IDDM, the BB rat and NOD mouse, spontaneously develop autoimmune diabetes with

characteristics similar to IDDM in humans (Buschard, 1996; Cheta, 1998). Male and female diabetes-prone (dp) BB rats develop insulitis at 50–70 days of age, which is followed by destruction of insulin-secreting β cells and the development of clinical diabetes at 120 days (Buschard, 1996). Similar to human IDDM, the absence of insulin treatment results in ketoacidosis and death (Buschard, 1996). Through inbreeding, a diabetes-resistant (dr) strain of BB rats has been developed in which fewer than 1% develop autoimmune diabetes. Similarly, NOD mice spontaneously develop diabetes, but diabetes in this model is gender specific. Approximately 80% of the females develop insulitis and diabetes, whereas only ~10–20% of the males develop disease (Kikutani and Makino, 1992). In these mice, peri-insulitis is first observed at 4–6 weeks of age, and the mice progress to diabetes at 12–15 weeks of age. In contrast to the BB rat model, NOD mice are able to survive in the absence of insulin treatment for several months. In both the dpBB rat and NOD mouse models of autoimmune diabetes, insulitis is first characterized by infiltration of monocytes or macrophages, followed by the appearance of T and B lymphocytes and natural killer (NK) cells (Burkart and Kolb, 1996; Crisa *et al.,* 1992). Destruction of β cells during the development of diabetes in BB rats and NOD mice appears to require both T lymphocytes and macrophages, as selective depletion of these cell types prevents disease progression (Bach, 1994; Lacy, 1994; Wicker *et al.,* 1986). The mechanism(s) by which T cells and macrophages mediate β-cell damage have remained elusive; however, macrophage and T-cell-derived soluble mediators, such as cytokines and nitric oxide, appear to play an important role.

In the BB rat, the expression of IL-1β, IFN-γ, TNF, IL-12, IL-2, and iNOS in islets is observed under conditions in which insulitis is associated with β-cell destruction (Chung *et al.,* 1997; Jiang and Woda, 1991; Kolb *et al.,* 1996; Rabinovitch *et al.,* 1996a; Zipris, 1996). Systemic administration of IL-1β, TNF, IFN-γ, and IL-2 has been shown to delay or decrease the incidence of diabetes in BB rats (Nicoletti *et al.,* 1998; Satoh *et al.,* 1990; Sobel *et al.,* 1998; Takahashi *et al.,* 1993; Wilson *et al.,* 1990; Zielasek *et al.,* 1990). *In vitro,* treatment of isolated dpBB rat islets with IL-1 for 40 hours results in an eightfold increase in nitrite accumulation in the medium, which is inhibited by coincubation with L-NAME (Dunger *et al.,* 1996). L-NAME also prevents the inhibitory effects of IL-1 on glucose-stimulated insulin secretion by isolated dpBB rat islets, suggesting that the inhibitory effects are mediated by nitric oxide (Dunger *et al.,* 1996). Increased nitric oxide formation is also observed in macrophages isolated from dpBB rats (Lau *et al.,* 1998). These findings implicate increased cytokine expression and cytokine-induced iNOS expression and nitric oxide production in the development of diabetes in BB rats.

In vivo, increased iNOS expression correlates with the natural progression of diabetes in BB rats. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of BB rat pancreata indicate that iNOS mRNA expression is increased in prediabetic and diabetic BB rats (Kolb *et al.,* 1996; Kleemann *et al.,* 1993). Immunohistochemical colocalization of iNOS with ED1-positive macrophages suggests that the majority of iNOS-expressing cells are infiltrating macrophages, with a smaller subset of iNOS-expressing β cells located within a proximal area of iNOS-positive macrophages (Kleemann *et al.,* 1993). Because activated macrophages express iNOS, produce nitric oxide, and release high levels of IL-1, it is possible that activated macrophages in close proximity to β cells during islet insulitis in the BB rat may stimulate β-cell expression of iNOS by releasing IL-1 in this microenvironment. iNOS inhibitors, L-NMMA and AG, delay the onset of diabetes in BB rats by 13–15 days without altering the incidence of diabetes (Wu, 1995); however, oral administration of L-NAME reduces the incidence of diabetes in these rats (Lindsay *et al.,* 1995). In addition, prevention of spontaneous diabetes by subcutaneous insulin injection correlates with a decrease in iNOS expression (Kolb *et al.,* 1997) and nitric oxide formation (Stevens *et al.,* 1997). These findings suggest that increased iNOS expression and nitric oxide formation correlate with the development of diabetes in dpBB rats.

In NOD mice, the expression of cytokines, IL-1, TNF, IFN-γ, IL-12, and IL-18 are associated with increased iNOS expression, destructive β-cell insulitis, and disease progression (Pilstrom *et al.,* 1995, 1997; Rabinovitch *et al.,* 1996b,c; Rothe *et al.,* 1996, 1997a; M. Welsh *et al.,* 1995). Inhibition of IL-1, by soluble IL-1 receptor or anti-IL-1β antibodies, prevents cyclophosphamide (CY)-induced diabetes in NOD mice (Cailleau *et al.,* 1997; Nicoletti *et al.,* 1994). Diabetes is delayed in NOD mice deficient in IFN-γ (Hultgren *et al.,* 1996), and monoclonal antisera specific for IFN-γ attenuates the development of diabetes in NOD mice (Campbell *et al.,* 1991; Debray-Sachs *et al.,* 1991). Similarly, diabetes is prevented in NOD mice deficient in TNF (TNF receptor knockout) or by systemic administration of TNF at 4 weeks of age (Hunger *et al.,* 1997; Yang and McDevitt, 1994; Yang *et al.,* 1994). Attenuation of diabetes in NOD or CY-treated NOD mice by IL-18 and IL-12 antagonist IL-12p40 administration, respectively, is associated with decreased IFN-γ and iNOS expression in islets (Rothe *et al.,* 1999, 1997b). *In vitro,* IFN-γ, in combination with IL-1β or TNF, stimulates iNOS expression and nitric oxide formation by isolated NOD mouse islets (Heitmeier *et al.,* 1999b; Thomas *et al.,* 1999). In addition, a short incubation (e.g., 30 min) of NOD mouse islets with IFN-γ primes NOD mouse islets for IL-1-induced iNOS expression and nitric oxide formation (Heitmeier *et al.,* 1999b). These findings suggest that macrophage and T-cell-derived cytokines induce iNOS expression and nitric oxide formation by NOD mouse islets *in vitro,* and increased cytokine and iNOS expression correlates with the development of diabetes *in vivo.*

Although iNOS expression correlates with disease progression in NOD mice, the exact role of nitric oxide is unclear. Inhibitors of iNOS, AG and L-NMMA, fail to prevent spontaneous diabetes in NOD mice, as does the genetic absence of iNOS (Bowman *et al.,* 1996; Pakala *et al.,* 1999). We have shown, however, that administration of AG delays the onset of diabetes induced by the adoptive transfer of splenocytes isolated from diabetic females to irradiated non-

diabetic male NOD mice (Corbett *et al.,* 1993b). Islets isolated from male mice following the adoptive transfer of splenocytes from diabetic female NOD mice produce high levels of nitric oxide and exhibit impaired insulin secretion in a time-dependent manner (Corbett *et al.,* 1993b). Immunohistochemical colocalization studies in pancreatic sections prepared from male NOD mice at day 9 after splenocyte transfer indicate that the β cell is one islet cellular source of iNOS in this animal model. iNOS immunoreactivity (red fluorescence) colocalizes with insulin-containing cells (green fluorescence), as evidenced by the yellow fluorescence on double exposure. (Fig. 9). A small number of non-insulin-containing cells, which are believed to be macrophages, also appear to express iNOS under these conditions (red fluorescent cells on double exposure, Fig. 9). Immunohistochemical analysis of inflammatory macrophages and islet cells isolated from NOD mice at the onset of diabetes indicates that 40–50% of both cell types express iNOS (Rabinovitch *et al.,* 1996c). Similar results were obtained in studies examining the immunohistochemical localization of iNOS in pancreatic sections prepared from NOD mice at diabetes onset (Reddy *et al.,* 1997, 1999). These findings indicate that iNOS is expressed and nitric oxide is produced in NOD islets during the progression to diabetes, and that inhibition of iNOS activity delays disease onset in the adoptive transfer NOD mouse model.

Incubation of islet-specific $CD8^+$ T-cell clones with islets isolated from female NOD mice have been shown to kill NOD mouse islet cells, and the cell death is dependent on the extent of islet inflammation in the isolated islets (Gurlo *et al.,* 1999). In effect, this study showed that islet destruction was dependent on preexisting islet infiltrate, identified as macrophages. The iNOS inhibitor L-NIL prevents $CD8^+$ T-cell killing of infiltrated islets in a concentration-dependent manner, suggesting that $CD8^+$ T-cell-mediated islet destruction is dependent on nitric oxide production (Gurlo *et al.,* 1999). $CD8^+$ T-cell-mediated islet destruction is also dependent on IFN-γ production, as antiserum to IFN-γ prevents T-cell-mediated islet killing. The amount of IFN-γ produced by the T-cell clones varied depending on the extent of islet macrophage infiltrate (Gurlo *et al.,* 1999). It is unclear from these studies whether macrophage production of nitric oxide directly mediates islet destruction, or whether macrophage-derived soluble mediators, such as IL-1 and TNF, mediate islet destruction by inducing iNOS expression and nitric oxide formation by β cells. Importantly, NOD mouse β cells require IFN-γ in addition to IL-1 to express iNOS (Thomas *et al.,* 1999), and the T-cell clones in the Gurlo study produce high levels of IFN-γ (Gurlo *et al.,* 1999). We have shown that local release of IL-1 in islets by resident macrophages mediates β-cell damage by releasing IL-1 in islets followed by IL-1 (or IL-1 plus IFN-γ)-induced iNOS expression by β cells (Arnush *et al.,* 1998b; Corbett and McDaniel, 1995). These results implicate nitric oxide in T-cell- and macrophage-mediated islet destruction during the development of IDDM in NOD mice, and they suggest that activated macrophages may participate in β-cell destruction either by producing nitric oxide, by releasing proinflammatory cytokines, or by both mechanisms.

Direct evidence to support the hypothesis that β-cell production of nitric oxide mediates β-cell damage leading to the development of diabetes has been obtained from a study that examined the development of diabetes in transgenic mice expressing iNOS under control of the rat insulin promoter (β-cell-specific iNOS expression). These mice have been shown to develop hyperinsulinemia within the first week of birth and develop hypoinsulinemic diabetes by 4 weeks of age (Takamura *et al.,* 1998). Importantly, diabetes develops in these mice in a nitric oxide-dependent manner, as AG administration (200 mg/kg two times daily) prevents the spontaneous development of diabetes (Takamura *et al.,* 1998). This study demonstrates that iNOS expression by β

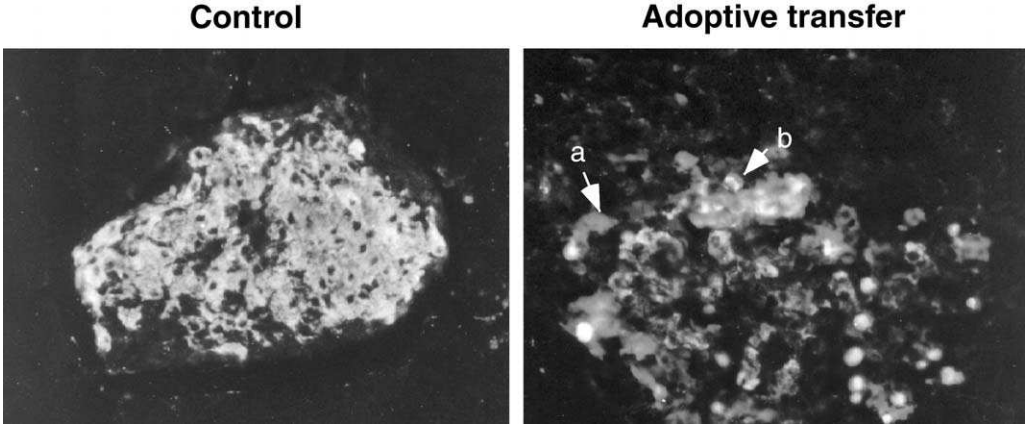


Figure 9 Immunohistochemical analysis of iNOS expression in NOD mouse pancreata following adoptive transfer. Pancreata were isolated either from irradiated male NOD mice 9 days after the transfer of spleen cells from a diabetic female or from irradiated male control NOD mice. Insulin (green) and iNOS (red) expression was evaluated by immunofluorescence. iNOS expression is absence in islets of control NOD mice; however, iNOS is expressed in the pancreas of irradiated NOD mice 9 days after spleen cell transfer. iNOS expression localizes with insulin-containing cells (a) as well as a second population of non-insulin-containing cells believed to be macrophages (b). See color insert.

cells and β-cell production of nitric oxide induce β-cell dysfunction and death, resulting in the development of autoimmune diabetes.

Virus-Induced Diabetes and Nitric Oxide

Viruses have been implicated as one environmental factor that may initiate or trigger an autoimmune reaction that targets and destroys β cells in genetically susceptible individuals. Evidence to support this hypothesis includes (1) seasonal variation in the onset of acute IDDM, with the highest prevalence of disease in autumn; (2) anecdotal reports of a temporal association of viral infection with the onset of IDDM; (3) virus isolation from pancreata of acutely diabetic deceased patients and the identification of virus-specific IgM antibodies in newly diagnosed diabetic patients; and (4) induction of diabetes in genetically susceptible strains of mice, rats, and primates by infection with encephalomyocarditis (EMC) virus, Coxsackie B4 virus, Kilham's rat virus (KRV), rubella virus, and retrovirus (Bach, 1994; Yoon, 1990; Yoon *et al.,* 1987, 1989).

A wide array of viruses stimulate the development of autoimmune diabetes in both rat and mouse models (Bach, 1994; Rossini *et al.,* 1985; von Herrath *et al.,* 1997; Yoon, 1992; Yoon *et al.,* 1989). As a general theme, viral-induced diabetes requires genetically susceptible strains of mice or rats. This is consistent with the development of IDDM in genetically predisposed individuals. Studies have focused on effector mechanisms by which viral infection stimulates β-cell damage. KRV-induced diabetes in drBB rats appears to require macrophages, and it is associated with increased expression of the macrophage-derived cytokines IL-1 and TNF. Yoon and co-workers have shown that macrophage depletion (by treatment with liposome-encapsulated dichloromethylene diphosphonate) prevents KRV-induced diabetes in BB rats (Chung *et al.,* 1997). In addition, the development of viral-induced diabetes in BB rats correlates with increased mRNA expression of the macrophage-derived cytokines IL-12, IL-1β, and TNF and with $CD4^+$ T-cell expression of IFN-γ (Chung *et al.,* 1997).

Similar to KRV-induced diabetes in BB rats, EMC viral-induced diabetes in DBA/2 mice correlates with an increased level of IL-1β and TNF mRNA expression in islets as determined by *in situ* hybridization of pancreatic sections (Hirasawa *et al.,* 1997). EMC viral-induced diabetes is characterized by an early infiltration of macrophages into islets followed by the presence of T cells at intermediate and late stages (Baek and Yoon, 1991). Depletion of T cells fails to alter disease progression in DBA/2 mice (Yoon *et al.,* 1985); however, macrophage depletion by treatment with anti-Mac 1 monoclonal antibodies (Hirasawa *et al.,* 1996) or silica (Baek and Yoon, 1991) prevents EMC viral-induced diabetes in DBA/2 mice.

Cytokine release followed by cytokine-induced β-cell damage is one mechanism by which macrophages may participate in the development of viral-induced diabetes. Hirasawa *et al.* (1997) have shown that daily administration of antiserum specific for IL-1β or TNF-α (0.5 mg/mouse) starting on the day of viral infection attenuates EMC virus-induced diabetes in DBA/2 mice. In addition, EMC virus stimulates iNOS mRNA expression in islets at early stages of infection, and iNOS expression persists until the onset of diabetes in DBA/2 mice. Daily administration of the iNOS inhibitor AG (at 2 mg/mouse/day) significantly attenuates the development of EMC virus-induced diabetes (Hirasawa *et al.,* 1997). In combination, daily administration of AG and neutralizing antisera specific for IL-1β and TNF is the most effective therapeutic strategy to attenuate EMC virus-induced diabetes in DBA/2 mice (Hirasawa *et al.,* 1997). These findings implicate macrophage-derived soluble mediators (IL-1 and TNF) and nitric oxide in the development of viral-induced diabetes.

It has been difficult to ascertain the mechanisms by which viral infection induces autoimmune diabetes because viruses from both RNA and DNA families (single-stranded and double-stranded viruses) have been implicated in disease onset (Bach, 1994; Yoon, 1990). One common determinant of a viral infection is the formation of dsRNA during virus replication. Many single-stranded viral RNAs also contain secondary structure that is double stranded in nature. dsRNA appears to be the active component of a viral infection that stimulates antiviral responses in infected cells, including type 1 interferon expression, nitric oxide production, macrophage IL-1 release, and a general inhibition of protein translation (Clemens, 1997; Proud, 1995; Robertson and Mathews, 1996; Williams, 1997). Importantly, *in vivo* administration of dsRNA [in the form of poly(IC)] stimulates the development of diabetes in drBB rats, and it accelerates the development of diabetes in dpBB rats (Ewel *et al.,* 1992; Sobel *et al.,* 1992, 1994).

Because macrophages appear to play a primary role in the development of virus-induced diabetes and because dsRNA appears to be an active component of a viral infection that activates the antiviral response (Clemens, 1997; Proud, 1995; Williams, 1997), we have examined the effects of dsRNA on macrophage activation. The combination of dsRNA and IFN-γ stimulates iNOS expression, nitric oxide production, and IL-1 release by primary macrophages isolated from CD-1 mice (Heitmeier *et al.,* 1998). Individually, dsRNA and IFN-γ fail to activate primary mouse peritoneal macrophages. These studies suggest that one mechanism by which viral infection stimulates macrophage activation is by dsRNA-induced iNOS expression, nitrite production, and IL-1 release in the presence of IFN-γ.

The direct effects of dsRNA and IFN-γ on iNOS expression and nitric oxide formation by rat and human islets have also been examined. Treatment of rat (Heitmeier *et al.,* 1999a) and human islets (M. R. Heitmeier and J. A. Corbett, 2000, unpublished observation) with dsRNA plus IFN-γ results in the time- and concentration-dependent expression of iNOS and production of nitric oxide. dsRNA plus IFN-γ-induced nitric oxide production by rat islets results in a potent inhibition of insulin secretion and islet degeneration.

AG prevents the inhibitory effects of dsRNA plus IFN-γ on β-cell function, indicating that the destructive effects are mediated by nitric oxide. Resident macrophage activation is not required for the inhibitory and destructive effects of dsRNA plus IFN-γ on rat islet insulin secretion and islet degeneration, as macrophage depletion (by a 7-day culture at 24°C) fails to prevent dsRNA plus IFN-γ-induced iNOS expression. In addition, dsRNA plus IFN-γ stimulates iNOS expression and nitric oxide formation by FACS-purified β cells, and this results in an inhibition of glucose-stimulated insulin secretion in a nitric oxide-dependent manner (Heitmeier *et al.,* 1999a). These findings indicate that a viral infection, or dsRNA, can directly inhibit β-cell function by stimulating β-cell production of nitric oxide.

In summary, these findings provide novel insights into potential mechanisms by which viral infection results in β-cell damage during the development of autoimmune diabetes. These studies suggest that viral infection stimulates macrophage activation, resulting in the local production of IL-1 in islets. IL-1 released locally within islets stimulates β-cell expression of iNOS. In addition, the viral replicative intermediate dsRNA also stimulates β-cell expression of iNOS in the presence of IFN-γ. β-Cell expression of iNOS and production of nitric oxide cause β-cell damage, resulting in the release of potential β-cell autoantigens leading to T-cell-dependent destruction of the remaining β cells and the development of autoimmune diabetes (Fig. 10).

Nitric Oxide and Type II Diabetes

Obesity, the most common disease in the United States, often leads to the development of type 2 diabetes. Peripheral insulin resistance characterizes the initial stages of type 2 diabetes; however, normal blood glucose levels are maintained because β cells compensate by secreting increased levels of insulin (DeFronzo, 1992; Lebovitz, 1999). Diabetes develops when the β cell is no longer able to compensate for insulin resistance, resulting in elevated blood glucose levels. The mechanisms by which β cells lose their ability to compensate for the increased insulin demand are unknown; however, postmortem studies on NIDDM patients have revealed a reduction in β-cell mass (Rahier *et al.,* 1983). Much of what is known concerning β-cell damage during the development of type 2 diabetes has been obtained from the Zucker diabetic fatty rat (ZDF). These rats display prediabetic obese (7 weeks of age) and diabetic obese (14 weeks of age) stages of diabetes (Bray, 1977). β-Cell damage has been attributed to fat deposition in islets of obese diabetic ZDF rats. This ''lipotoxicity'' is associated with high plasma levels of free fatty acids (FFA) and with an increased capacity for lipogenesis (Unger, 1995).

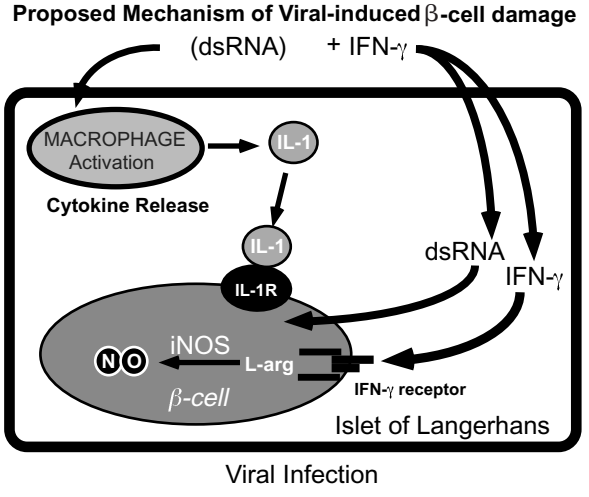


Figure 10 Mechanism of viral-induced β-cell damage. dsRNA, formed during viral replication, activates macrophages, stimulating both iNOS and IL-1 expression. IL-1, released from activated macrophages, directly stimulates β-cell expression of iNOS and production of nitric oxide. dsRNA in combination with IFN-γ is also capable of directly inducing iNOS expression by β cells. β-Cell production of nitric oxide, either in response to IL-1 released from macrophages or following the induction of iNOS in response to dsRNA plus IFN-γ, stimulates β-cell damage.

Unger and co-workers (Shimabukuro *et al.,* 1997a) examined whether nitric oxide may contribute to β-cell damage in ZDF rats. Initial experiments examined the effects of FFA on the expression of iNOS and production of nitric oxide by islets isolated from prediabetic ZDF rats, from lean nondiabetic ZDF rats, and from control Wistar rats. FFA (2:1 mixture of oleote and palmitate) stimulates iNOS expression and nitrite production by islets isolated from each species of rat. However, iNOS expression and nitrite production were significantly higher in islets isolated from lean nondiabetic ZDF rats as compared to islets isolated from Wistar rats (Shimabukuro *et al.,* 1997a). Importantly, islets isolated from prediabetic ZDF rats produced the highest levels of nitric oxide in response to FFA. Functionally, FFA stimulate glucose-induced insulin secretion by islets isolated from Wistar rats, whereas they attenuate insulin secretion by islets isolated from both lean nondiabetic and prediabetic ZDF rats. The iNOS-selective inhibitor AG prevents FFA-induced nitrite production and the inhibitory actions of FFA on glucose-stimulated insulin secretion by islets isolated from both lean nondiabetic and prediabetic ZDF rats (Shimabukuro *et al.,* 1997a). In prediabetic ZDF rats, plasma FFA begin to accumulate at 7–9 weeks of age, and diabetes develops by 11–14 weeks of age (Lee *et al.,* 1994). Administration of AG to prediabetic obese ZDF rats by daily intraperitoneal injections of 400 mg/kg body weight beginning at 6 weeks of age prevents the development of diabetes and the loss of insulin immunoreactivity in islets without decreasing plasma FFA levels (Shimabukuro *et al.,* 1997a).

Regulation of FFA metabolism appears to be controlled by the action of leptin, which has been shown to inhibit esterification of FFA by enhancing intracellular oxidation (Shimabukuro *et al.,* 1998). ZDF rats are leptin resistant because of a Glu-269 to Pro mutation in the leptin receptor (Phillips *et al.,* 1996). This defect is associated with an increased triglyceride (TG) content in islets isolated from homozygous ZDF rats (fa/fa) (Lee *et al.,* 1997). Elevated β-cell triglyceride content has been implicated in the lipotoxicity of β cells (Shimabukuro *et al.,* 1997b). In response to IL-1,

fat-laden β cells produce greater than twofold higher levels of nitrite than wild-type control islets (Shimabukuro *et al.,* 1997c). Importantly, reductions in islet triglyceride either by adenoviral expression of the wild-type leptin receptor in β cells or by treatment with troglitazone attenuates IL-1-induced iNOS expression and islet damage in ZDF rat islets (Shimabukuro *et al.,* 1997c). These findings have been interpreted to indicate that reductions in islet TG content, by either functional leptin receptor expression in islets or by troglitazone administration, prevent β-cell lipotoxicity associated with the development of type II diabetes in ZDF rats (Shimabukuro *et al.,* 1997b,c).

Troglitazone is a high-affinity agonist of the peroxisome proliferator-activated receptor-γ (PPAR-γ). Agonists of this receptor, specifically the thiazolidenediones, are potent insulin sensitizers that are currently being used to treat insulin resistance in type 2 diabetes (Auwerx, 1999; Spiegelman, 1998; Spiegelman *et al.,* 1997; Vamecq and Latruffe, 1999; Vanden Heuvel, 1999). Naturally occurring PPAR-γ agonists such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-$\Delta^{12,14}$-PGJ_2) and the thiazolidenediones have been shown to have anti-inflammatory properties. Troglitazone and 15-d-$\Delta^{12,14}$-PGJ_2, as well as other PPAR-γ agonists, prevent LPS- and LPS plus IFN-γ-stimulated iNOS expression, nitrite formation, and proinflammatory cytokine expression by RAW 264.7 cells, mouse peritoneal macrophages, and microglia (Huang *et al.,* 1999; Jiang *et al.,* 1998; Petrova *et al.,* 1999; Ricote *et al.,* 1998, 1999). In addition, these PPAR-γ agonists also prevent resident macrophage IL-1 expression and release following TNF plus LPS stimulation of rat islets (Kwon *et al.,* 1999; Maggi *et al.,* 2000). Inhibition of iNOS and IL-1 mRNA transcription appears to be one mechanism by which troglitazone and 15-D-$\Delta^{12,14}$-PGJ_2 function as anti-inflammatory agents (Jiang *et al.,* 1998; Maggi *et al.,* 2000; Ricote *et al.,* 1998). The anti-inflammatory actions of these agonists on iNOS expression suggest that the ability of troglitazone to prevent β-cell lipotoxicity in the ZDF rat islets may also be a function of the ability of this PPAR-γ agonist to inhibit iNOS expression in addition to its TG lowering actions.

Although troglitazone and 15-d-$\Delta^{12,14}$-PGJ_2 are PPAR-γ agonists, troglitazone has a much higher affinity for PPAR-γ than 15-d-$\Delta^{12,14}$-PGJ_2, but it is over 10-fold less effective at inhibiting iNOS expression and nitrite production by mouse peritoneal macrophages and RINm5F cells (Maggi *et al.,* 2000). These findings suggest that receptors other than PPAR-γ may mediate the anti-inflammatory actions of these agents. Consistent with this interpretation, Vaidya *et al.* (1999) have shown that the inhibitory actions of 15-d-$\Delta^{12,14}$-PGJ_2 on the integrin-dependent oxidative burst of neutrophils are independent of PPAR-γ. In this study, 15-d-$\Delta^{12,14}$-PGJ_2 was shown to prevent adhesion-dependent H_2O_2 production, an effect that was not modulated by a selective PPAR-γ agonist, AD-5075. In addition, AD-5075 had no effect on adhesion-dependent H_2O_2 (Vaidya *et al.,* 1999). Taken together, these studies suggest that the inhibitory actions of PPAR-γ agonists such as troglitazone and 15-d-$\Delta^{12,14}$-PGJ_2 may be mediated by a yet to be defined receptor.

Conclusions

In this chapter, we have provided a summary of the evidence supporting a role for nitric oxide in the development of autoimmune diabetes and in β-cell damage in type 2 diabetes. It is clear from these studies that nitric oxide inhibits insulin secretion (both human and rodent) and kills β cells. Does nitric oxide mediate β-cell death during the development of autoimmune diabetes? This question has yet to be definitively addressed. Controversy concerning whether nitric oxide mediates the inhibitory effects of cytokines on human islet function continues. In addition, the development of diabetes in transgenic mice deficient in the iNOS gene has generated further skepticism. While this healthy skepticism exists, a large body of evidence supports a role for nitric oxide in the development of diabetes. Is iNOS a viable target for therapeutic intervention designed to prevent diabetes in predisposed individuals? It is too early to determine. It is clear that nitric oxide can impair β-cell function and mediate β-cell death; however, the precise stage(s) in the diabetogenic process in which nitric oxide may mediate β-cell damage and the potential roles that nitric oxide may play in the regulation of islet inflammation are unclear.

We favor an early role for nitric oxide in the development of autoimmune diabetes, functioning in the precipitating events that result in the initial damage to β cells. In this setting, β-cell damage may result in the release of autoantigens and further T-cell-dependent destruction of β cells. We also believe that macrophages may play a critical role in the regulation of iNOS expression in islets under precipitating conditions. This regulation may be by the local release of cytokines such as IL-1 and TNF in islets. In addition, islet macrophages may also participate in $CD8^+$ T-cell-mediated β-cell damage that is dependent on nitric oxide production (Gurlo *et al.,* 1999). Viral infection is one precipitating event that may trigger the initial destruction of β cells during the development of autoimmune diabetes. Importantly, inhibitors of iNOS and neutralization of macrophage-derived cytokines prevent viral-induced diabetes. Although these studies provide evidence to support a role for macrophages, macrophage-derived cytokines, and nitric oxide in the development of autoimmune diabetes, continued studies into the potential roles of nitric oxide in the pathogenesis of diabetes will be required to determine whether iNOS or nitric oxide are viable therapeutic targets for disease prevention.

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References

Akabane, A., Kato, I., Takasawa, S., Unno, M., Yonekura, H., *et al.* (1995). Nicotinamide inhibits IRF-1 mRNA induction and prevents IL-1β-induced nitric oxide synthase expression in pancreatic beta cells. *Biochem. Biophys. Res. Commun.* **215,** 524–530.

Albina, J. E. (1995). On the expression of nitric oxide synthase by human macrophages. Why no NO? *J. Leukocyte Biol.* **58,** 643–649.

Albina, J. E., and Reichner, J. S. (1998). Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. *Cancer Metastasis Rev.* **17,** 39–53.

Andersen, H. U., Larsen, P. M., Fey, S. J., Karlsen, A. E., Mandrup-Poulsen, T., and Nerup, J. (1995). Two-dimensional gel electrophoresis of rat islet proteins. Interleukin 1β-induced changes in protein expression are reduced by L-arginine depletion and nicotinamide. *Diabetes* **44,** 400–407.

Arend, W. P. (1993). Interleukin-1 receptor antagonist. *Adv. Immunol.* **54,** 167–227.

Arend, W. P., Malyak, M., Guthridge, C. J., and Gabay, C. (1998). Interleukin-1 receptor antagonist: Role in biology. *Annu. Rev. Immunol.* **16,** 27–55.

Arnush, M., Heitmeier, M. R., Scarim, A. L., Marino, M. H., Manning, P. T., and Corbett, J. A. (1998a). IL-1 produced and released endogenously within human islets inhibits β cell function. *J. Clin. Invest.* **102,** 516–526.

Arnush, M., Scarim, A. L., Heitmeier, M. R., Kelly, C. B., and Corbett, J. A. (1998b). Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. *J. Immunol.* **160,** 2684–2691.

Auwerx, J. (1999). PPAR-γ, the ultimate thrifty gene. *Diabetologia* **42,** 1033–1049.

Bach, J. F. (1994). Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr. Rev.* **15,** 516–542.

Baek, H. S., and Yoon, J. W. (1991). Direct involvement of macrophages in destruction of β-cells leading to development of diabetes in virus-infected mice. *Diabetes* **40,** 1586–1597.

Baeuerle, P. A., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* **12,** 141–179.

Bai, J. Z., Saafi, E. L., Zhang, S., and Cooper, G. J. (1999). Role of Ca^{2+} in apoptosis evoked by human amylin in pancreatic islet β-cells. *Biochem. J.* **343**(Part 1), 53–61.

Bain, S. C., Mijovic, C. V., and Barnett, A. H. (1997). ''Genetic Factors in the Pathogenesis of Insulin-Dependent Diabetes Mellitus,'' pp. 13.1–13.13. Blackwell Science, Cambridge, Massachusetts.

Balon, T. W. (1998). Role of nitric oxide in contraction induced glucose transport. *Adv. Exp. Med. Biol.* **441,** 87–95.

Baynes, J. W., and Thorpe, S. R. (1999). Role of oxidative stress in diabetic complications: A new perspective on an old paradigm. *Diabetes* **48,** 1–9.

Beck, K. F., and Sterzel, R. B. (1996). Cloning and sequencing of the proximal promoter of the rat iNOS gene: Activation of NF-κB is not sufficient for transcription of the iNOS gene in rat mesangial cells. *FEBS Lett.* **394,** 263–267.

Bellmann, K., Wenz, A., Radons, J., Burkart, V., Kleemann, R., and Kolb, H. (1995). Heat shock induces resistance in rat pancreatic islet cells against nitric oxide, oxygen radicals and streptozotocin toxicity in vitro. *J. Clin. Invest.* **95,** 2840–2845.

Bellmann, K., Jaattela, M., Wissing, D., Burkart, V., and Kolb, H. (1996). Heat shock protein hsp70 overexpression confers resistance against nitric oxide. *FEBS Lett.* **391,** 185–188.

Bendtzen, K., Mandrup-Poulsen, T., Nerup, J., Nielsen, J. H., Dinarello, C. A., and Svenson, M. (1986). Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. *Science* **232,** 1545–1547.

Benhamou, P. Y., Mullen, Y., Clare-Salzler, M., Sangkharat, A., Benhamou, C., *et al.* (1995). Essential fatty acid deficiency prevents autoimmune diabetes in nonobese diabetic mice through a positive impact on antigen-presenting cells and Th2 lymphocytes. *Pancreas* **11,** 26–37.

Bergman, B., and Haskins, K. (1997). Autoreactive T-cell clones from the nonobese diabetic mouse. *Proc. Soc. Exp. Biol. Med.* **214,** 41–48.

Bigdeli, N., Niemann, A., Sandler, S., and Eizirik, D. L. (1994). Dissociation between interleukin-1 β-induced expression of mRNA for superoxide dismutase and nitric oxide synthase in insulin-producing cells. *Biochem. Biophys. Res. Commun.* **203,** 1542–1547.

Bleich, D., Chen, S., Wen, Y., and Nadler, J. L. (1997). The stress-activated c-Jun protein kinase (JNK) is stimulated by lipoxygenase pathway product 12-HETE in RIN m5F cells. *Biochem. Biophys. Res. Commun.* **230,** 448–451.

Bonner-Weir, S. (1988). Morphological evidence for pancreatic polarity of β-cell within islets of Langerhans. *Diabetes* **37,** 616–621.

Bonner-Weir, S., and Orci, L. (1982). New perspectives on the microvasculature of the islets of Langerhans in the rat. *Diabetes* **31,** 883–889.

Borg, L. A., and Eizirik, D. L. (1990). Short-term exposure of rat pancreatic islets to human interleukin-1 β increases cellular uptake of calcium. *Immunol. Lett.* **26,** 253–258.

Borg, L. A., Cagliero, E., Sandler, S., Welsh, N., and Eizirik, D. L. (1992). Interleukin-1 β increases the activity of superoxide dismutase in rat pancreatic islets. *Endocrinology* **130,** 2851–2857.

Bowman, M. A., Simell, O. G., Peck, A. B., Cornelius, J., Luchetta, R., *et al.* (1996). Pharmacokinetics of aminoguanidine administration and effects on the diabetes frequency in nonobese diabetic mice. *J. Pharmacol. Exp. Ther.* **279,** 790–794.

Bray, G. A. (1977). The Zucker-fatty rat: A review. *Fed. Proc.* **36,** 148–153.

Burkart, V., and Kolb, H. (1996). Macrophages in islet destruction in autoimmune diabetes mellitus. *Immunobiology* **195,** 601–613.

Burkart, V., Wang, Z. Q., Radons, J., Heller, B., Herceg, Z., *et al.* (1999). Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic β-cell destruction and diabetes development induced by streptozocin. *Nat. Med.* **5,** 314–319.

Buschard, K. (1996). Diabetic animal models. *APMIS* **104,** 609–614.

Cailleau, C., Diu-Hercend, A., Ruuth, E., Westwood, R., and Carnaud, C. (1997). Treatment with neutralizing antibodies specific for IL-1β prevents cyclophosphamide-induced diabetes in nonobese diabetic mice. *Diabetes* **46,** 937–940.

Campbell, I. L., and Harrison, L. C. (1990). Molecular pathology of type 1 diabetes. *Mol. Biol. Med.* **7,** 299–309.

Campbell, I. L., Kay, T. W., Oxbrow, L., and Harrison, L. C. (1991). Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J. Clin. Invest.* **87,** 739–742.

Caprio, S., and Tamborlane, W. V. (1999). Metabolic impact of obesity in childhood. *Endocrinol. Metab. Clin. North. Am.* **28,** 731–747.

Cetkovic-Cvrlje, M., and Eizirik, D. L. (1994). TNF-α and IFN-γ potentiate the deleterious effects of IL-1β on mouse pancreatic islets mainly via generation of nitric oxide. *Cytokine* **6,** 399–406.

Cheta, D. (1998). Animal models of type I (insulin-dependent) diabetes mellitus. *J. Pediatr. Endocrinol. Metab.* **11,** 11–19.

Chu, S. C., Wu, H. P., Banks, T. C., Eissa, N. T., and Moss, J. (1995). Structural diversity in the 5'-untranslated region of cytokine-stimulated human inducible nitric oxide synthase mRNA. *J. Biol. Chem.* **270,** 10625–10630.

Chung, Y. H., Jun, H. S., Kang, Y., Hirasawa, K., Lee, B. R. *et al.* (1997). Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant BioBreeding rats. *J. Immunol.* **159,** 466–471.

Clemens, M. J. (1997). PKR—a protein kinase regulated by double-stranded RNA. *Int. J. Biochem. Cell Biol.* **29,** 945–9.

Comens, P. G., Wolf, B. A., Unanue, E. R., Lacy, P. E., and McDaniel, M. L. (1987). Interleukin 1 is potent modulator of insulin secretion from isolated rat islets of Langerhans. *Diabetes* **36,** 963–970.

Cooper, J. T., Stroka, D. M., Brostjan, C., Palmetshofer, A., Bach, F. H., and Ferran, C. (1996). A20 blocks endothelial cell activation through a NF-κB-dependent mechanism. *J. Biol. Chem.* **271,** 18068–18073.

Corbett, J. A., and McDaniel, M. L. (1992). Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM. *Diabetes* **41,** 897–903.

Corbett, J. A., and McDaniel, M. L. (1994). Reversibility of interleukin-1 β-induced islet destruction and dysfunction by the inhibition of nitric oxide synthase. *Biochem. J.* **299,** 719–724.

Corbett, J. A., and McDaniel, M. L. (1995). Intraislet release of interleukin 1 inhibits β cell function by inducing beta cell expression of inducible nitric oxide synthase. *J. Exp. Med.* **181,** 559–568.

Corbett, J. A., Lancaster, J. R., Jr., Sweetland, M. A., and McDaniel, M. L. (1991). Interleukin-1 β-induced formation of EPR-detectable iron–nitrosyl complexes in islets of Langerhans. Role of nitric oxide in

interleukin-1β-induced inhibition of insulin secretion. *J. Biol. Chem.* **266,** 21351–21354.

Corbett, J. A., Tilton, R. G., Chang, K., Hasan, K. S., Ido, Y., *et al.* (1992a). Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* **41,** 552–556.

Corbett, J. A., Wang, J. L., Sweetland, M. A., Lancaster, J. R., Jr., and McDaniel, M. L. (1992b). Interleukin 1β induces the formation of nitric oxide by β-cells purified from rodent islets of Langerhans. Evidence for the β-cell as a source and site of action of nitric oxide. *J. Clin. Invest.* **90,** 2384–91.

Corbett, J. A., Sweetland, M. A., Wang, J. L., Lancaster, J. R., Jr., and McDaniel, M. L. (1993a). Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 1731–1735.

Corbett, J. A., Mikhael, A., Shimizu, J., Frederick, K., Misko, T. P., *et al.* (1993b). Nitric oxide production in islets from nonobese diabetic mice: Aminoguanidine-sensitive and -resistant stages in the immunological diabetic process. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 8992–8995.

Corbett, J. A., Kwon, G., Hill, J. R., and L., M. M. (1995). ''Nitric Oxide Mediates Cytokine-Induced Islet Destruction,'' Vol. 9, pp. 265–294. Elsevier Science, Amsterdam.

Corbett, J. A., Kwon, G., Marino, M. H., Rodi, C. P., Sullivan, P. M., *et al.* (1996). Tyrosine kinase inhibitors prevent cytokine-induced expression of iNOS and COX-2 by human islets. *Am. J. Physiol.* **270,** C1581–C1587.

Crisa, L., Mordes, J. P., and Rossini, A. A. (1992). Autoimmune diabetes mellitus in the BB rat. *Diabetes Metab. Rev.* **8,** 4–37.

Cunningham, J. M., and Green, I. C. (1994). Cytokines, nitric oxide and insulin secreting cells. *Growth Regul.* **4,** 173–180.

Darnell, J. E., Jr. (1998). Studies of IFN-induced transcriptional activation uncover the Jak–Stat pathway. *J. Interferon Cytokine Res.* **18,** 549–554.

Darville, M. I., and Eizirik, D. L. (1998). Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* **41,** 1101–1108.

Debray-Sachs, M., Carnaud, C., Boitard, C., Cohen, H., Gresser, I., *et al.* (1991). Prevention of diabetes in NOD mice treated with antibody to murine IFN-γ. *J. Autoimmun.* **4,** 237–248.

DeFronzo, R. A. (1992). Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: A balanced overview. *Diabetologia* **35,** 389–397.

Delaney, C. A., and Eizirik, D. L. (1996). Intracellular targets for nitric oxide toxicity to pancreatic β-cells. *Braz. J. Med. Biol. Res.* **29,** 569–579.

Delaney, C. A., Green, M. H., Lowe, J. E., and Green, I. C. (1993). Endogenous nitric oxide induced by interleukin-1β in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay. *FEBS Lett.* **333,** 291–295.

Delaney, C. A., Tyrberg, B., Bouwens, L., Vaghef, H., Hellman, B., and Eizirik, D. L. (1996). Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. *FEBS Lett.* **394,** 300–306.

Delaney, C. A., Pavlovic, D., Hoorens, A., Pipeleers, D. G., and Eizirik, D. L. (1997). Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* **138,** 2610–2614.

Dimmeler, S., Rippmann, V., Weiland, U., Haendeler, J., and Zeiher, A. M. (1997). Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. *Circ. Res.* **81,** 970–976.

Drapier, J. C., and Hibbs, J. B., Jr. (1996). Aconitases: A class of metalloproteins highly sensitive to nitric oxide synthesis. *Methods Enzymol.* **269,** 26–36.

Dunger, A., Augstein, P., Schmidt, S., and Fischer, U. (1996). Identification of interleukin 1-induced apoptosis in rat islets using in situ specific labelling of fragmented DNA. *J. Autoimmun.* **9,** 309–313.

Eberhardt, W., Kunz, D., Hummel, R., and Pfeilschifter, J. (1996). Molecular cloning of the rat inducible nitric oxide synthase gene promoter. *Biochem. Biophys. Res. Commun.* **223,** 752–756.

Eizirik, D. L., Sandler, S., Hallberg, A., Bendtzen, K., Sener, A., and Malaisse, W. J. (1989). Differential sensitivity to β-cell secretagogues in cultured rat pancreatic islets exposed to human interleukin-1β. *Endocrinology* **125,** 752–759.

Eizirik, D. L., Cagliero, E., Bjorklund, A., and Welsh, N. (1992). Interleukin-1β induces the expression of an isoform of nitric oxide synthase in insulin-producing cells, which is similar to that observed in activated macrophages. *FEBS Lett.* **308,** 249–252.

Eizirik, D. L., Sandler, S., Welsh, N., Cetkovic-Cvrlje, M., Nieman, A., *et al.* (1994a). Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *J. Clin. Invest.* **93,** 1968–1974.

Eizirik, D. L., Pipeleers, D. G., Ling, Z., Welsh, N., Hellerstrom, C., and Andersson, A. (1994b). Major species differences between humans and rodents in the susceptibility to pancreatic β-cell injury. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 9253–9256.

Eizirik, D. L., Flodstrom, M., Karlsen, A. E., and Welsh, N. (1996a). The harmony of the spheres: Inducible nitric oxide synthase and related genes in pancreatic β cells. *Diabetologia* **39,** 875–890.

Eizirik, D. L., Delaney, C. A., Green, M. H., Cunningham, J. M., Thorpe, J. R., *et al.* (1996b). Nitric oxide donors decrease the function and survival of human pancreatic islets. *Mol. Cell. Endocrinol.* **118,** 71–83.

Erwig, L. P., Kluth, D. C., Walsh, G. M., and Rees, A. J. (1998). Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines. *J. Immunol.* **161,** 1983–1988.

Ewel, C. H., Sobel, D. O., Zeligs, B. J., and Bellanti, J. A. (1992). Poly I:C accelerates development of diabetes mellitus in diabetes-prone BB rat. *Diabetes* **41,** 1016–1021.

Fehsel, K., Jalowy, A., Qi, S., Burkart, V., Hartmann, B., and Kolb, H. (1993). Islet cell DNA is a target of inflammatory attack by nitric oxide. *Diabetes* **42,** 496–500.

Ferran, C., Stroka, D. M., Badrichani, A. Z., Cooper, J. T., Wrighton, C. J., *et al.* (1998). A20 inhibits NF-κB activation in endothelial cells without sensitizing to tumor necrosis factor-mediated apoptosis. *Blood* **91,** 2249–2258.

Flodstrom, M., and Eizirik, D. L. (1997). Interferon-γ-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. *Endocrinology* **138,** 2747–2753.

Flodstrom, M., Welsh, N., and Eizirik, D. L. (1996). Cytokines activate the nuclear factor kappa B (NF-κB) and induce nitric oxide production in human pancreatic islets. *FEBS Lett.* **385,** 4–6.

Flodstrom, M., Tyrberg, B., Eizirik, D. L., and Sandler, S. (1999). Reduced sensitivity of inducible nitric oxide synthase-deficient mice to multiple low-dose streptozotocin-induced diabetes. *Diabetes* **48,** 706–713.

Förstermann, U., and Kleinert, H. (1995). Nitric oxide synthase: Expression and expressional control of the three isoforms. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352,** 351–364.

Fries, K. L., Miller, W. E., and Raab-Traub, N. (1996). Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. *J. Virol.* **70,** 8653–8659.

Gaster, B., and Hirsch, I. B. (1998). The effects of improved glycemic control on complications in type 2 diabetes. *Arch. Intern. Med.* **158,** 134–140.

Gepts, W. (1965). Pathologic anatomy of the pancrease in juvenile diabetes mellitus. *Diabetes* **14,** 619–633.

Ghosh, S. (1999). Regulation of inducible gene expression by the transcription factor NF-κB. *Immunol. Res.* **19,** 183–189.

Grey, S. T., Arvelo, M. B., Hasenkamp, W., Bach, F. H., and Ferran, C. (1999). A20 inhibits cytokine-induced apoptosis and nuclear factor κB-dependent gene activation in islets. *J. Exp. Med.* **190,** 1135–1146.

Gurlo, T., Kawamura, K., and von Grafenstein, H. (1999). Role of inflammatory infiltrate in activation and effector function of cloned islet reactive nonobese diabetic $CD8^+$ T cells: Involvement of a nitric oxide-dependent pathway. *J. Immunol.* **163,** 5770–5780.

Hadjivassiliou, V., Green, M. H., James, R. F., Swift, S. M., Clayton, H. A., and Green, I. C. (1998). Insulin secretion, DNA damage, and apoptosis in human and rat islets of Langerhans following exposure to nitric oxide, peroxynitrite, and cytokines. *Nitric Oxide* **2,** 429–441.

Hanenberg, H., Kolb-Bachofen, V., Kantwerk-Funke, G., and Kolb, H. (1989). Macrophage infiltration precedes and is a prerequisite for lymphocytic insulitis in pancreatic islets of pre-diabetic BB rats. *Diabetologia* **32,** 126–134.

Hao, W., Myhre, A. P., and Palmer, J. P. (1999). Nitric oxide mediates IL-1β stimulation of heat shock protein but not IL-1β inhibition of glutamic acid decarboxylase. *Autoimmunity* **29,** 93–101.

Harada, H., Taniguchi, T., and Tanaka, N. (1998). The role of interferon regulatory factors in the interferon system and cell growth control. *Biochimie* **80,** 641–650.

Hart, S. P., Haslett, C., and Dransfield, I. (1996). Recognition of apoptotic cells by phagocytes. *Experientia* **52,** 950–956.

Hayward, A. R., Shriber, M., Cooke, A., and Waldmann, H. (1993). Prevention of diabetes but not insulitis in NOD mice injected with antibody to CD4. *J. Autoimmun.* **6,** 301–310.

Heitmeier, M. R., Scarim, A. L., and Corbett, J. A. (1997). Interferon-γ increases the sensitivity of islets of Langerhans for inducible nitric-oxide synthase expression induced by interleukin 1. *J. Biol. Chem.* **272,** 13697–13704.

Heitmeier, M. R., Scarim, A. L., and Corbett, J. A. (1998). Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF-κB activation. *J. Biol. Chem.* **273,** 15301–15307.

Heitmeier, M. R., Scarim, A. L., and Corbett, J. A. (1999a). Double-stranded RNA inhibits β-cell function and induces islet damage by stimulating β-cell production of nitric oxide. *J. Biol. Chem.* **274,** 12531–12536.

Heitmeier, M. R., Scarim, A. L., and Corbett, J. A. (1999b). Prolonged STAT1 activation is associated with interferon-γ priming for interleukin-1-induced inducible nitric-oxide synthase expression by islets of Langerhans. *J. Biol. Chem.* **274,** 29266–29273.

Heller, B., Wang, Z. Q., Wagner, E. F., Radons, J., Burkle, A., *et al.* (1995). Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J. Biol. Chem.* **270,** 11176–11180.

Helqvist, S., Zumsteg, U. W., Spinas, G. A., Palmer, J. P., Mandrup-Poulsen, T., *et al.* (1991). Repetitive exposure of pancreatic islets to interleukin-1β. An in vitro model of pre-diabetes? *Autoimmunity* **10,** 311–318.

Heurtier, A. H., and Boitard, C. (1997). T-cell regulation in murine and human autoimmune diabetes: The role of TH1 and TH2 cells. *Diabetes Metab.* **23,** 377–385.

Hickey, M. J., and Kubes, P. (1997). Role of nitric oxide in regulation of leucocyte–endothelial cell interactions. *Exp. Physiol.* **82,** 339–348.

Hirasawa, K., Tsutsui, S., Takeda, M., Mizutani, M., Itagaki, S., and Doi, K. (1996). Depletion of Mac1-positive macrophages protects DBA/2 mice from encephalomyocarditis virus-induced myocarditis and diabetes. *J. Gen. Virol.* **77,** 737–741.

Hirasawa, K., Jun, H. S., Maeda, K., Kawaguchi, Y., Itagaki, S., *et al.* (1997). Possible role of macrophage-derived soluble mediators in the pathogenesis of encephalomyocarditis virus-induced diabetes in mice. *J. Virol.* **71,** 4024–4031.

Hohmeier, H. E., Thigpen, A., Tran, V. V., Davis, R., and Newgard, C. B. (1998). Stable expression of manganese superoxide dismutase (MnSOD) in insulinoma cells prevents IL-1β-induced cytotoxicity and reduces nitric oxide production. *J. Clin. Invest.* **101,** 1811–1820.

Holstad, M., Jansson, L., and Sandler, S. (1997). Inhibition of nitric oxide formation by aminoguanidine: An attempt to prevent insulin-dependent diabetes mellitus. *Gen. Pharmacol.* **29,** 697–700.

Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., *et al.* (1999). Interleukin-4-dependent production of PPAR-γ ligands in macrophages by 12/15-lipoxygenase. *Nature* **400,** 378–382.

Hughes, J. H., Colca, J. R., Easom, R. A., Turk, J., and McDaniel, M. L. (1990). Interleukin 1 inhibits insulin secretion from isolated rat pancreatic islets by a process that requires gene transcription and mRNA translation. *J. Clin. Invest.* **86,** 856–863.

Hultgren, B., Huang, X., Dybdal, N., and Stewart, T. A. (1996). Genetic absence of γ-interferon delays but does not prevent diabetes in NOD mice. *Diabetes* **45,** 812–817.

Hunger, R. E., Carnaud, C., Garcia, I., Vassalli, P., and Mueller, C. (1997). Prevention of autoimmune diabetes mellitus in NOD mice by transgenic expression of soluble tumor necrosis factor receptor p55. *Eur. J. Immunol.* **27,** 255–261.

Ido, Y., Kilo, C., and Williamson, J. R. (1997). Cytosolic NADH/NAD^+, free radicals, and vascular dysfunction in early diabetes mellitus. *Diabetologia* **40**(Suppl. 2), S115–S117.

Ingelsson, E., Saldeen, J., and Welsh, N. (1998). Islet expression of perforin, Fas/Apo-1 and interleukin-1-converting enzyme (ICE) in non-obese diabetic (NOD) mice. *Immunol. Lett.* **63,** 125–129.

Jaattela, M., Mouritzen, H., Elling, F., and Bastholm, L. (1996). A20 zinc finger protein inhibits TNF and IL-1 signaling. *J. Immunol.* **156,** 1166–1173.

Jansson, L., and Andersson, A. (1997). Nitric oxide and pancreatic islet blood flow after induced portal hypertension in rats. *Pancreas* **14,** 280–284.

Jiang, C., Ting, A. T., and Seed, B. (1998). PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. *Nature* **391,** 82–86.

Jiang, Z., and Woda, B. A. (1991). Cytokine gene expression in the islets of the diabetic BioBreeding/Worcester rat. *J. Immunol.* **146,** 2990–2994.

Jovanovic, L., and Gondos, B. (1999). Type 2 diabetes: The epidemic of the new millennium. *Ann Clin Lab. Sci.* **29,** 33–42.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., *et al.* (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* **263,** 1612–1615.

Kastern, W., Lang, F., and Kryspin-Sorensen, I. (1990). The genetics of insulin-dependent diabetes in the BB rat. *Curr. Top. Microbiol. Immunol.* **156,** 87–102.

Kerwin, J. F., Jr., Lancaster, J. R., Jr., and Feldman, P. L. (1995). Nitric oxide: A new paradigm for second messengers. *J. Med. Chem.* **38,** 4343–4362.

Kikutani, H., and Makino, S. (1992). The murine autoimmune diabetes model: NOD and related strains. *Adv. Immunol.* **51,** 285–322.

Kim, Y. M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Kleemann, R., Rothe, H., Kolb-Bachofen, V., Xie, Q. W., Nathan, C., *et al.* (1993). Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats. *FEBS Lett.* **328,** 9–12.

Kolb, H. (1997). Benign versus destructive insulitis. *Diabetes Metab. Rev.* **13,** 139–146.

Kolb, H., Worz-Pagenstert, U., Kleemann, R., Rothe, H., Rowsell, P., and Scott, F. W. (1996). Cytokine gene expression in the BB rat pancreas: Natural course and impact of bacterial vaccines. *Diabetologia* **39,** 1448–1454.

Kolb, H., Worz-Pagenstert, U., Kleemann, R., Rothe, H., Rowsell, P., *et al.* (1997). Insulin therapy of prediabetes suppresses TH1 associated gene expression in BB rat pancreas. *Autoimmunity* **26,** 1–6.

Kroncke, K. D., Kolb-Bachofen, V., Berschick, B., Burkart, V., and Kolb, H. (1991a). Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation. *Biochem. Biophys. Res. Commun.* **175,** 752–758.

Kroncke, K. D., Funda, J., Berschick, B., Kolb, H., and Kolb-Bachofen, V. (1991b). Macrophage cytotoxicity towards isolated rat islet cells: Neither lysis nor its protection by nicotinamide are β-cell specific. *Diabetologia* **34,** 232–238.

Kubes, P. (1995). Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. *Microcirculation* **2,** 235–244.

Kwon, G., Corbett, J. A., Rodi, C. P., Sullivan, P., and McDaniel, M. L. (1995). Interleukin-1 β-induced nitric oxide synthase expression by rat pancreatic β-cells: Evidence for the involvement of nuclear factor κ B in the signaling mechanism. *Endocrinology* **136,** 4790–4795.

Kwon, G., Xu, G., Marshall, C. A., and McDaniel, M. L. (1999). Tumor necrosis factor α-induced pancreatic β-cell insulin resistance is mediated by nitric oxide and prevented by 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 and aminoguanidine. A role for peroxisome proliferator-activated receptor γ activation and inos expression. *J. Biol. Chem.* **274,** 18702–18708.

Kwon, N. S., Lee, S. H., Choi, C. S., Kho, T., and Lee, H. S. (1994). Nitric oxide generation from streptozotocin. *FASEB J.* **8,** 529–533.

Lacy, P. E. (1994). The intraislet macrophage and type I diabetes. *Mt. Sinai J. Med.* **61,** 170–174.

Lacy, P. E., and Finke, E. H. (1991). Activation of intraislet lymphoid cells causes destruction of islet cells. *Am. J. Pathol.* **138,** 1183–1190.

Lafaille, J. J. (1998). The role of helper T cell subsets in autoimmune diseases. *Cytokine Growth Factor Rev.* **9,** 139–151.

Lancaster, J. R., Jr. (1997). A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* **1,** 18–30.

Larsen, C. M., Wadt, K. A., Juhl, L. F., Andersen, H. U., Karlsen, A. E., *et al.* (1998). Interleukin-1β-induced rat pancreatic islet nitric oxide synthesis requires both the p38 and extracellular signal-regulated kinase ½ mitogen-activated protein kinases. *J. Biol. Chem.* **273,** 15294–15300.

Lau, A., Ramanathan, S., and Poussier, P. (1998). Excessive production of nitric oxide by macrophages from DP-BB rats is secondary to the T-lymphopenic state of these animals. *Diabetes* **47,** 197–205.

Lebovitz, H. E. (1999). Type 2 diabetes: An overview. *Clin. Chem.* **45,** 1339–1345.

Lee, Y., Hirose, H., Ohneda, M., Johnson, J. H., McGarry, J. D., and Unger, R. H. (1994). Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: Impairment in adipocyte–β-cell relationships. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 10878–10882.

Lee, Y., Hirose, H., Zhou, Y. T., Esser, V., McGarry, J. D., and Unger, R. H. (1997). Increased lipogenic capacity of the islets of obese rats: A role in the pathogenesis of NIDDM. *Diabetes* **46,** 408–413.

Lefkowith, J. B., Jakschik, B. A., Stahl, P., and Needleman, P. (1987). Metabolic and functional alterations in macrophages induced by essential fatty acid deficiency. *J. Biol. Chem.* **262,** 6668–6675.

Lefkowith, J., Schreiner, G., Cormier, J., Handler, E. S., Driscoll, H. K., *et al.* (1990). Prevention of diabetes in the BB rat by essential fatty acid deficiency. Relationship between physiological and biochemical changes. *J. Exp. Med.* **171,** 729–743.

Li, J., and Billiar, T. R. (1999). The anti-apoptotic actions of nitric oxide in hepatocytes. *Cell Death Differ.* **6,** 952–955.

Like, A. A., Weringer, E. J., Holdash, A., McGill, P., Atkinson, D., and Rossini, A. A. (1985). Adoptive transfer of autoimmune diabetes mellitus in BioBreeding/Worcester (BB/W) inbred and hybrid rats. *J. Immunol.* **134,** 1583–1587.

Lindsay, R. M., Smith, W., Rossiter, S. P., McIntyre, M. A., Williams, B. C., and Baird, J. D. (1995). N^{ω}-nitro-L-arginine methyl ester reduces the incidence of IDDM in BB/E rats. *Diabetes* **44,** 365–368.

McConkey, D. J. (1998). Biochemical determinants of apoptosis and necrosis. *Toxicol. Lett.* **99,** 157–168.

McDaniel, M. L., Hughes, J. H., Wolf, B. A., Easom, R. A., and Turk, J. W. (1988). Descriptive and mechanistic considerations of interleukin 1 and insulin secretion. *Diabetes* **37,** 1311–1315.

McDaniel, M. L., Kwon, G., Hill, J. R., Marshall, C. A., and Corbett, J. A. (1996). Cytokines and nitric oxide in islet inflammation and diabetes. *Proc. Soc. Exp. Biol. Med.* **211,** 24–32.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.

Maggi, L. B., Sadeghi, H., Weigand, C., Scarim, A. L., Heitmeier, M. R., and Corbett, J. A. (2000). Anti-inflammatory actions of 15-D-$\Delta^{12,14}$-prostaglandin J2 and troglitazone: Evidence for heat shock-dependent and -independent inhibition of cytokine-induced iNOS expression. *Diabetes* in press.

Mamane, Y., Heylbroeck, C., Genin, P., Algarte, M., Servant, M. J., *et al.* (1999). Interferon regulatory factors: The next generation. *Gene* **237,** 1–14.

Mandrup-Poulsen, T. (1996). The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* **39,** 1005–1029.

Mandrup-Poulsen, T., Bendtzen, K., Nielsen, J. H., Bendixen, G., and Nerup, J. (1985). Cytokines cause functional and structural damage to isolated islets of Langerhans. *Allergy* **40,** 424–429.

Margulis, B. A., Sandler, S., Eizirik, D. L., Welsh, N., and Welsh, M. (1991). Liposomal delivery of purified heat-shock protein hsp70 into rat pancreatic islets as protection against interleukin 1β-induced impaired β-cell function. *Diabetes* **40,** 1418–1422.

Marre, M. (1999). Genetics and the prediction of complications in type 1 diabetes. *Diabetes Care* **22** (Suppl. 2), B53–B58.

Martin, E., Nathan, C., and Xie, Q. W. (1994). Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med.* **180,** 977–984.

Masutani, M., Suzuki, H., Kamada, N., Watanabe, M., Ueda, O., *et al.* (1999). Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 2301–2304.

Mauricio, D., and Mandrup-Poulsen, T. (1998). Apoptosis and the pathogenesis of IDDM: A question of life and death. *Diabetes* **47,** 1537–1543.

Medici, F., Hawa, M., Ianari, A., Pyke, D. A., and Leslie, R. D. (1999). Concordance rate for type II diabetes mellitus in monozygotic twins: Actuarial analysis. *Diabetologia* **42,** 146–150.

Misler, S., Barnett, D. W., Gillis, K. D., and Pressel, D. M. (1992). Electrophysiology of stimulus–secretion coupling in human β-cells. *Diabetes* **41,** 1221–1228.

Mizuno, N., Yoshitomi, H., Ishida, H., Kuromi, H., Kawaki, J., *et al.* (1998). Altered bcl-2 and bax expression and intracellular Ca^{2+} signaling in apoptosis of pancreatic cells and the impairment of glucose-induced insulin secretion. *Endocrinology* **139,** 1429–1439.

Must, A., Spadano, J., Coakley, E. H., Field, A. E., Colditz, G., and Dietz, W. H. (1999). The disease burden associated with overweight and obesity. *JAMA* **282,** 1523–1529.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6,** 3051–3064.

Nicoletti, F., Di Marco, R., Barcellini, W., Magro, G., Schorlemmer, H. U. *et al.* (1994). Protection from experimental autoimmune diabetes in the non-obese diabetic mouse with soluble interleukin-1 receptor. *Eur. J. Immunol.* **24,** 1843–1847.

Nicoletti, F., Zaccone, P., Di Marco, R., Magro, G., Grasso, S., *et al.* (1998). Paradoxical antidiabetogenic effect of γ-interferon in DP-BB rats. *Diabetes* **47,** 32–38.

Nicotera, P., Leist, M., Single, B., and Volbracht, C. (1999). Execution of apoptosis: Converging or diverging pathways? *Biol. Chem.* **380,** 1035–1040.

Olmos, P., A'Hern, R., Heaton, D. A., Millward, B. A., Risley, D., *et al.* (1988). The significance of the concordance rate for type 1 (insulin-dependent) diabetes in identical twins. *Diabetologia* **31,** 747–750.

Oschilewski, U., Kiesel, U., and Kolb, H. (1985). Administration of silica prevents diabetes in BB-rats. *Diabetes* **34,** 197–199.

Pakala, S. V., Chivetta, M., Kelly, C. B., and Katz, J. D. (1999). In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor α. *J. Exp. Med.* **189,** 1053–1062.

Pathak, D. S., and Burke, T. A. (1998). Economic evaluations and diabetes. *Pharm. Pract. Manag. Q.* **17,** 17–24.

Pavlovic, D., Chen, M. C., Bouwens, L., Eizirik, D. L., and Pipeleers, D. (1999a). Contribution of ductal cells to cytokine responses by human pancreatic islets. *Diabetes* **48,** 29–33.

Pavlovic, D., Chen, M. C., Gysemans, C. A., Mathieu, C., and Eizirik, D. L. (1999b). The role of interferon regulatory factor-1 in cytokine-induced mRNA expression and cell death in murine pancreatic β-cells. *Eur. Cytokine. Netw.* **10,** 403–412.

Petrova, T. V., Akama, K. T., and Van Eldik, L. J. (1999). Cyclopentenone prostaglandins suppress activation of microglia: Down-regulation of inducible nitric-oxide synthase by 15-deoxy-Δ 12,14-prostaglandin J2. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 4668–4673.

Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., *et al.* (1996). Leptin receptor missense mutation in the fatty Zucker rat. *Nat. Genet.* **13,** 18–19.

Pieper, A. A., Brat, D. J., Krug, D. K., Watkins, C. C., Gupta, A., *et al.* (1999). Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 3059–3064.

Pilstrom, B., Bjork, L., and Bohme, J. (1995). Demonstration of a TH1 cytokine profile in the late phase of NOD insulitis. *Cytokine* **7,** 806–814.

Pilstrom, B., Bjork, L., and Bohme, J. (1997). Monokine-producing cells predominate in the recruitment phase of NOD insulitis while cells producing Th1-type cytokines characterize the effector phase. *J. Autoimmun.* **10,** 147–155.

Pipeleers, D. G., in't Veld, P. A., Van de Winkel, M., Maes, E., Schuit, F. C., and Gepts, W. (1985). A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* **117,** 806–816.

Proud, C. G. (1995). PKR: A new name and new roles. *Trends Biochem. Sci.* **20,** 241–246.

Rabinovitch, A. (1998). An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus. *Diabetes Metab. Rev.* **14,** 129–151.

Rabinovitch, A., and Suarez-Pinzon, W. L. (1998). Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. *Biochem. Pharmacol.* **55,** 1139–1149.

Rabinovitch, A., Suarez-Pinzon, W. L., Shi, Y., Morgan, A. R., and Bleackley, R. C. (1994a). DNA fragmentation is an early event in cytokine-induced islet β-cell destruction. *Diabetologia* **37,** 733–738.

Rabinovitch, A., Suarez-Pinzon, W. L., Strynadka, K., Schulz, R., Lakey, J. R., *et al.* (1994b). Human pancreatic islet β-cell destruction by cytokines is independent of nitric oxide production. *J. Clin. Endocrinol. Metab.* **79,** 1058–1062.

Rabinovitch, A., Suarez-Pinzon, W., El-Sheikh, A., Sorensen, O., and Power, R. F. (1996a). Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: Expression of Th1 cytokines correlates with β-cell destructive insulitis and IDDM. *Diabetes* **45,** 749–754.

Rabinovitch, A., Suarez-Pinzon, W. L., and Sorensen, O. (1996b). Interleukin 12 mRNA expression in islets correlates with β-cell destruction in NOD mice. *J. Autoimmun.* **9,** 645–651.

Rabinovitch, A., Suarez-Pinzon, W. L., Sorensen, O., and Bleackley, R. C. (1996c). Inducible nitric oxide synthase (iNOS) in pancreatic islets of nonobese diabetic mice: Identification of iNOS-expressing cells and relationships to cytokines expressed in the islets. *Endocrinology* **137,** 2093–2099.

Radons, J., Heller, B., Burkle, A., Hartmann, B., Rodriguez, M. L., *et al.* (1994). Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD^+ depletion. *Biochem. Biophys. Res. Commun.* **199,** 1270–1277.

Rahier, J., Goebbels, R. M., and Henquin, J. C. (1983). Cellular composition of the human diabetic pancreas. *Diabetologia* **24,** 366–371.

Rathmell, J. C., and Thompson, C. B. (1999). The central effectors of cell death in the immune system. *Annu. Rev. Immunol.* **17,** 781–828.

Reddy, S., Kaill, S., Poole, C. A., and Ross, J. (1997). Inducible nitric oxide synthase in pancreatic islets of the non-obese diabetic mouse: A light and confocal microscopical study of its ontogeny, co-localization and up-regulation following cytokine administration. *Histochem. J.* **29,** 53–64.

Reddy, S., Yip, S., Karanam, M., Poole, C. A., and Ross, J. M. (1999). An immunohistochemical study of macrophage influx and the co-localization of inducible nitric oxide synthase in the pancreas of non-obese diabetic (NOD) mice during disease acceleration with cyclophosphamide. *Histochem. J.* **31,** 303–314.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998). The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. *Nature* **391,** 79–82.

Ricote, M., Huang, J. T., Welch, J. S., and Glass, C. K. (1999). The peroxisome proliferator-activated receptor (PPARγ) as a regulator of monocyte/macrophage function. *J. Leukocyte Biol.* **66,** 733–739.

Robertson, H. D., and Mathews, M. B. (1996). The regulation of the protein kinase PKR by RNA. *Biochimie* **78,** 909–914.

Rossini, A. A., Mordes, J. P., and Like, A. A. (1985). Immunology of insulin-dependent diabetes mellitus. *Annu. Rev. Immunol.* **3,** 289–320.

Rothe, H., and Kolb, H. (1999). Strategies of protection from nitric oxide toxicity in islet inflammation. *J. Mol. Med.* **77,** 40–44.

Rothe, H., Burkart, V., Faust, A., and Kolb, H. (1996). Interleukin-12 gene expression is associated with rapid development of diabetes mellitus in non-obese diabetic mice. *Diabetologia* **39,** 119–122.

Rothe, H., Hibino, T., Itoh, Y., Kolb, H., and Martin, S. (1997a). Systemic production of interferon-γ inducing factor (IGIF) versus local IFN-γ expression involved in the development of Th1 insulitis in NOD mice. *J. Autoimmun.* **10,** 251–256.

Rothe, H., O'Hara, R. M., Jr., Martin, S., and Kolb, H. (1997b). Suppression of cyclophosphamide induced diabetes development and pancreatic Th1 reactivity in NOD mice treated with the interleukin (IL)-12 antagonist IL-12(p40)2. *Diabetologia* **40,** 641–646.

Rothe, H., Hausmann, A., Casteels, K., Okamura, H., Kurimoto, M., *et al.* (1999). IL-18 inhibits diabetes development in nonobese diabetic mice by counterregulation of Th1-dependent destructive insulitis. *J. Immunol.* **163,** 1230–1236.

Saldeen, J., and Welsh, N. (1994). Interleukin-1β induced activation of NF-κB in insulin producing RINm5F cells is prevented by the protease inhibitor N^{α}-*p*-tosyl-L-lysine chloromethylketone. *Biochem. Biophys. Res. Commun.* **203,** 149–155.

Samali, A., Gorman, A. M., and Cotter, T. G. (1996). Apoptosis—The story so far. *Experientia* **52,** 933–941.

Sandler, S., Eizirik, D. L., Svensson, C., Strandell, E., Welsh, M., and Welsh, N. (1991). Biochemical and molecular actions of interleukin-1 on pancreatic β-cells. *Autoimmunity* **10,** 241–253.

Satoh, J., Seino, H., Shintani, S., Tanaka, S., Ohteki, T., *et al.* (1990). Inhibition of type 1 diabetes in BB rats with recombinant human tumor necrosis factor-α. *J. Immunol.* **145,** 1395–1399.

Scarim, A. L., Heitmeier, M. R., and Corbett, J. A. (1997). Irreversible inhibition of metabolic function and islet destruction after a 36-hour exposure to interleukin-1β. *Endocrinology* **138,** 5301–5307.

Scarim, A. L., Heitmeier, M. R., and Corbett, J. A. (1998). Heat shock inhibits cytokine-induced nitric oxide synthase expression by rat and human islets. *Endocrinology* **139,** 5050–5057.

Schindler, C. (1999). Cytokines and JAK–STAT signaling. *Exp. Cell Res.* **253,** 7–14.

Schreiner, G. F., Flye, W., Brunt, E., Korber, K., and Lefkowith, J. B. (1988). Essential fatty acid depletion of renal allografts and prevention of rejection. *Science* **240,** 1032–1033.

Schwab, E., Burkart, V., Freytag, G., Kiesel, U., and Kolb, H. (1986). Inhibition of immune-mediated low-dose streptozotocin diabetes by agents which reduce vascular permeability. *Immunopharmacology* **12,** 17–21.

Setum, C. M., Serie, J. R., and Hegre, O. D. (1991). Confocal microscopic analysis of the nonendocrine cellular component of isolated adult rat islets of Langerhans. *Transplantation* **51,** 1131–1133.

Shimabukuro, M., Ohneda, M., Lee, Y., and Unger, R. H. (1997a). Role of nitric oxide in obesity-induced β cell disease. *J. Clin. Invest.* **100,** 290–295.

Shimabukuro, M., Koyama, K., Chen, G., Wang, M. Y., Trieu, F., *et al.* (1997b). Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 4637–4641.

Shimabukuro, M., Koyama, K., Lee, Y., and Unger, R. H. (1997c). Leptin- or troglitazone-induced lipopenia protects islets from interleukin 1β cytotoxicity. *J. Clin. Invest.* **100,** 1750–1754.

Shimabukuro, M., Zhou, Y. T., Lee, Y., and Unger, R. H. (1998). Troglitazone lowers islet fat and restores β cell function of Zucker diabetic fatty rats. *J. Biol. Chem.* **273,** 3547–3550.

Signore, A., Pozzilli, P., Gale, E. A., Andreani, D., and Beverley, P. C. (1989). The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia* **32,** 282–289.

Sobel, D. O., Newsome, J., Ewel, C. H., Bellanti, J. A., Abbassi, V., *et al.* (1992). Poly I:C induces development of diabetes mellitus in BB rat. *Diabetes* **41,** 515–520.

Sobel, D. O., Ewel, C. H., Zeligs, B., Abbassi, V., Rossio, J., and Bellanti, J. A. (1994). Poly I:C induction of α-interferon in the diabetes-prone BB and normal Wistar rats. Dose–response relationships. *Diabetes* **43,** 518–522.

Sobel, D. O., Creswell, K., Yoon, J. W., and Holterman, D. (1998). α-Interferon administration paradoxically inhibits the development of diabetes in BB rats. *Life Sci.* **62,** 1293–1302.

Southern, C., Schulster, D., and Green, I. C. (1990). Inhibition of insulin secretion by interleukin-1-β and tumour necrosis factor-α via an -L-arginine-dependent nitric oxide generating mechanism. *FEBS Lett.* **276,** 42–44.

Spiegelman, B. M. (1998). PPAR-γ: Adipogenic regulator and thiazolidinedione receptor. *Diabetes* **47,** 507–514.

Spiegelman, B. M., Hu, E., Kim, J. B., and Brun, R. (1997). PPAR γ and the control of adipogenesis. *Biochimie* **79,** 111–112.

Spinas, G. A., Laffranchi, R., Francoys, I., David, I., Richter, C., and Reinecke, M. (1998). The early phase of glucose-stimulated insulin secretion requires nitric oxide. *Diabetologia* **41,** 292–299.

Steiner, L., Kroncke, K., Fehsel, K., and Kolb-Bachofen, V. (1997). Endothelial cells as cytotoxic effector cells: Cytokine-activated rat islet endothelial cells lyse syngeneic islet cells via nitric oxide. *Diabetologia* **40,** 150–155.

Stephens, L. A., Thomas, H. E., and Kay, T. W. (1997). Protection of NIT-1 pancreatic β-cells from immune attack by inhibition of NF-κB. *J. Autoimmun.* **10,** 293–298.

Stevens, R. B., Sutherland, D. E., Ansite, J. D., Saxena, M., Rossini, T. J., *et al.* (1997). Insulin down-regulates the inducible nitric oxide synthase pathway: Nitric oxide as cause and effect of diabetes? *J. Immunol.* **159,** 5329–5335.

Strandell, E., Buschard, K., Saldeen, J., and Welsh, N. (1995). Interleukin-1β induces the expression of hsp70, heme oxygenase and Mn-SOD in FACS-purified rat islet β-cells, but not in α-cells. *Immunol. Lett.* **48,** 145–148.

Suschek, C., Fehsel, K., Kroncke, K. D., Sommer, A., and Kolb-Bachofen, V. (1994). Primary cultures of rat islet capillary endothelial cells. Constitutive and cytokine-inducible macrophagelike nitric oxide synthases are expressed and activities regulated by glucose concentration. *Am. J. Pathol.* **145,** 685–695.

Suschek, C. V., Bonmann, E., and Kolb-Bachofen, V. (1999). A regulatory defect of constitutive NO synthase in islet endothelial cells correlates with probability of disease manifestation in BBdp rats. *Diabetologia* **42,** 457–464.

Swidan, S. Z., and Montgomery, P. A. (1998). Effect of blood glucose concentrations on the development of chronic complications of diabetes mellitus. *Pharmacotherapy* **18,** 961–972.

Takahashi, K., Satoh, J., Seino, H., Zhu, X. P., Sagara, M., *et al.* (1993). Prevention of type I diabetes with lymphotoxin in BB rats. *Clin. Immunol. Immunopathol.* **69,** 318–323.

Takamura, T., Kato, I., Kimura, N., Nakazawa, T., Yonekura, H., *et al.* (1998). Transgenic mice overexpressing type 2 nitric-oxide synthase in pancreatic β cells develop insulin-dependent diabetes without insulitis. *J. Biol. Chem.* **273,** 2493–2496.

Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., *et al.* (1998). Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* **273,** 15148–15156.

Thomas, H. E., Darwiche, R., Corbett, J. A., and Kay, T. W. (1999). Evidence that β cell death in the nonobese diabetic mouse is Fas independent. *J. Immunol.* **163,** 1562–1569.

Todd, J. A. (1999). From genome to aetiology in a multifactorial disease, type 1 diabetes. *BioEssays* **21,** 164–174.

Trissler, R. J. (1999). Type 2 diabetes on the rise in children: Is the American lifestyle coming home to roost? *J. Am. Diet Assoc.* **99,** 1354.

Turk, J., Corbett, J. A., Ramanadham, S., Bohrer, A., and McDaniel, M. L. (1993). Biochemical evidence for nitric oxide formation from streptozotocin in isolated pancreatic islets. *Biochem. Biophys. Res. Commun.* **197,** 1458–1464.

Unger, R. H. (1995). Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* **44,** 863–870.

Vaidya, S., Somers, E. P., Wright, S. D., Detmers, P. A., and Bansal, V. S. (1999). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 inhibits the β_2-integrin-dependent oxidative burst: Involvement of a mechanism distinct from peroxisome proliferator-activated receptor γ ligation. *J. Immunol.* **163,** 6187–6192.

Vamecq, J., and Latruffe, N. (1999). Medical significance of peroxisome proliferator-activated receptors. *Lancet* **354,** 141–148.

Vanden Heuvel, J. P. (1999). Peroxisome proliferator-activated receptors: A critical link among fatty acids, gene expression and carcinogenesis. *J. Nutr.* **129,** 575S–580S.

von Herrath, M. G., Homann, D., Gairin, J. E., and Oldstone, M. B. (1997). Pathogenesis and treatment of virus-induced autoimmune diabetes: Novel insights gained from the RIP-LCMV transgenic mouse model. *Biochem. Soc. Trans.* **25,** 630–635.

Vouldoukis, I., Becherel, P. A., Riveros-Moreno, V., Arock, M., da Silva, O., *et al.* (1997). Interleukin-10 and interleukin-4 inhibit intracellular killing of *Leishmania infantum* and *Leishmania major* by human macrophages by decreasing nitric oxide generation. *Eur. J. Immunol.* **27,** 860–865.

Wang, B., Gonzalez, A., Benoist, C., and Mathis, D. (1996). The role of $CD8^+$ T cells in the initiation of insulin-dependent diabetes mellitus. *Eur. J. Immunol.* **26,** 1762–1769.

Wang, Y., and Marsden, P. A. (1995). Nitric oxide synthases: Gene structure and regulation. *Adv. Pharmacol.* **34,** 71–90.

Welsh, M., Welsh, N., Bendtzen, K., Mares, J., Strandell, E., *et al.* (1995). Comparison of mRNA contents of interleukin-1β and nitric oxide synthase in pancreatic islets isolated from female and male nonobese diabetic mice. *Diabetologia* **38,** 153–160.

Welsh, N., and Sandler, S. (1992). Interleukin-1β induces nitric oxide production and inhibits the activity of aconitase without decreasing glucose oxidation rates in isolated mouse pancreatic islets. *Biochem. Biophys. Res. Commun.* **182,** 333–340.

Welsh, N. (1996). Interleukin-1β-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH_2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. *J. Biol. Chem.* **271,** 8307–8312.

Welsh, N., Eizirik, D. L., Bendtzen, K., and Sandler, S. (1991). Interleukin-1β-induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. *Endocrinology* **129,** 3167–3173.

Welsh, N., Eizirik, D. L., and Sandler, S. (1994). Nitric oxide and pancreatic β-cell destruction in insulin dependent diabetes mellitus: Don't take NO for an answer. *Autoimmunity* **18,** 285–290.

Welsh, N., Margulis, B., Borg, L. A., Wiklund, H. J., Saldeen, J., *et al.* (1995). Differences in the expression of heat-shock proteins and antioxidant enzymes between human and rodent pancreatic islets: Implications for the pathogenesis of insulin-dependent diabetes mellitus. *Mol. Med.* **1,** 806–820.

Wicker, L. S., Miller, B. J., and Mullen, Y. (1986). Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes* **35,** 855–860.

Williams, B. R. (1997). Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.* **25,** 509–513.

Wilson, C. A., Jacobs, C., Baker, P., Baskin, D. G., Dower, S., *et al.* (1990). IL-1β modulation of spontaneous autoimmune diabetes and thyroiditis in the BB rat. *J. Immunol.* **144,** 3784–3788.

Wright, J. R., Jr., and Lacy, P. E. (1989). Silica prevents the induction of diabetes with complete Freund's adjuvant and low-dose streptozotocin in rats. *Diabetes Res.* **11,** 51–54.

Wright, J. R., Jr., Lefkowith, J. B., Schreiner, G., and Lacy, P. E. (1988). Essential fatty acid deficiency prevents multiple low-dose streptozotocin-induced diabetes in CD-1 mice. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6137–6141.

Wu, G. (1995). Nitric oxide synthesis and the effect of aminoguanidine and N^G-monomethyl-L-arginine on the onset of diabetes in the spontaneously diabetic BB rat. *Diabetes* **44,** 360–364.

Xie, Q. W., Whisnant, R., and Nathan, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J. Exp. Med.* **177,** 1779–1784.

Yamamoto, H., Uchigata, Y., and Okamoto, H. (1981). Streptozocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature* **294,** 284–286.

Yang, X. D., and McDevitt, H. O. (1994). Role of TNF-α in the development of autoimmunity and the pathogenesis of insulin-dependent diabetes mellitus in NOD mice. *Circ. Shock* **43,** 198–201.

Yang, X. D., Tisch, R., Singer, S. M., Cao, Z. A., Liblau, R. S., *et al.* (1994). Effect of tumor necrosis factor α on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J. Exp. Med.* **180,** 995–1004.

Yoon, J. W. (1990). The role of viruses and environmental factors in the induction of diabetes. *Curr. Top. Microbiol. Immunol.* **164,** 95–123.

Yoon, J. W. (1992). Induction and prevention of type 1 diabetes mellitus by viruses. *Diabetes Metab.* **18,** 378–386.

Yoon, J. W., McClintock, P. R., Bachurski, C. J., Longstreth, J. D., and Notkins, A. L. (1985). Virus-induced diabetes mellitus. No evidence for immune mechanisms in the destruction of β-cells by the D-variant of encephalomyocarditis virus. *Diabetes* **34,** 922–925.

Yoon, J. W., Kim, C. J., Pak, C. Y., and McArthur, R. G. (1987). Effects of environmental factors on the development of insulin-dependent diabetes mellitus. *Clin. Invest. Med.* **10,** 457–469.

Yoon, J. W., Ihm, S. H., and Kim, K. W. (1989). Viruses as a triggering factor of type 1 diabetes and genetic markers related to the susceptibility to the virus-associated diabetes. *Diabetes Res. Clin. Pract.* **7**(Suppl. 1), S47–S58.

Yoon, J. W., Jun, H. S., and Santamaria, P. (1998). Cellular and molecular mechanisms for the initiation and progression of β cell destruction resulting from the collaboration between macrophages and T cells. *Autoimmunity* **27,** 109–122.

Zhou, Y. P., Teng, D., Dralyuk, F., Ostrega, D., Roe, M. W., *et al.* (1998). Apoptosis in insulin-secreting cells. Evidence for the role of intracellular Ca^{2+} stores and arachidonic acid metabolism. *J. Clin. Invest.* **101,** 1623–1632.

Zielasek, J., Burkart, V., Naylor, P., Goldstein, A., Kiesel, U., and Kolb, H. (1990). Interleukin-2-dependent control of disease development in spontaneously diabetic BB rats. *Immunology* **69,** 209–214.

Zipris, D. (1996). Evidence that Th1 lymphocytes predominate in islet inflammation and thyroiditis in the BioBreeding (BB) rat. *J. Autoimmun.* **9,** 315–319.

CHAPTER 50

Role of Inducible Nitric Oxide Synthase in Autoimmune or Other Immune-Mediated Diseases

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IN AUTOIMMUNE OR IMMUNE-MEDIATED DISEASES, THE IMMUNE DEFENSE SYSTEM TURNS AGAINST ITS OWN ORGANISM, SOMETIMES LEADING TO A CHRONIC AND LONG-LASTING IMBALANCE OF IMMUNE REACTIONS. IMMUNE DEFENSES ARE EITHER TH1-TYPE RESPONSES IN CONJUNCTION WITH PREDOMINANTLY CELLULAR IMMUNE REACTIONS, OR TH2-LIKE REACTIONS, MAINLY ASSOCIATED WITH HUMORAL RESPONSES. EXPRESSION OF THE INDUCIBLE NO SYNTHASE (INOS) ALWAYS CONCURS WITH A TH1-TYPE IMMUNE REACTION, BUT THE IMPACT OF NO ON IMMUNE FUNCTION AND ITS REGULATION IS COMPLEX AND OFTEN APPEARS CONTRADICTORY. NO CAN EXERT CYTOTOXIC ACTIVITY BUT MAY ALSO PROTECT CELLS FROM A TOXIC INSULT. IT IS KNOWN TO INDUCE APOPTOSIS, BUT IT CAN ALSO EXHIBIT PROMINENT ANTIAPOPTOTIC ACTIVITY. NO ACTS LIKE AN ANTIOXIDANT BUT MAY ALSO COMPROMISE THE CELLULAR REDOX STATE VIA OXIDATION OF THIOLS SUCH AS GLUTATHIONE. NO MAY ACTIVATE SPECIFIC SIGNAL TRANSDUCTION PATHWAYS BUT IS ALSO REPORTED TO INHIBIT THEM, AND LAST BUT NOT LEAST NO MAY ACTIVATE BUT ALSO INHIBIT GENE TRANSCRIPTION. THE SITUATION APPEARS EVEN MORE COMPLICATED, AS, DEPENDING ON ITS CONCENTRATION, NO MAY REACT WITH O_2 OR O_2^- TO YIELD SPECIES THAT ARE MUCH MORE REACTIVE AND EXHIBIT A BROADER CHEMICAL REACTION SPECTRUM THAN NO ITSELF. PROLONGED INOS ACTIVITY MAY THUS RESULT IN DIRECT CYTOTOXICITY AND LOCAL TISSUE DESTRUCTION, BUT ON THE OTHER HAND MAY ALSO SERVE TO DOWNREGULATE TH1 RESPONSES, WHICH SERVES THE IMPORTANT TASK OF LIMITING THE EXTENT OF INFLAMMATION. IN ADDITION, INOS ACTIVITY MAY BE PROTECTIVE. THUS, THE ACTIVITY OF INOS EXPRESSED DURING INFLAMMATORY REACTIONS HAS TO BE EVALUATED IN THE CONTEXT OF TIMING AND DURATION OF NO SYNTHESIS AS WELL AS STAGES AND SPECIFIC EVENTS IN INFLAMMATION.

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Introduction

In this chapter we will outline our current understanding of the role inducible nitric oxide synthase (iNOS) plays in autoimmune or immune-mediated diseases. Our perception of the impact of NO in these illnesses has dramatically changed. We now have a much broader understanding of the role NO plays as a regulatory, protective, and destructive agent in autoimmune or immune-mediated diseases.

Definition of Autoimmunity

In order to understand the proven or possible roles of NO in autoimmune disease, a short definition of these processes is necessary. Figure 1 lists the key characteristics of autoimmunity, a process Paul Ehrlich envisaged as *Horror autotoxicus.*

First and above all, in autoimmune and immune-mediated diseases the immune defense system turns against its own body and recognizes one or several molecules as possibly hazardous, that is, it recognizes ''self.'' This is not dangerous per se, and it often appears to occur during local infections or other short lasting diseases. Normally, these anti-self reactivities will be rapidly downregulated and abolished. Second, the anti-self attacks have to be chronic, long-term processes in order to mediate clinically recognizable disease. Thus, the normally operating counterbalancing forces, leading to time-restricted immune responses, fail. The immune system remains locked in one state of aggression.

All of the different immune reactions operative in autoimmunity are those that work in normal immune defenses. We have to remind ourselves that there is not a special means of immune attack at work in autoimmune reactions, but rather a long-lasting imbalance of a normal response.

One important feature to distinguish autoimmune processes from other diseases is the ability to transfer the disease by antibodies or lymphocytes, for instance, to nude mice, which cannot mount an immune attack themselves. As this is not always proven or may not always be possible for various reasons, we prefer to call diseases thought to be autoimmune, but lacking this final proof, immune-mediated diseases.

One reason for such a failure in immune balance can be seen in local long-lasting and subclinical infections by virus or bacteria. Such an infection can be regarded as a trigger for autoimmune processes. Other factors contributing to the onset of autoimmunity are found in our environment. Thus, certain foods or exposure to ultraviolet irradiation can be regarded as environmental conditions adding to the risk for developing autoimmune diseases. However, not every person suffering from a chronic infection or exposed to environmental hazards will develop autoimmune reactions; rather, a genetic predisposition is essential for the development of disease. These reside in the major histocompatibility complex (MHC), which regulates immune responses, but genes coding for other features of normal body function outside the MHC loci are also often involved. Important risk factors contributing to the onset of self-destructive immunity are listed in Fig. 2.

Autoimmunity (Horror autotoxicus)

- ➨**Specific immune reaction against "SELF"**
- ➨**Chronic and concomitant destruction of tissue**
- ➨**Principal mechanisms are identical with those used in protective immunity**
- ➨**Adoptive transfer with antibodies or reactive T-lymphocytes (not always possible)**

Figure 1 Main features of autoimmune diseases. Autoimmune destruction of body tissue had been envisaged by Paul Ehrlich, who named his vision *Horror autotoxicus.*

Introducing the Th1/Th2 Balance

Immune defense commences in two different ways. We have long known that infections will be fought either by producing specific antibodies or by using cellular defense mechanisms. We now understand that these two different ways of immune attack can be distinguished by the different cytokines expressed. These different sets of communication-mediating proteins serve as additional signals either mounting and helping cellular defenses or steering the immune responses toward antibody production. The two states are named the Th1 response, corresponding to a cytokine profile typically found in inflammatory reactions, and the Th2 reaction, which is mainly associated with a cytokine profile driving B-lymphocyte differentiation. A list of the most prominent cytokines found in the two different states is provided in Fig. 3.

In accord with the earlier statement that autoimmune processes are normal immune reactions, albeit directed against self, we find both states of immune reaction in different autoimmune diseases. Systemic lupus erythematosus is an autoantibody-mediated disease, in contrast to insulin-dependent, ''autoimmune'' diabetes. In animals developing insulin-dependent autoimmune diabetes mellitus (also termed type 1 diabetes), organ destruction is invariably associated

Risk Factors

- ➨**Genetic risk factors, predominately in the MHC loci**
- ➨**Additional risk factors in somatic genes**
- ➨**Environmental factors**
- ➨**Long-lasting sub-clinical or fulminant infections**

Figure 2 Main risks that contribute to manifestation of autoimmune diseases.

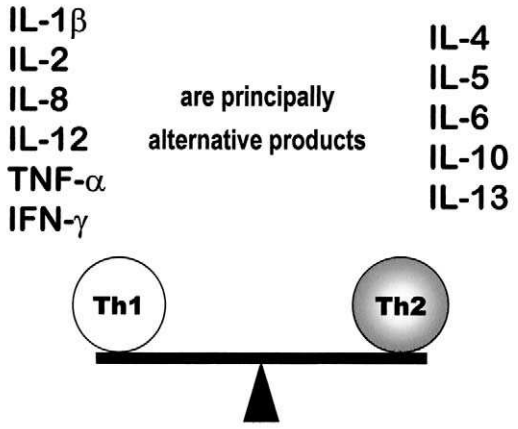


Figure 3 Immune reactions can take two different routes. One type of reaction is generally named the Th1 type or inflammatory reaction that will commence by cellular reactions mostly, and during this state the cytokines listed on the left-hand side are produced. The alternative, called a Th2 type of reaction, will use antibody production predominantly and is characterized by the cytokine profile listed on the right-hand side.

with a Th1 cytokine profile. In type 1 diabetes the importance of the Th1 immune regulatory state can be demonstrated most impressively, as shown in Fig. 4. Here, in a mouse developing this disease, lymphocytes are first found around the insulin-producing islets of Langerhans in the pancreas (Fig. 4A, B). At this stage we find mostly a local expression of Th2 cytokines, and animals can remain at this stage without ever becoming diabetic. If a conversion toward a Th1 cytokine profile occurs, lymphocytes start to infiltrate the islet, and organ destruction and manifestation of the disease will result (Fig. 4C, D). This is also associated with expression of iNOS in the infiltrate. However, in human disease processes, and also in some animal models for various diseases, this Th1/Th2 imbalance is not an absolute one, and the reverse cytokines may also be expressed; they are either inefficient in counterbalancing or are time-restricted in a vain attempt to counterbalance.

After having introduced the main topics that help us to understand the immune processes at work in autoimmunity, we will now look into the role of iNOS and the local production of NO in autoimmune diseases.

Expression of iNOS as a Marker for a Th1 Cytokine Expression Profile

Expression of iNOS has been found in human immune-mediated or autoimmune diseases such as rheumatoid arthritis and multiple sclerosis, as well as in a variety of chronically inflammatory diseases of the airways, bowel, skin, blood vessels, heart, kidney, apex of the teeth, and other organs of the body (see Table I). Owing to ethical problems, data do not exist concerning iNOS expression in the most prevalent human immune-mediated disease, type 1 diabetes.

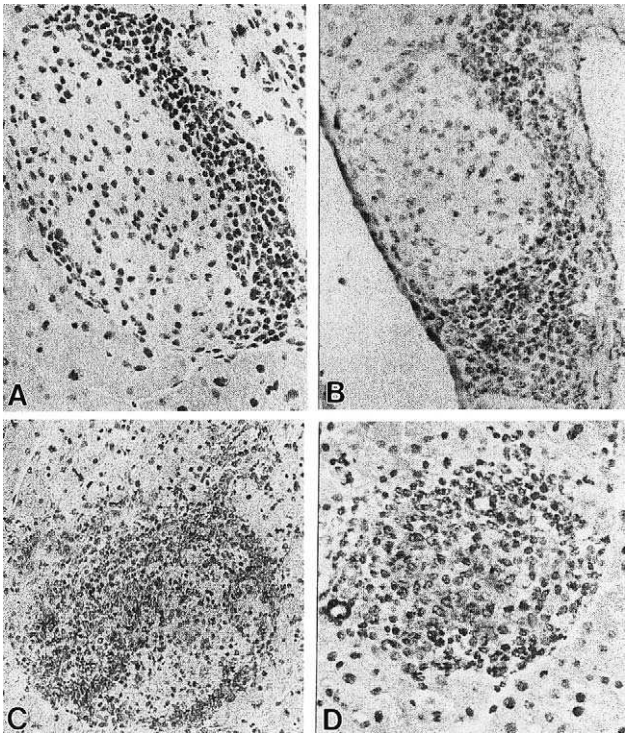


Figure 4 Autoimmunity is associated with local imbalance of the Th1/Th2 reactions. For example, in animals developing insulin-dependent diabetes, two local states of immune reaction occur. (A and B): Islets are often found with surrounding infiltrate that is not in a Th1 state [no γ-interferon (IFN-γ) (A) nor iNOS (B) expression seen], and animals can stay this way without disease; therefore, this state is also called benign insulitis. (C and D): When immunocytes start to invade the islet and destroy the insulin-producing cells, a conversion to the Th1 state is found, as depicted by strong expression of IFN-γ (in C) and iNOS (in D). This state is also called destructive insulitis and will lead to disease manifestation. See color insert. (Micrographs courtesy of Hubert Kolb, Düsseldorf, Germany.)

However, studies of animals developing this disease unequivocally show a role of iNOS expression for disease progression and manifestation (see Fig. 4). In all diseases associated with iNOS expression, as listed in Table I, we find a local cytokine expression profile that invariably correlates with the presence of the proinflammatory Th1-type cytokines (also compare Table I to cytokine list in Fig. 3). Although localization of iNOS immunoreactivity varies somewhat, as it is sometimes found in macrophages or in macrophage-like cells, in most cases it is associated with epithelial cells around inflammatory foci. Despite the ever-growing list of pathological conditions with iNOS expression, it is not yet known whether iNOS expression is only an epiphenomenon, due to the presence of the proinflammatory cytokines, or whether iNOS activity serves important roles. The NO produced by macrophages and epithelial cells may predominantly contribute to local destruction of tissue. Alternatively, NO can also serve to limit inflammation or local overshooting immune reactions and thus limit tissue destruction during Th1-driven immune reactions, as will be outlined in the next two sections.

Table I Local Th-1 Cytokine Production in Various Human Diseases Is Invariably Associated with iNOS Expression

Disease	Predominant cytokine profile[a]	Localization of iNOS[b]
Rheumatoid arthritis	IL-1, IL-6, IL-8, TNF-α, GM-CSF	Synovial lining cells, EC, mononuclear cells, fibroblasts, VSMC
Multiple sclerosis	IL-1, IL-2, IL-6, TNF-α, IFN-γ	Mϕ, microglia, astrocytes
Sjögren's syndrome	IL-1β, IL-6, TNF-α, IFN-γ	Acinar and ductal epithelial cells
Asthma	IL-1β, IL-8, TNF-α, GM-CSF	Epithelium, EC, inflammatory cells
Bronchiectasis	IL-1β, IL-8, TNF-α	Mϕ
Idiopathic pulmonary fibrosis	IL-1β, IL-6, IL-8, TNF-α, IFN-γ, TGF-β	Mϕ, neutrophils, airway and alveolar epithelium
Ulcerative colitis	IL-1, TNF-α, IFN-γ	Epithelial cells, inflammatory infiltrate
Crohn's disease	IL-1, TNF-α, IFN-γ	Epithelial cells, inflammatory infiltrate
Necrotizing enterocolitis	IL-6, TNF-α, IFN-γ	Epithelial cells
Celiac disease	IL-2, IL-6, TNF-α, IFN-γ	Epithelial cells, Mϕ
Psoriasis	IL-1β, IL-8	Keratinocytes, EC, inflammatory infiltrate
Cutaneous lupus erythematosus	IL-1β, IL-6, TNF-α	Basal epidermal layer, EC, inflammatory infiltrate
Contact dermatitis	IL-1, TNF-α, IFN-γ	EC, inflammatory cells
Atherosclerotic plaques	IL-1, IL-6, IL-12, TNF-α, IFN-γ	Mϕ, foam cells, VSMC
Dilated cardiomyopathy	IL-6, IL-8, TNF-α	Myocytes, EC, VSMC, skeletal muscle
Glomerulonephritis	TNF-α, IFN-γ	Mϕ, mesangial cells, epithelial cells
Periapical periodontitis	IL-1β, IL-6, TNF-α, IFN-γ	Epithelial cells, EC, fibroblasts, Mϕ, PMNL
Kikuchi's disease	IL-2, IL-6, IFN-γ	Histiocytes
Systemic sclerosis	IL-1β, IL-6, IL-8, TNF-α	EC, fibroblasts, Mϕ

[a] IL, interleukin; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, γ-interferon.
[b] EC, endothelial cells; VSMC, vascular smooth muscle cells; Mϕ, macrophages; PMNL, polymorphonuclear leukocytes.

High-Output NO Synthesis Will Downregulate Local Th1 Responses

In the previous section we have seen that in various diseases the production of Th1 cytokines will be invariably associated with iNOS expression. We will now discuss the consequences of local high-output NO synthesis as far as we currently understand it. We know that the local effects of NO can be very different, ranging from cytotoxicity contributing to organ destruction, or in contrast providing protection from cell death, to altering the gene expression patterns in various cells.

NO at the site of high-output production can serve the task of self-limiting downregulation of the expression of proinflammatory cytokines, thus limiting the extent of inflammation under normal conditions. Evidence for this effect comes from two different lines of investigations. The first consists of studying the effect of NO on immune cells in *in vitro* studies. Owing to altered gene expression in the presence of NO, lymphocytes will not initiate the production of IL-2, a key cytokine for T-lymphocyte proliferation. It was also found that when IL-4, a component of the Th2 system, is upregulated by NO and the formation of the IL-12 $(p40)_2$ dimer, an endogenous inhibitor of IL-12 is favored. All these findings are in excellent agreement with the very early finding that the suppression of *in vitro* lymphocyte proliferation by activated syngeneic macrophages (the so-called ''suppressor macrophages'') is due to the NO production by these latter cells. In Fig. 5 schematic drawings of these regulatory effects are shown. Additional support for this effect comes from examining the immune system of mice lacking a functional iNOS gene, that is, from iNOS knockout mice. These animals show a slight but consistent imbalance favoring the Th1 cytokine profile. Moreover, when backcrossing of the iNOS gene defect into animal models for various diseases, most of the Th1 diseases are unaffected, and some illnesses, especially the experimentally induced encephalitis model, show a severe exacerbation when compared to littermates with a functional iNOS gene.

In conclusion, iNOS is expressed in the presence of a proinflammatory cytokine profile (Th1 response). One of its effects is that it represents a means to downregulate the inflammation as effector in an autoregulatory feedback loop, and this loop appears to be nonfunctional in some autoimmune conditions.

High-Output NO Synthesis May Induce Local Tissue Destruction

High-output NO synthesis is currently thought to have evolved for protection of the host from infection, owing to its cytotoxic potential. However, damage of normal host cells confers on iNOS the same protective/destructive duality inherent in every major component of the protective immune response. Thus high-output NO production can result

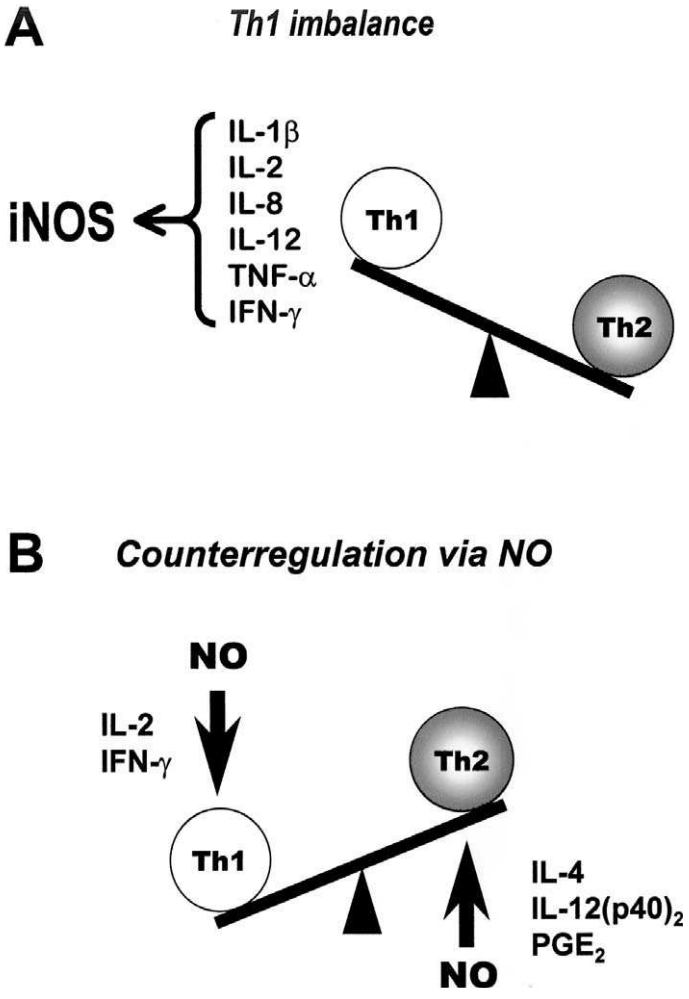


Figure 5 (A) A Th1 imbalance will induce the expression of iNOS, as most of the respective cytokines are known inducers for this enzyme. (B) On expression and functional activity of the iNOS enzyme, the NO produced will normally act as a suppressor for Th1-like activity, as it will downregulate IL-1β and IFN-γ as well as upregulate IL-4, the endogenous IL-12 inhibitor IL-12(p40)$_2$, as well as prostaglandin E_2 (PGE_2). It appears that in prolonged states of chronic imbalance this feedback loop is faulty in function.

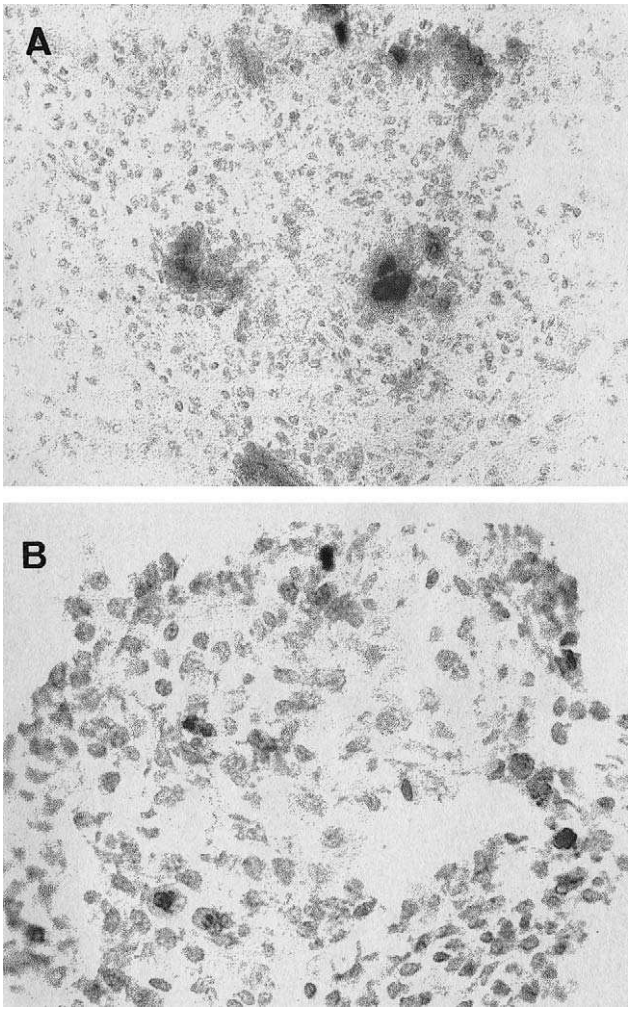


Figure 6 (A) In pancreatic tissue sections of prediabetic rats, an iNOS-specific antiserum detects iNOS-expressing macrophages and endothelial cells in the islets of Langerhans. (B) The dark brown staining in some cells indicates DNA strand breaks as an early marker for cell death. See color insert.

in direct cytotoxicity toward tumor cells and diverse infectious agents, but at sites of prolonged iNOS expression the neighboring tissue is also exposed to its cytotoxic activity.

Cell types that are particularly sensitive toward NO include a large subpopulation of thymocytes, certain neurons, pancreatic β cells (which produce the hormone insulin), and, of note, the macrophages themselves. In animal models of type 1 diabetes, macrophages are found within the islets activating the T-cell response according to the Th1 cytokine profile (see Fig. 4). Immunohistochemical staining for iNOS in islets with ongoing tissue destruction reveals positively stained macrophages and endothelial cells. In the same islets, pancreatic cells stain positive for DNA strand breaks (Fig. 6). As a further indication that NO produced by iNOS indeed contributes to disease manifestation, *in vitro* investigations have shown the following: (i) activated syngeneic macrophages effectively kill islet β cells, and this process is arginine dependent and can be inhibited by arginine analogs; (ii) incubation with various NO donor agents will show exactly the same result with the identical morphology, proving that NO alone suffices for islet cell killing; (iii) endothelial cells isolated and cultured from islet capillaries will also effectively kill β cells, if they had been challenged to express iNOS prior to coculture with the hormone-producing cells; and finally (iv) feeding mice developing the disease with an arginine analog in the drinking water will suspend this process, even though all the destructive infiltrating immune cells are sitting within the islets.

Another killing effect of iNOS that may also contribute to immune imbalance in autoimmunity is found in the thymus, the organ where the decisive steps of T-lymphocyte maturation and selection occur. Again, a large subpopulation of the developing T cells within the thymus are highly susceptible to NO-induced cell death. On a systemic challenge,

as with circulating lipopolysaccharide (LPS), thymic endothelia and some macrophages become positive for iNOS, and simultaneously large numbers of thymocytes will undergo apoptosis and cell death (Fig. 7). Again, *in vitro* studies confirm the effector function of endothelial cells. Such a process may contribute to immune bias in some stages of autoimmune disease.

The evidence so far appears simple and clear, demonstrating that in autoimmune processes the prolonged expression of iNOS is deleterious (as schematically shown in Fig. 8). However, investigations of mice lacking a functional iNOS gene have not confirmed the disease-propagating activity of this enzyme as essential for development of clinically overt illnesses, indicating that NO may just add to destruction or in some diseases, such as like autoimmune encephalitis, even protect from cell death, as will be outlined in the following section.

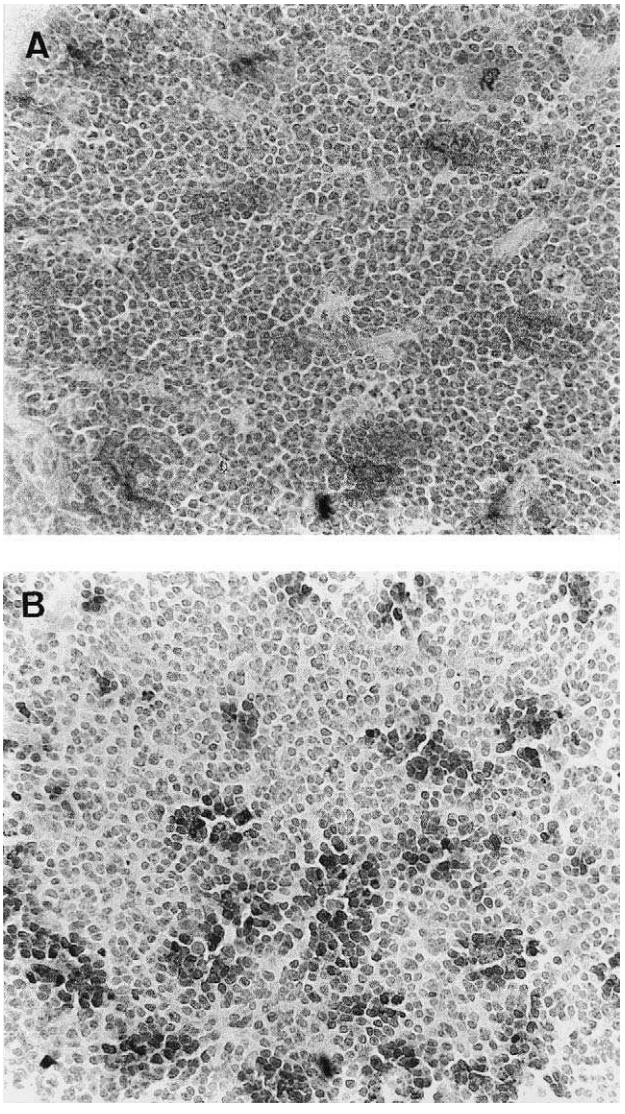


Figure 7 (A) Injection of a mouse with bacterial endotoxin will lead to expression of iNOS protein in the thymus within 18 hours. (B) Detection of DNA strand breaks as revealed by brown staining shows induction of apoptosis in many thymocytes located near the iNOS-positive areas. See color insert.

Local NO Synthesis May Also Protect from Cell Death

Apoptosis is an event essential for normal development as well as physiological cell turnover. Both excess apoptosis and a failure to undergo apoptosis will result in pathological disorders. Apoptosis, or programmed cell death, is a cascade of specific biochemical and structural events (Fig. 8) that finally lead to orderly packaged cell pieces, ready to be swallowed by neighboring cells or phagocytes. Thus pathway and morphology are distinct from lytic or necrotic cell death. The morphological changes characteristic for cells undergoing apoptosis are most easily recognizable by condensation of the nucleus followed by nuclear fragmentation (Fig. 9).

Although NO is a known cytotoxic effector molecule, as outlined in the previous section, it is evident that in certain cell types endogenously produced or exogenously added NO can act protectively against apoptosis induced by different stimuli (Fig. 10). Studies on the antiapoptotic mechanisms of NO have identified a series of interactions with the ever-growing list of molecular events in the apoptotic machinery.

One of the very first steps in the apoptosis cascade appears to be the release of cytochrome *c* from mitochondria into the cytoplasm, followed by activation of proteolytic enzymes belonging to the caspase family. These are all

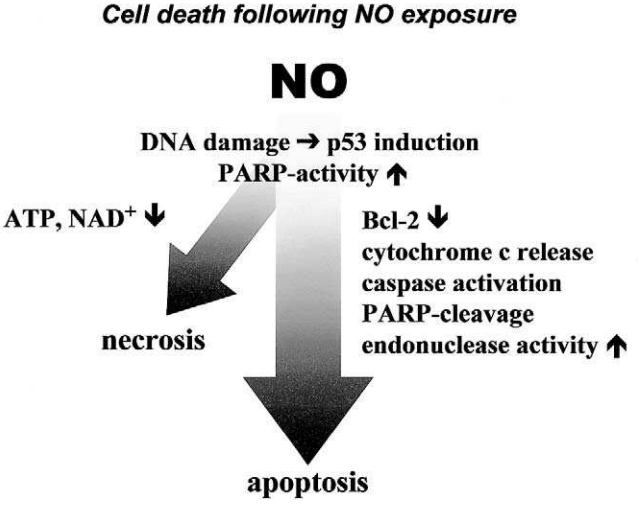


Figure 8 NO may induce apoptosis or necrosis. For instance, NO can induce DNA damage leading to simultaneous activation of poly(ADP-ribose) polymerase (PARP) and induction of p53. At this stage the cell will check whether repair is possible or whether the damage is beyond repair. In some cells activation of PARP may lead to severe depletion of NAD^+ and ATP, and subsequently necrosis will occur. However, if the damage is beyond repair, the cell death program (apoptosis) is activated, leading to degradation of Bcl-2, release of cytochrome *c* from mitochondria, cleavage and thus inactivation of PARP, and activation of endonucleases. Whether a cell dies by necrosis or apoptosis largely depends on the cell type and the NO concentration.

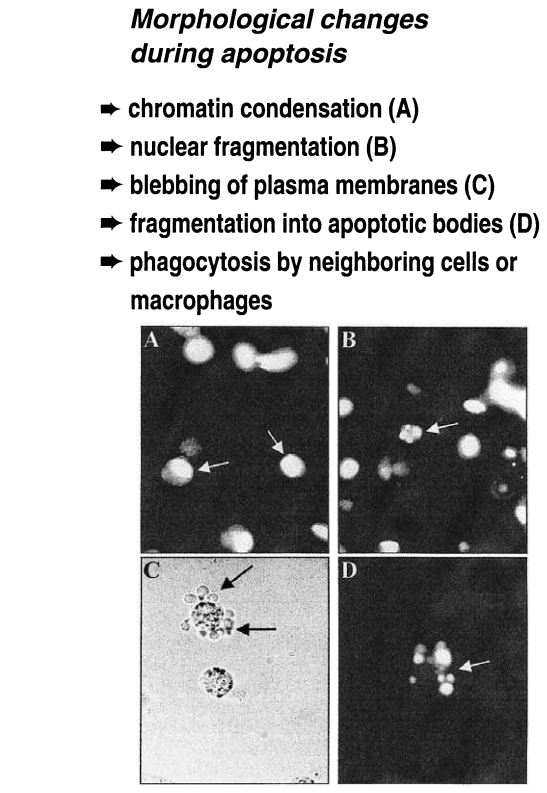


Figure 9 Shown are the main characteristics of apoptotic cell death. (A) The earliest change to be seen with appropriate nuclear staining is condensation of the nuclear chromatin. (B) This will then commence to fragmentation into smaller nuclear pieces. (C) Around this time the cell membrane begins to form sacs (blebs). (D) Finally, the cell breaks up into pieces still surrounded by a membrane.

present as proenzyme forms that require proteolytic cleavage for activation. Caspases will propagate the apoptotic cascade of events by cleaving/activating other caspases, some of which will execute the terminal events in apoptosis by cleaving various substrates. For example, caspase-9

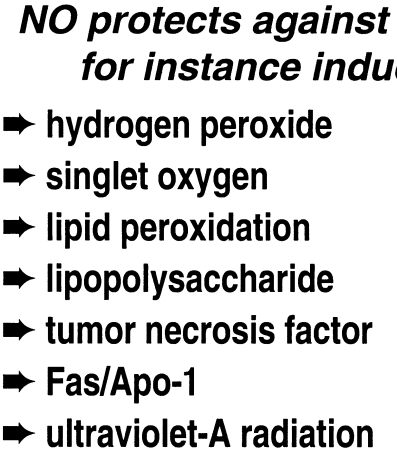


Figure 10 Some factors that may induce apoptosis in various cell types.

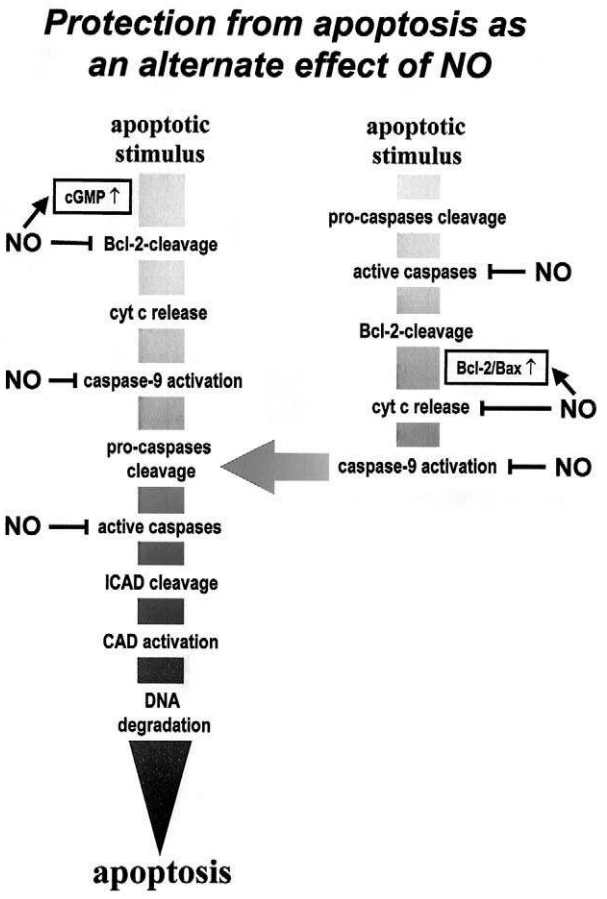


Figure 11 NO suppresses apoptosis by different mechanisms. Via activation of soluble guanylate cyclase, NO increases cGMP levels, which may inhibit signal transduction pathways early in apoptosis induction. NO may also directly inhibit caspase activation as well as inhibit caspase enzyme activities by S-nitrosylation. This results in preventing degradation of proteins of the Bcl-2 family and thus inhibits release of cytochrome *c* (cyt *c*) from mitochondria. Additionally, NO inhibits cytochrome *c* release by increasing the Bcl-2/Bax ratio. This prevents activation of downstream caspases and the terminal events in apoptosis such as the activation of caspase-activated DNase (CAD) and the cleavage of its inhibitor (ICAD).

cleaves and thereby activates caspase-3, and caspase-3 cleaves cytoplasmic and nuclear proteins, among which are fodrin and lamin, poly(ADP-ribose) polymerase (PARP), DNA-dependent kinases, and the inhibitor of the caspase-dependent activated deoxyribonuclease (ICAD) (Fig. 11).

Thus, cytochrome *c* release from mitochondria is a key event in the activation of caspases, and this step is under control of proteins in the Bcl-2 family that regulate apoptosis either positively (e.g., by Bax) or negatively (e.g., by Bcl-2). Both Bax and Bcl-2 are found in the outer mitochondrial membrane, but also in the endoplasmatic reticulum and the nuclear envelope. Bax and related proteins form channels in lipid membranes and thus facilitate the leakage of cytochrome *c* from the mitochondrial outer compartment into the cytosol. In contrast, Bcl-2 and related proteins inhibit the Bax channel-forming activity, thus preventing the release of cytochrome *c* (Fig. 12).

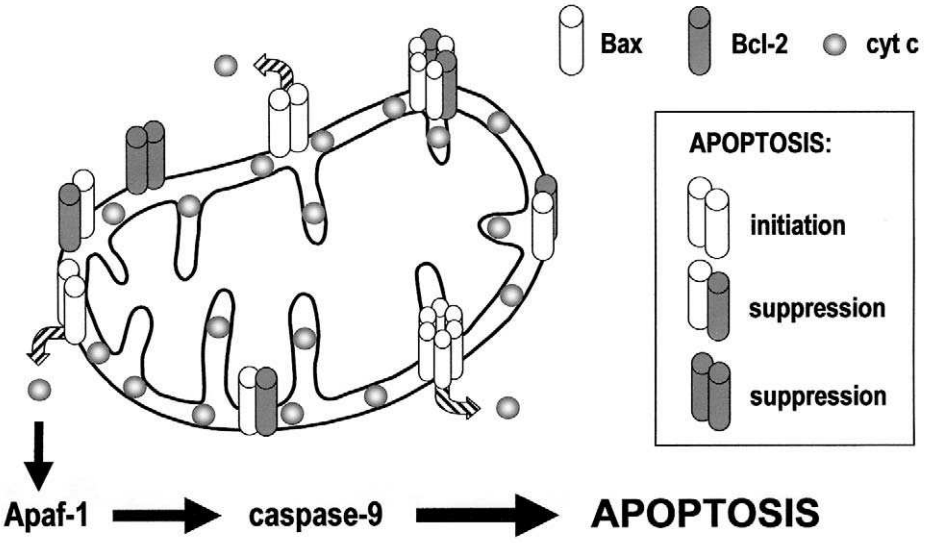


Figure 12 Role of Bax and Bcl-2 in apoptosis. Cytochrome *c* (cyt *c*) release from mitochondria is thought to represent a key event in the activation of caspases and thus in the onset of apoptosis. Proteins of the Bcl-2 family contribute to regulation of cyt *c* release. Overexpression of Bax facilitates leakage of cyt *c,* whereas Bcl-2 forms complexes with Bax, thus preventing the release of cyt *c.* Once in the cytoplasm, cyt *c* will activate apoptotic protease activating factor-1 (Apaf-1), which will then activate caspase-9.

This life–death rheostat is influenced by NO as it regulates the gene expression of Bcl-2. For instance, we found that not only endogenous NO synthesis but also exogenously applied NO fully protects against ultraviolet A (UVA)-induced apoptosis in the skin by inducing a strong increase in Bcl-2 protein expression and inhibition of UVA-induced increases in Bax expression (Fig. 11).

Although several endogenous inhibitors of caspase activation and activity have been described, none has been shown to be more prevalent than NO. NO activates guanylate cyclase, which generates cGMP from GTP. Increased cGMP levels decrease the intracellular free Ca^{2+} concentration. High Ca^{2+} concentrations, being another key signal of apoptosis and NO/cGMP inhibition of apoptosis, involve the cGMP-dependent inhibition of caspase activation. Furthermore, all caspase proteases contain a single cysteine at the enzyme catalytic site essential for enzyme activity, and this thiol is susceptible to NO-mediated redox modification, leading to caspase inhibition. Additionally, as Bcl-2 itself is a substrate for caspase-3, NO can thus inhibit Bcl-2 cleavage and subsequent cytochrome *c* release.

Moreover, by its capacity to alter gene expression, NO will induce the expression of protective proteins such as heat-shock proteins, metallothionein, or superoxide dismutase, all of which will add to protection from apoptotic cell death. Finally, protection from both apoptosis and necrosis is found as a result of NO-mediated quenching of reactive oxygen intermediates and inhibition of lipid peroxidation. These very direct effects will suppress not only apoptotic signal transduction, but also toxic plasma membrane damage as the initial event in necrotic cell lysis (Fig. 13).

The decision for a cell to undergo apoptosis is the result of a shift in the balance between the antiapoptotic and proapoptotic forces within the cell, and physiologically relevant levels of NO contribute to this balance.

NO Will Alter Cellular Gene Expression

The intricate task of regulating gene expression in the many differentiated cell types of multicellular organisms is

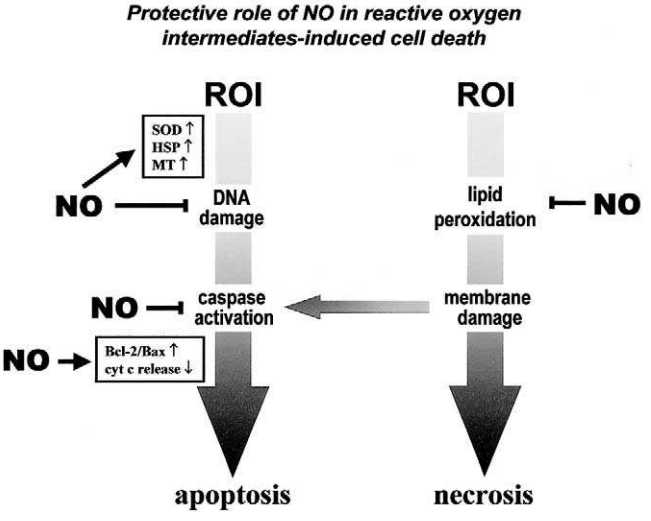


Figure 13 NO may protect from reactive oxygen intermediate (ROI)-induced apoptosis or necrosis. NO-mediated protection may occur via induction of protective proteins such as superoxide dismutase (SOD), heat-shock proteins (HSP), or metallothionein (MT), but it may also occur via direct quenching of ROIs or via inhibition of lipid peroxidation. This may lead to suppression of signal transduction pathways leading to apoptosis or to inhibition of plasma damage, an initial event of necrosis.

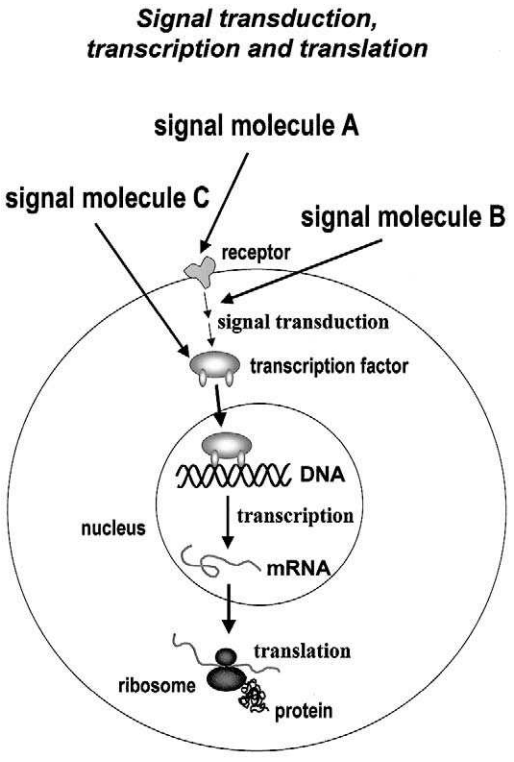


Figure 14 The pathway of signal transduction from the initial signal to cellular protein synthesis. A signal molecule may bind to a receptor in the cell membrane, and this initial event will lead to a signal transduction and activation of transcription factors in the cytoplasm. Alternatively, signal molecules may enter the cell and directly activate signal transduction pathways or transcription factors. These activated transcription factors now enter the nucleus and specifically bind to promoter or enhancer regions of the genome. This leads to specific transcription of genes into messenger RNA, which then leaves the nucleus and will be translated into protein.

accomplished primarily by the combined actions of multiple different transcription factors, which are specific proteins required for RNA polymerase II to initiate transcription. These transcription factors contain two domains, one that specifically binds to DNA and another that activates transcription by interacting with other components of the transcriptional machinery (transactivating domain). To induce cells to synthesize new proteins, signals from the surroundings must meet the target cell and must bind to specific receptors expressed at the cell surface, inducing several signal transduction pathways, which leads to activation of transcription factors. Alternatively, signals may also directly activate signal transduction pathways or transcription factors. After activation, transcription factors enter the nucleus and bind to specific DNA sequences in the promoter region of individual genes. This results in the formation of an initiation complex that binds and activates RNA polymerase II. The gene is then transcribed into messenger RNA, which leaves the nucleus and subsequently is translated into protein on the ribosomes (Fig. 14).

High-output NO (''nitrosative stress'') will lead to altered gene expression. NO reacts with selected molecular targets within a cell only, these are the heme group, free thiols, iron–sulfur (Fe–S) clusters, and zinc–sulfur (Zn–S) clusters. Whereas Fe–S clusters in proteins serve mostly as catalytic centers, Zn–S clusters are structural elements that provide a specific protein conformation essential for specific binding to DNA, RNA, or other proteins. Transcription factors bind to DNA via various DNA binding domains, for example, via zinc fingers, helix–turn–helix or helix–loop–helix motifs, or leucine zippers. Zinc fingers are by far the most prevalent motif and contain repeats of cysteine and/or histidine residues that bind Zn^{2+}, thereby folding looped structures in proteins. These loops often contain the DNA binding domain. NO will temporarily destroy such a structure via S-nitrosylation and subsequent ejection of Zn^{2+}, as outlined in Fig. 15. Zinc finger-dependent gene transcription will thus be inhibited by NO (Fig. 16A). However, NO will not irreversibly destroy zinc finger proteins, since the zinc finger structures can be restored by cellular redox systems, if the

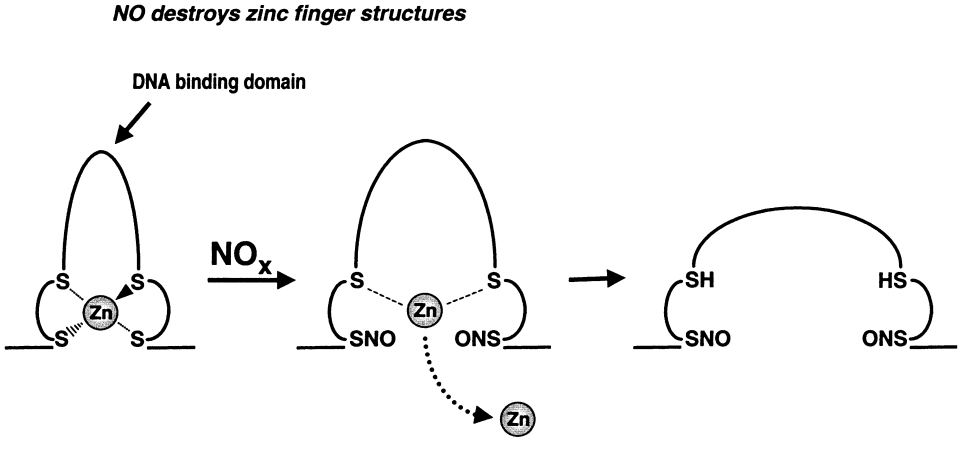


Figure 15 Destruction of zinc finger structures by NO. The most prevalent DNA binding motif of proteins are so-called zinc fingers, where a Zn^{2+} ion determines a special protein conformation necessary for specific binding to DNA, RNA, or proteins. Under aerobic conditions, NO leads to S-nitrosylation and subsequent ejection of the Zn^{2+}, and this destroys the zinc finger structure and thus the DNA binding domain.

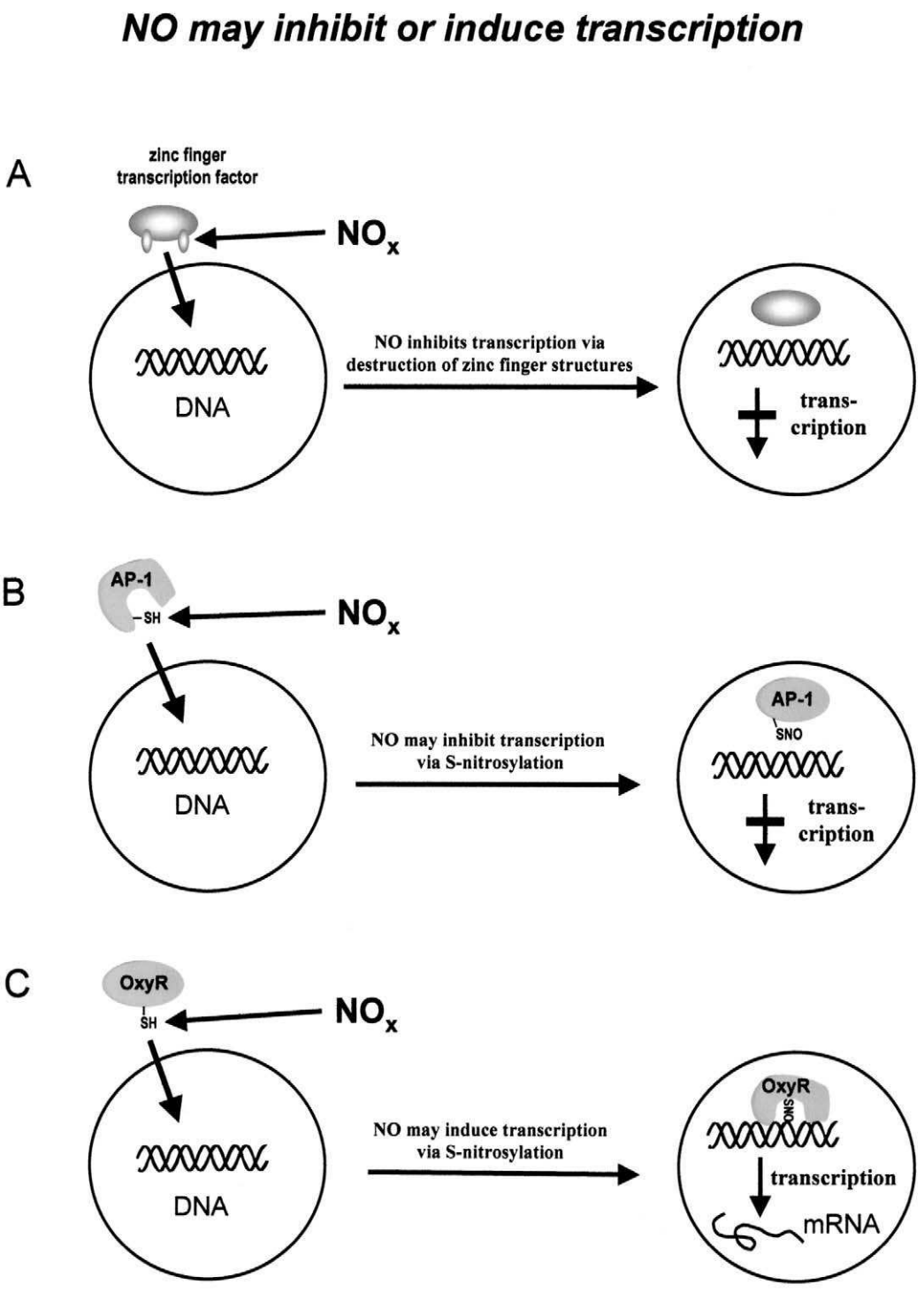


Figure 16 Inhibition or induction of transcription by NO. (A) Destruction of zinc finger structures by NO leads to inhibition of the DNA binding activity of the respective transcription factor and thus transcription. (B) The same holds true for other redox-sensitive transcription factors such as AP-1, where a cysteine residue within the DNA binding domain can be S-nitrosylated by NO, which will also result in inhibition of DNA binding. (C) However, NO may also activate transcription factors, for example, the bacterial stress sensor OxyR. In this case, S-nitrosylation leads to a conformational change in the protein that induces DNA binding of OxyR and subsequent transcription.

individual redox capacity of the cell is not overtaxed. Restoring zinc finger structures after nitrosative stress may allow a more finely tuned and specific regulatory action of NO. Importantly, once zinc finger transcription factors have bound to responsive DNA elements, they are much more resistant toward NO, suggesting that NO inhibits transcription during its initiation only.

Besides zinc finger transcription factors, NO also inhibits the DNA binding activity of redox-sensitive non-zinc finger transcription factors such as nuclear factor κB (NF-κB) and

activator protein 1 (AP-1) (Fig. 16B), which both contain a cysteine residue within or close to the DNA binding domain. However, transcription factors with zinc fingers are probably the most sensitive targets. In contrast, transcription factors that lack cysteine residues essential for DNA binding or transactivating activity will not be inhibited by NO. This means that nitrosative stress unspecifically but nevertheless selectively inhibits the DNA binding activity of redox-sensitive transcription factors and thus leads to inhibition or reduction of gene expression.

In contrast, NO may also enhance transcription by reacting with other transcription factors. An example is the transcription factor OxyR, a bacterial redox-stress sensor capable of recognizing both oxidative and nitrosative stress and inducing transcription of genes for protective enzymes such as catalase or glutathione reductase. OxyR contains a critical cysteine residue, and S-nitrosylation of this cysteine activates OxyR, thus leading to specific transcription of genes (Fig. 16C). Transcriptional regulation of OxyR by S-nitrosylation thus serves as a switch to regulate gene expression of bacteria under nitrosative stress.

These few examples show that NO may regulate gene expression in either a positive or a negative direction depending on the transcription factors involved.

Conclusions

In this chapter we have listed a number of apparent contrasting ways in which NO will act on its production and release by the iNOS in tissues or cells. We have shown that NO induces apoptosis but also may kill cells via necrosis. On the other hand, we have presented evidence showing that NO will very efficiently protect cells from apoptosis and necrosis. Now, what are we going to predict, if we know of iNOS expression in a certain disease? And this is exactly the problem: We are currently not able to predict anything. Disease outcome appears to depend on the amount of NO released within a given period of time, and it also appears to depend on the exact timing of iNOS-produced NO. As an example, we have found that in human skin keratinocytes will become iNOS positive on ultraviolet irradiation. This is apparently a normal response, as healthy volunteers all show this reaction, which is detectable at the first day and lasts for another day or two. In contrast to this normal response, patients with an immune-mediated disease triggered by ultraviolet light (cutaneous lupus erythematosus) show no iNOS staining within the first 2 days and will belatedly express this enzyme from day 3 on for up to 25 days or more, which is the period during which these patients develop the characteristic skin lesions. This tells us that iNOS expression may not be much of a disease marker (as suggested in Table 1); rather, it is the inappropriate timing of its expression that is disease related. As NO can regulate gene expression in cells, this may explain why the timing of NO formation is crucial.

Another factor, which we can rarely determine in ongoing diseases, is the amount of NO locally released. There is increasing evidence for the relative concentration of NO being crucial for the outcome, with various levels of NO resulting in protection, in induction of apoptosis, or in direct necrotic effects. Moreover, these concentration ranges will be different for different cell types, as already mentioned.

Additional Reading

Bruch-Gerharz, D., Ruzicka, T., and Kolb-Bachofen, V. (1998). Nitric oxide and its implications in skin homeostasis and disease. *Arch. Dermatol. Res.* **290,** 643–651.

Kolb, H., and Kolb-Bachofen, V. (1998). NO in autoimmune disease: Cytotoxic or regulatory mediator? *Immunol. Today* **19,** 556–561.

Kröncke, K.-D., Fehsel, K., and Kolb-Bachofen, V. (1997). Nitric oxide: Cytotoxicity versus cytoprotection—How, why, when and where? *Nitric Oxide Chem. Biol.* **1,** 107–120.

Kröncke, K.-D., Fehsel, K., and Kolb-Bachofen, V. (1998). Inducible nitric oxide synthase in human diseases. *Clin. Exp. Immunol.* **113,** 147–156.

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CHAPTER 51

Nitric Oxide and Platelet Function

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PLATELETS ARE ANUCLEATE BLOOD ELEMENTS THAT PLAY A CRUCIAL ROLE IN VASCULAR HEMOSTASIS. PLATELETS ADHERE TO THE SITE OF VASCULAR INJURY, AGGREGATE, AND SEAL THE RENT IN THE VASCULAR WALL. PLATELET FUNCTION IS REGULATED VIA THE RECIPROCAL INTERACTIONS BETWEEN AGENTS THAT STIMULATE AND INHIBIT PLATELET ACTIVATION. NITRIC OXIDE (NO) IS AN IMPORTANT CONTRIBUTOR TO THE PLATELET INHIBITORY REACTIONS. NITRIC OXIDE, WHEN GENERATED BY PLATELETS AND THE ENDOTHELIUM, INHIBITS ADHESION, INHIBITS AGGREGATION, AND HELPS TO MAINTAIN VASCULAR INTEGRITY, ACTING MOSTLY THROUGH A CGMP-DEPENDENT STIMULATION OF PROTEIN PHOSPHORYLATION. VASCULAR PATHOLOGY IS OFTEN ASSOCIATED WITH ABERRATIONS OF NO GENERATION, METABOLISM, AND ACTION. RESTORATION OF THE DISTURBED BALANCE BETWEEN PLATELET MEDIATORS AND THE DEVELOPMENT OF PLATELET-SELECTIVE NO DONOR AGENTS MAY BE USED TO TREAT VASCULAR PATHOLOGIES ASSOCIATED WITH PLATELET DYSFUNCTION.

Introduction

Vascular hemostasis is a physiological process aimed at preservation of both blood fluidity and vascular integrity. The fluid state of flowing blood must be carefully balanced against another important homeostatic property of blood that is meant to protect the vascular system from the loss of fluid due to an accidental breach of vascular integrity. These diverse yet coordinated reactions form the cornerstone of physiological hemostasis. Thrombosis is a pathological extension of hemostasis that occurs when the regulatory mechanisms of physiological hemostasis are inadequate (Radomski and Radomski, 1999).

Stages of Hemostasis

When a blood vessel is injured the first discernible effect resulting from damage is the recruitment of platelets to the site of injury. This recruitment results in formation of a primary hemostatic plug, often referred to by earlier scholars as ''white aggregate.'' The effectiveness of this primary vascular wall-sealing device depends on its reinforcement with a wirelike network of the strong protein fibrin, which is the final product of the protein coagulation cascade promoted by platelets. The fibrin-rich ''red aggregate'' is subsequently remodeled by the action of the blood fibrinolytic system, leading to vessel wall repair processes and a return to the physiological status (Fig. 1). The complexity of hemostatic reactions requires flawless performance of the regulatory checkpoints that operate at various levels of hemostasis. A failure of hemostasis in maintaining self-control may lead to thrombosis.

Blood Platelets

MORPHOLOGY AND FUNCTION

Platelets are small (~2 μm in diameter), anucleate cell fragments first described by Italian researcher Bizozzero at the end of the nineteenth century. Platelets are generated following fragmentation of the cytoplasm of large progenitor

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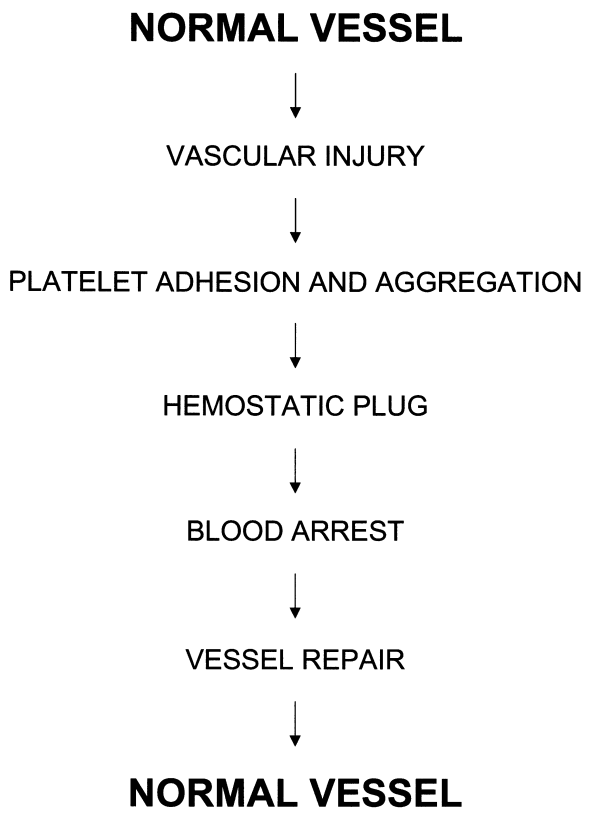


Figure 1 The main stages of vascular hemostasis.

cells, the megakaryocytes. Fragmentation of one megakaryocyte results in formation of a few thousand platelets.

Gross features of platelets can be observed using phase-contrast microscopy *ex vivo* (Fig. 2); however, closer scrutiny requires the use of electron microscopy (Fig. 3). The most striking ultrastructural features are the lack of nucleus (hence, by classic definition, platelets should be considered as cell fragments rather than cells) and the presence of numerous granules (Read *et al.,* 1985). Platelet granules contain various proactivator and mitogenic agents, including adenine nucleotides, calcium, protein growth factors, and fibrinogen.

As described earlier, vessel wall injury leads to a chain of platelet reactions. It is convenient to separate platelet reactions into three major components: adhesion, that is, the ability of a platelet to attach to a natural or foreign surface; aggregation, the ability of platelets to attach to each other; and platelet procoagulant activity, the ability to accelerate fibrin meshwork formation (Coller, 1992; Salas *et al.,* 1997a).

It is important to realize that *in vivo* platelet reactions take place in flowing blood. Blood flow determines the fundamental parameters of platelet behavior. The rheological (spatial) configuration of blood elements inside the vessel depends on the shear rate, that is, the forces generated as a result of pulsatile propulsion of blood by the working heart in the biological tubes. Red blood cells, more numerous and larger than platelets, occupy the central axial position in the vessel, forcing platelets to stream toward the endothelial lining (Radomski and Radomski, 1999). Thus, under physiological conditions platelets are in the proximal vicinity of the endothelial cells (Fig. 4). This arrangement is particularly apparent under conditions of laminar flow best seen in the microvasculature. In contrast, a turbulent flow, as detected at vessel branching or in pathological derangement of vasculature, changes this natural configuration of blood elements and facilitates thrombosis.

Platelet adhesion takes place when platelets attach and spread (similar to an egg on a frying pan) on natural surfaces (the vessel wall constituents) and on foreign surfaces (e.g., blood bank containers and the extracorporeal circuits). The platelet–surface interactions are mediated via the expression of platelet receptors (Table I). These are adhesive glycoproteins that act in gluing platelets to their stratum.

Platelet aggregation results from formation of bridges between the adjacent platelets. This process depends mainly on the expression of glycoprotein integrin IIb/IIIa by platelets, and this receptor binds fibrinogen and other plasma ligands to form the aggregate. The process of activation (a change from the low- to high-affinity conformation) of the IIb/IIIa receptor is paramount for platelet aggregation to occur. Platelet aggregation triggers structural changes in the surface membrane organization, exposing to the lumen of the vessel a highly procoagulant lipid surface that facilitates thrombin activation and fibrin formation (Coller, 1992).

Platelet aggregation is conveniently studied *in vitro* using aggregometry (Fig. 5). Both the theory and practice of platelet aggregation studies and their application to NO research were reviewed by Radomski *et al.* (1996).

PATHWAYS OF PLATELET REGULATION

Activator Reactions Research over the past 40 years has provided the biochemical rationale for these dramatic alterations in basal behavior of platelets. It is now well established that the formation of a platelet plug is supported by the activity of at least three platelet-derived activator systems.

The release of adenosine diphosphate (ADP) from platelet granules and the interactions of ADP with purinergic receptors form the foundation of the first system. *In vivo,* a platelet-derived pool of ADP may be supplemented with ADP released from red blood cells (Radomski and Radomski, 1999).

The discovery of the mechanism of action of aspirin and its congeners by Sir J. Vane (1971) formed the foundations for the discovery of the proaggregating metabolites of arachidonic acid in platelets, namely, cyclic endoperoxides and thromboxane A_2 (Hamberg *et al.,* 1974). Thromboxane A_2 is synthesized by the sequential action of platelet cyclooxygenase and thromboxane synthase, and once formed, it acts on its receptors to amplify aggregation (Moncada, 1982).

Inhibition of the generation of and the action of ADP (e.g., by ticlopidine) and thromboxane (e.g., by aspirin) is not sufficient per se to abolish platelet aggregation stimulated by such potent agonists as thrombin. In 1997, we discovered that human platelets express matrix metalloproteinase-2 (MMP-2) that, when released, stimulates platelet aggrega-

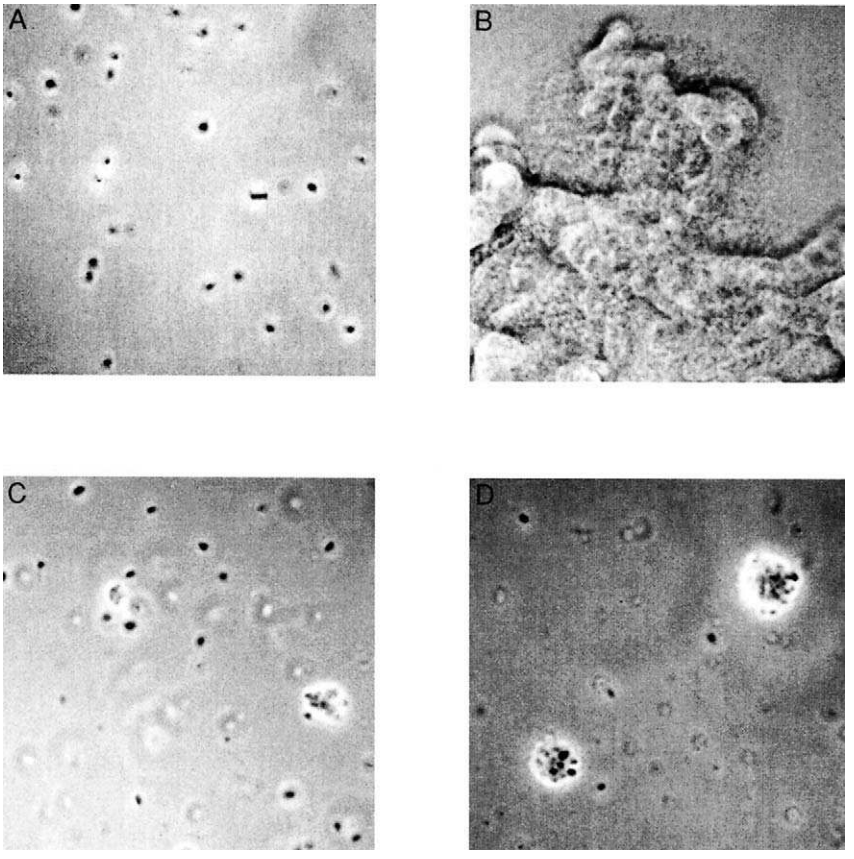


Figure 2 Phase-contrast microscopy examination of reactions of human washed platelets. (A) Resting platelets. (B) Platelet aggregate resulting from stimulation of platelets with collagen (10 μg/ml). (C and D) Inhibition of platelet aggregation by 10 μ*M S*-nitrosoglutathione (GSNO) and 10 μ*M S*-nitroso-DL-acetylpenicillamine (SNAP), respectively (200×).

tion in a nonthromboxane-, non-ADP-dependent manner (Sawicki *et al.,* 1997) (Fig. 6).

Inhibitor Reactions The vascular endothelium is a major contributor to the inhibitor reactions that control platelet activation.

Vane and associates first discovered that endothelial cells generate prostacyclin, a potent inhibitor of platelet aggregation and stimulator of platelet disaggregation (Moncada *et al.,* 1976). Prostacyclin is a biological opponent of thromboxane A_2 on platelets (Fig. 7) and the vessel wall resulting in inhibition of platelet aggregation and vasodilation (Moncada, 1982). Prostacyclin binds to its specific receptors present on platelets that are linked to the adenylyl cyclase. Stimulation of prostacyclin receptor leads to increased accumulation of the intracellular cAMP and downregulation of all pathways involved in amplification of platelet aggregation (Moncada, 1982). Prostacyclin exerts little influence on the process of platelet adhesion to the subendothelial components of the vessel wall (Radomski *et al.,* 1987a,b).

Prostacyclin acts as a paracrine inhibitor of platelet activation. It is released close to the endothelial surface in response to stimulation with various vasoactive mediators including angiotensins and bradykinin (Nowak *et al.,* 1981). Platelets themselves lack the capacity to synthesize prostacyclin; however, they may contribute to the endothelial synthesis of this eicosanoid by generating and releasing arachidonic acid cyclic endoperoxides, which may be taken up by the endothelial cells for prostacyclin synthesis (Moncada, 1982).

Some of lipoxygenase metabolites of polyunsaturated fatty acids, including 12-hydroperoxy and 13-hydroxy derivatives of arachidonic and linoleic acids, respectively, have been shown to inhibit the process of platelet activation (Aharony *et al.,* 1981; Buchanan and Brister, 1991). The precise mode of action of these metabolites on platelets is not known.

Nitric Oxide

Search for Noneicosanoid Regulators of Platelet Function

The discovery of prostacyclin, a major platelet-regulatory prostaglandin, intensified research aimed at understanding the mechanisms of platelet regulation. Indeed, it soon became apparent that the generation and release of this eicosanoid

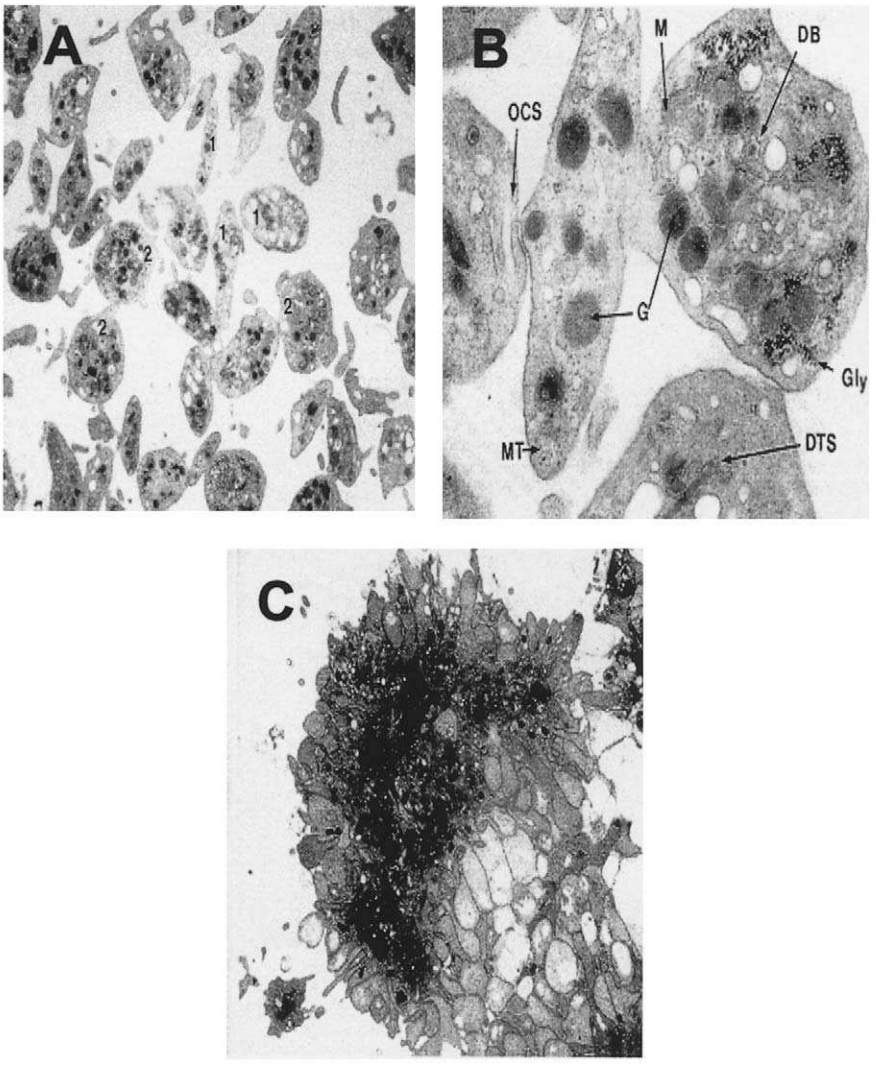


Figure 3 Ultrastructural features of human platelets. (A) Low-power (20,000×) examination of resting platelets. Physiologically, the shape of resting platelets is discoid (1). Isolation and preparation of platelet for the microscopy studies may result in the appearance of platelets with spherical shape (2). (B) High-power (40,000×) examination of resting platelets. OCS, open canalicular system; MT, microtubuli; G, α granules; DB, dense bodies; M, mitochondria; Gly, glycogen; DTS, dense tubular system. (C) Platelet aggregate. Platelets in the aggregate show various degree of degranulation.

can only account in part for nonthrombogenic properties of vascular endothelium. The endothelial cells can express the antiaggregatory activity even under conditions of complete inhibition of prostaglandin generation (Radomski *et al.,* 1987c,d).

The discovery of endothelium-dependent relaxation (Furchgott and Zawadzki, 1980) and the fact that endothelium-derived relaxing factor (EDRF) appeared not to be a metabolite of arachidonic acid has added new impetus to this search. The study of Azuma and colleagues (1986) suggested that EDRF might be acting as an inhibitor of platelet aggregation. In 1986–1987, we investigated noneicosanoid, platelet-inhibitory properties of endothelial cells and found that these could be accounted for by the release of EDRF. Moreover, the pharmacological properties of EDRF were identical with those of nitric oxide (NO) gas on platelets. Furthermore, the measurement of NO released during platelet regulation by the endothelium showed that its generation could account for the platelet-inhibitory activities of EDRF on platelet adhesion and aggregation (Radomski *et al.,* 1987a–d). Interestingly, the endogenous NO proved to be identical in its antiplatelet spectrum with that generated during cigarette smoking and by pharmacologically related vasodilators, as demonstrated by Ignarro and co-workers (Mellion *et al.,* 1981).

The description of the L-arginine to NO pathway in endothelial cells (Palmer *et al.,* 1988) stimulated the search for the presence of nitric oxide synthase (NOS) in platelets. The rationale for this research was provided by previous observations that platelet activation was associated with increased generation of cyclic guanosine 3',5'-monophosphate (cGMP) (Goldberg *et al.,* 1975) and by the experiments of Ignarro

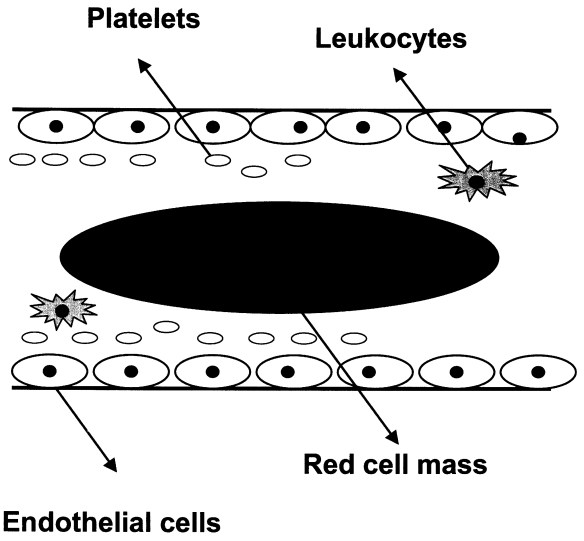


Figure 4 The arrangement of platelets in blood under the conditions of laminar flow.

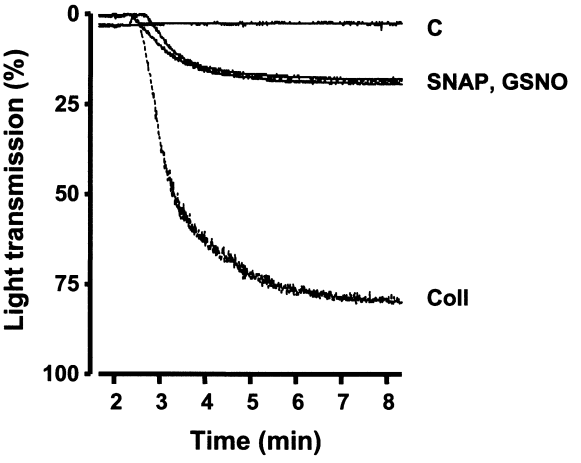


Figure 5 Superimposed traces obtained during aggregation of human washed platelets. C, control nonaggregated platelets; Coll, platelet aggregation stimulated with collagen (10 μg/ml); SNAP, inhibition of aggregation by 10 μ*M S*-nitroso-DL-acetylpenicillamine; GSNO, inhibition of aggregation by 10 μ*M S*-nitrosoglutathione.

and colleagues showing that NO gas inhibited aggregation in a cGMP-dependent manner (Mellion *et al.,* 1981). In 1990–1993, we provided biochemical and pharmacological characteristics of platelet NOS (Radomski *et al.,* 1990a,b), and we measured the activity of the enzyme during platelet stimulation using a selective porphyrinic microsensor (Malinski *et al.,* 1993). More recently, a number of groups reported the molecular characteristics of platelet NOS (Muruganandam and Mutus, 1994; Chen and Mehta, 1996; Wallerath *et al.,* 1997; Berkels *et al.,* 1997).

Molecular Biology of NO Generation by Platelets

In contrast to megakaryocytes, which contain large amounts of RNA and DNA, platelets contain trace amounts of DNA and small amounts of RNA. Therefore, the identification of DNA fragments coding NOS proteins requires application of reverse transcriptase-polymerase chain reaction (RT-PCR). A number of researchers extracted platelet RNA and amplified DNA fragments consistent with expression of endothelial NOS (eNOS) but not inducible NOS (iNOS) or neuronal NOS (nNOS) in platelets (Sase and Michel, 1995; Mehta *et al.,* 1995; Wallerath *et al.,* 1997).

Although the presence of eNOS mRNA in normal platelets (Sase and Michel, 1995; Mehta *et al.,* 1995) appears to be beyond dispute, the identification of iNOS mRNA has proved to be controversial. Chen and Mehta (1996) but not Wallerath and colleagues (1997) found mRNA for iNOS in normal platelets. It is possible that a short half-life iNOS

Table I Selected Platelet Receptors

Receptor	Ligand
Adhesion	
Integrins	
GPIa/IIa (VLA-2)	Collagen
GPIc/IIa (VLA-6)	Laminin
GPIc*/IIa (VLA-5)	Fibronectin
α_vIIIa	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin
GPIIb/IIIa	Fibrinogen, fibronectin, von Willebrand factor, vitronectin
Others	
P-selectin	Selectin counter receptors
GPIb	Von Willebrand factor
GPIV	Thrombospondin, collagen
Aggregation	
GPIIb/IIIa	Fibrinogen, fibronectin, von Willebrand factor

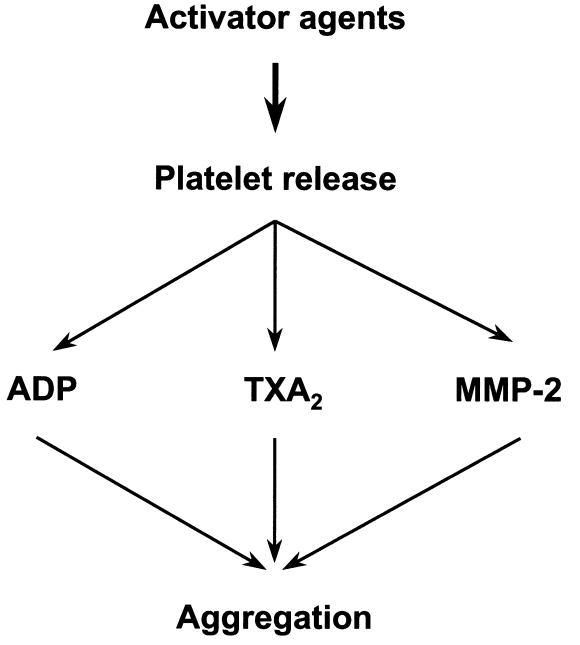


Figure 6 The activator pathways that amplify platelet aggregation.

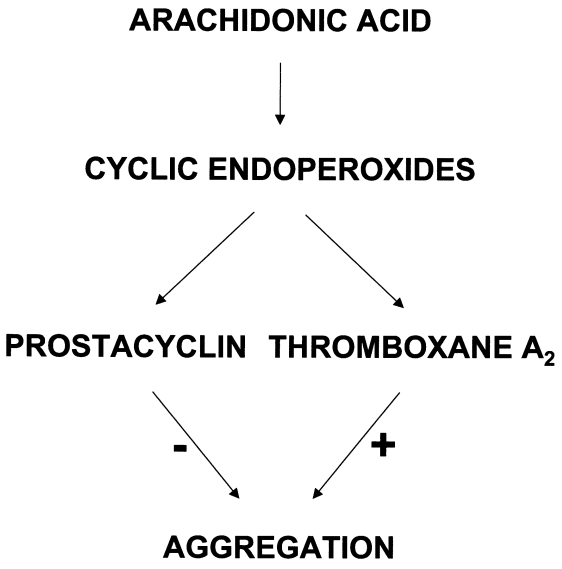


Figure 7 The prostacyclin–thromboxane balance in regulation of platelet aggregation. + denotes stimulation; − denotes inhibition.

mRNA (6 hours) (Geng and Lotz, 1995) accounts for this discrepancy.

Muruganandam and Mutus (1994) first purified to homogeneity platelet NOS protein. The isolated protein was cytosolic, and the molecular mass of the monomer was found to be approximately 80 kDa. Western blot, immunocytochemistry, and NADPH diaphorase staining have been used to study NOS protein isoforms in platelets. The immunological methods detected the presence of eNOS and iNOS immunoreactivity in normal human and porcine platelets (Chen and Mehta 1996; Wallerath *et al.,* 1997; Berkels *et al.,* 1997). In contrast to the studies by Muruganandam and Mutus (1994) and Mehta *et al.* (1995), other researchers found platelet eNOS, similar to endothelial eNOS, to be associated with the particulate fraction of the platelet (Wallerath *et al.,* 1997; Berkels *et al.,* 1997). It is possible that platelet eNOS undergoes intracellular translocation and activation during platelet activation (Radomski *et al.,* 1990a,b; Berkels *et al.,* 1997).

The significance of iNOS protein expression under physiological conditions in platelets remains to be elucidated. Platelets have a limited capacity to synthesize protein *de novo,* and they acquire most of their proteins from megakaryocytes. We and others have shown that cytokine-stimulated megakaryoblasts and bone marrow megakaryocytes express iNOS (Lelchuk *et al.,* 1992; Wallerath *et al.,* 1997). Megakaryocytopoiesis (growth and differentiation of megakaryocytes) and thrombopoiesis (platelet formation following megakaryocyte fragmentation) are physiological processes that are cytokine dependent and regulated by the stimulatory and inhibitory cytokines (Brown and Martin, 1994). It is possible that the changes in expression of iNOS in platelets occur physiologically as a result of interactions between factors controlling thrombopoiesis and megakaryocytopoiesis.

Factors Regulating NO Generation in Vascular Hemostasis

Nitric oxide available for platelet regulation is generated by both endothelium- and platelet-derived NOS (Fig. 8). Stimulation of platelet and endothelial function plays an important role in the generation of NO. Tonic release of NO from the endothelial cells is likely to be mediated by shear stress (Busse and Fleming, 1998). Resting platelets generate small amounts of NO (Zhou *et al.,* 1995; Malinski *et al.,* 1993). Platelet adhesion and aggregation stimulate platelet NOS, leading to the release of NO (Polanowska-Grabowska and Gear, 1994; Radomski *et al.,* 1990a; Malinski *et al.,* 1993; Lantoine *et al.,* 1995).

The substrate for NOS, L-arginine, is present in high concentrations in plasma and the intracellular compartment. These concentrations are many times higher than K_m for NOS, suggesting that under resting conditions L-arginine may not be a rate-limiting factor for NO generation. In contrast, endothelial and platelet activation lead to stimulation of NOS and generation of substantial amounts of NO (Radomski *et al.,* 1990a; Bode-Boger *et al.,* 1998).

Platelet- and endothelium-derived eNOS are NADPH, biopterin, and flavin dependent (Salas *et al.,* 1997a; Berkels *et al.,* 1997). The availability of calcium appears to be crucial for the activity of eNOS, as its removal abolishes the enzyme activity (Radomski *et al.,* 1990a). Interestingly, there is an apparent lack of correlation between changes in the intraplatelet calcium levels and activation of platelet-

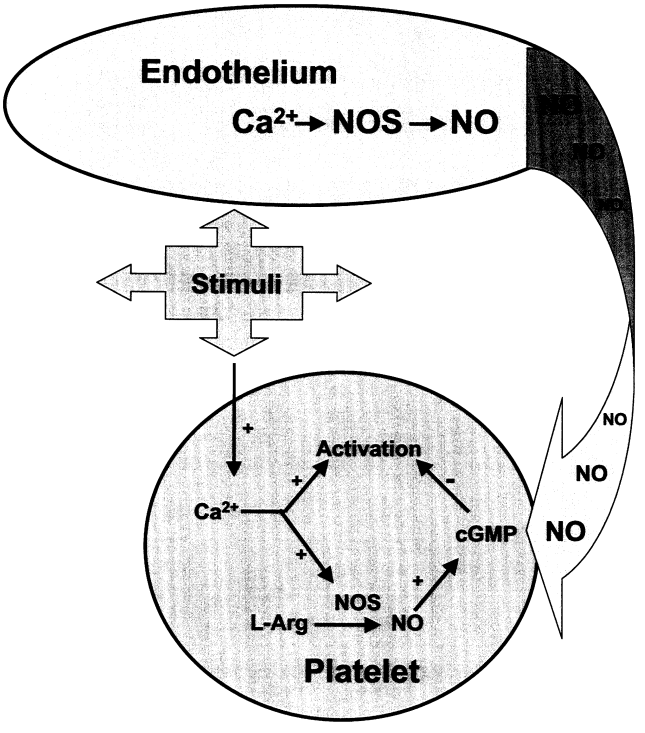


Figure 8 Generation and physiological effects of NO in the platelet–endothelium microenvironment. + denotes stimulation; − denotes inhibition.

derived eNOS (Malinski *et al.,* 1993; Lantoine *et al.,* 1995). The reasons for this discrepancy are unclear.

As explained in earlier, the rheological conditions, that is, the positioning of platelets against the vascular wall in flowing blood, exert strong influence on platelet function. Indeed, the placement of platelets in the proximal vicinity of the endothelial lining facilitates regulation of platelet function by the endothelium-derived NO (Radomski and Radomski, 1999). Moreover, red blood cells containing hemoglobin, a NO scavenger, stream toward the axial part of the vascular lumen.

Physiological Regulation of Platelet Function by NO

Early experiments examined the effects of NO on platelet adhesion and aggregation *in vitro.* Both basal (shear stress-dependent) and agonist-stimulated release of NO have been implicated in platelet regulation. Indeed, the coronary and pulmonary vasculatures generate NO to inhibit platelet adhesion under constant flow conditions (Venturini *et al.,* 1989; Pohl and Busse, 1989). Similarly, bradykinin-stimulated endothelial cells release NO in quantities sufficient to inhibit platelet adhesion (Radomski *et al.,* 1987b,c; Sneddon and Vane, 1988).

Platelet aggregation induced by a variety of agonists, as well as by shear stress, is inhibited by NO released from fresh or cultured endothelial cells (Radomski *et al.,* 1987c,d; Furlong *et al.,* 1987; Busse *et al.,* 1987; Macdonald *et al.,* 1988; Alheid *et al.,* 1989; Houston *et al.,* 1990; Broekman *et al.,* 1991). In addition to inhibition of adhesion and aggregation, NO disaggregates preformed platelet aggregates (Radomski *et al.,* 1987d) and inhibits platelet recruitment to the aggregate (Freedman *et al.,* 1997).

There is now convincing evidence that NO is an important regulator of platelet function *in vivo.* Animal studies have shown that basal or stimulated release of NO results in inhibition of platelet aggregation induced by some aggregating agents or endothelial injury (Rosenblum *et al.,* 1987; Bhardwaj *et al.,* 1988; Hogan *et al.,* 1988; Humphries *et al.,* 1990; Herbaczynska-Cedro *et al.,* 1991; May *et al.,* 1991; Golino *et al.,* 1992; Yao *et al.,* 1992; Houston and Buchanan, 1994). In addition, studies in humans evidenced that there is basal luminal release of NO from the vasculature, causing increases in the intraplatelet cGMP levels (N. P. Andrews *et al.,* 1994). In healthy volunteers, inhibition of NOS with N^G-monomethyl-L-arginine (L-NMMA) increased platelet aggregation granule release *ex vivo* (Bodzenta-Lukaszyk *et al.,* 1994) and shortened bleeding time (Simon *et al.,* 1995). In contrast to L-NMMA, L-arginine administration *in vivo* leads to the inhibition of platelet activation *ex vivo* (Caren and Corbo, 1973; Adams *et al.,* 1995; Bode-Boger *et al.,* 1998). Both the vasodilator (Houston and Buchanan, 1994) and platelet-inhibitory (Yao *et al.,* 1992; Golino *et al.,* 1992; Bodzenta-Lukaszyk *et al.,* 1994; Adams *et al.,* 1995; Bode-Boger *et al.,* 1998) components contribute to the hemostatic action of NO.

Early studies reviewed by Radomski and Moncada (1991) showed that platelet activation and thrombus formation occur as a result of synergistic interactions between proaggregating agents. In 1987, we found that synergistic interactions also take place between agents that inhibit platelet aggregation. Indeed, the endothelial cells generate and release NO and prostacyclin that act in concert to maximize the extent of platelet inhibition and vessel wall protection (Radomski *et al.,* 1987d) (Fig. 9). Similar synergistic interactions occur between NO and other inhibitors of platelet activation (for references, see Salas *et al.,* 1997a). The biological significance of the synergistic regulation of platelet function is to ensure a multilevel control of this process and minimize the impact of pathological alterations on hemostasis.

A biological counterpart for MMP-2 (Fig. 6) has been identified in the vessel wall and in platelets (Fernandez-Patron *et al.,* 1999). This is another matrix metalloproteinase enzyme (MMP-9) (Fig. 10). The interactions between NO, prostacyclin, and MMP-2 remain to be studied.

Mechanisms of Platelet Regulation by NO

Similar to many cell and tissue systems, the effects of NO on platelets are largely dependent on the stimulation of the soluble guanylyl cyclase (GC-S) and the resultant increase in the intraplatelet cGMP levels. However, some actions of NO on platelets may also be independent of GC-S activation.

SOLUBLE GUANYLYL CYCLASE

It is now generally accepted that NO activates soluble GC-S by binding to the heme moiety of the enzyme (Craven and DeRubertis, 1978). The binding of NO to GC-S results

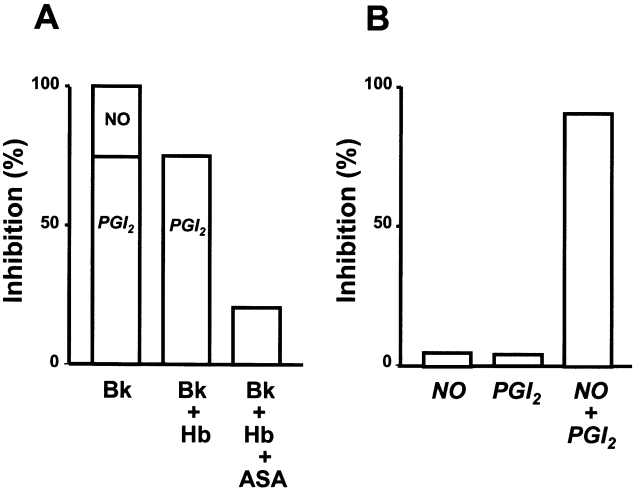


Figure 9 Synergistic interactions between NO and prostacyclin as inhibitors of platelet aggregation. (A) Endogenous NO and prostacyclin (PGI_2) released from cultured endothelial cells by bradykinin (Bk) maximally inhibit collagen-induced platelet aggregation *in vitro.* The NO-dependent part of this inhibition is abolished by hemoglobin (Hb), a NO scavenger. PGI_2-dependent inhibition is decreased by aspirin (ASA). (B) Synthetic NO and PGI_2 synergize as inhibitors of platelet aggregation. Graphs adapted from data in Radomski *et al.* (1987c,d).

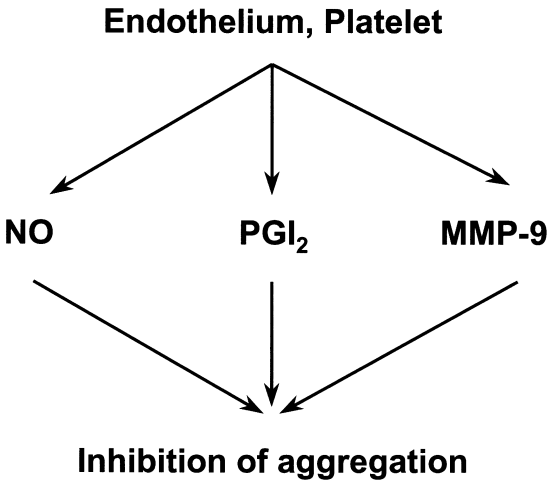


Figure 10 Factors involved in inhibition of platelet activation.

in the conversion of magnesium guanosine 5'-triphosphate to guanosine 3',5'-monophosphate (cGMP) (Mellion *et al.,* 1981). Indeed, platelet fractions containing GC-S readily sequester NO gas (Liu *et al.,* 1993).

Three proteins mediate the actions of cGMP on platelets: cGMP-dependent protein kinase, cGMP-binding cAMP phosphodiesterase, and cGMP-regulated ion channels (Walter, 1989). Stimulation of cGMP-dependent protein kinase results in phosphorylation of proteins (Walter, 1989) such as the 46- to 50-kDa vasodilator-stimulated phosphoprotein (VASP, Haffner *et al.,* 1995). During platelet adhesion VASP is associated with actin filaments and focal contact areas, that is, transmembrane junctions between microfilaments and the extracellular matrix (Reinhard *et al.,* 1992). The binding of VASP with the platelet cytoskeleton may mediate its inhibitory effect on the fibrinogen receptor (Horstrup *et al.,* 1994). More recently, it has been shown that cGMP mediates phosphorylation of the carboxyl terminus of thromboxane receptor (Wang *et al.,* 1998). The thromboxane receptor is a G-protein-coupled receptor, and its phosphorylation by cGMP-dependent protein kinase disrupts receptor–G-protein coupling and inhibits thromboxane-mediated platelet aggregation.

Cyclic GMP may also interfere with the transport of biogenic amines into platelets. Indeed, cGMP-induced protein phosphorylation may downregulate the uptake of serotonin by platelets (Launay *et al.,* 1994).

Cyclic GMP decreases basal and stimulated concentrations of intracellular Ca^{2+} (Nakashima *et al.,* 1986; Johansson and Haynes, 1992). A number of systems that regulate Ca^{2+} trafficking have been identified in platelets. These include the receptor-operated channels, the Ca^{2+}-ATPase extrusion pump, the Na^+/Ca^{2+} exchanger, the Ca^{2+}-accumulating ATPase pump of the dense tubular membrane (an intraplatelet membrane Ca^{2+} store), and passive leakage and receptor-operated Ca^{2+} channels in the dense tubular membrane. These systems could be affected by cGMP. It has been shown that cGMP increases the activity of the Ca^{2+}-ATPase extrusion pump and leakage across the plasma membrane (Johansson and Haynes, 1992). In addition, cGMP causes inhibition of Ca^{2+} mobilization from intraplatelet stores, including the dense tubular membrane (Nakashima *et al.,* 1986). All these effects of cGMP lead to decreased availability of Ca^{2+} within and in the proximal vicinity of platelets.

Metabolism of membrane phospholipids may be also a target for the action of cGMP. Indeed, the inhibition of phospholipases C and A_2 has been implicated in the mechanism of this action on platelets (Nakashima *et al.,* 1986; Sane *et al.,* 1989).

Cyclic GMP downregulates the function of some platelet receptors, including the fibrinogen receptor IIb/IIIa, and it downregulates protein kinase C-induced expression of P-selectin and the release of lysosomal protein CD63 (Salas *et al.,* 1994; Murohara *et al.,* 1995; Mendelsohn *et al.,* 1990; Michelson *et al.,* 1996). Interestingly, von Willebrand and fibronectin receptors appear not to be regulated by cGMP (Michelson *et al.,* 1996; Shahbazi *et al.,* 1994).

Cyclic GMP, by inhibiting cGMP-inhibited cAMP phosphodiesterase, may delay the hydrolysis of cAMP and enhance the biological effects of the latter nucleotide (Maurice and Haslam, 1990). However, the physiological and pharmacological relevance of this ''cross talk'' between cAMP and cGMP pathways is unclear (Radomski *et al.,* 1992).

Cyclic GMP-Independent Action of NO on Platelets

Some authors suggested that some actions of NO on platelets might be independent of the generation of cGMP. These could be direct actions of NO on calcium flux (Menshikov *et al.,* 1993), metabolism via inhibition of ADP ribosylation (Brune and Lapetina, 1989), and inhibition of 12-lipoxygenase (Nakatsuka and Osawa, 1994).

Studies suggest that some NO donor compounds such as *S*-nitrosoglutathione (GSNO) may inhibit platelet function in both a cGMP-dependent and cGMP-independent manner (Gordge *et al.,* 1998). Cyclic GMP-independent actions of GSNO would involve reactions dependent on the presence of thiols and copper.

Studies attempting to investigate the role of GC-S in mediation of the physiological effects of NO on platelets have been handicapped for a long time by the lack of a potent and selective inhibitor of this enzyme. Both methylene blue and LY83583, which were widely used to inhibit GC-S, lack selectivity and interact with a number of molecular targets beside GC-S (for references, see Moro *et al.,* 1996). We characterized 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) as a potent and selective inhibitor of GC-S (Moro *et al.,* 1996; Martinez-Cuesta and Radomski, 1998). Using this compound we have shown that both the antiaggregation and adhesion-inhibitory effects of NO *in vitro* are sensitive to inhibition by ODQ and thus are cGMP dependent.

The biological actions of cGMP are terminated by cGMP phosphodiesterase and by its efflux from platelets, and they may also depend on the activity of protein phosphatases (Walter, 1989). An overview of effects of NO on platelets is shown in Fig. 11.

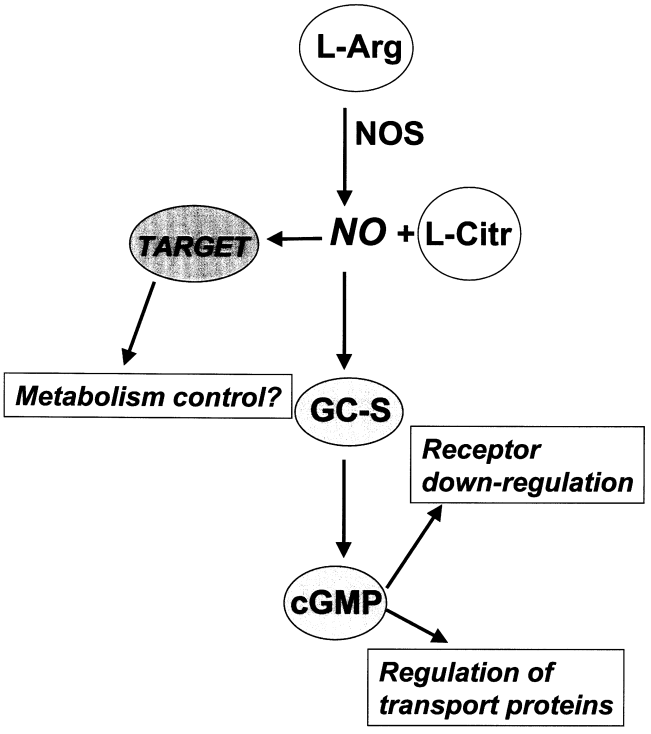


Figure 11 Cyclic GMP-dependent and -independent regulation of platelet activation.

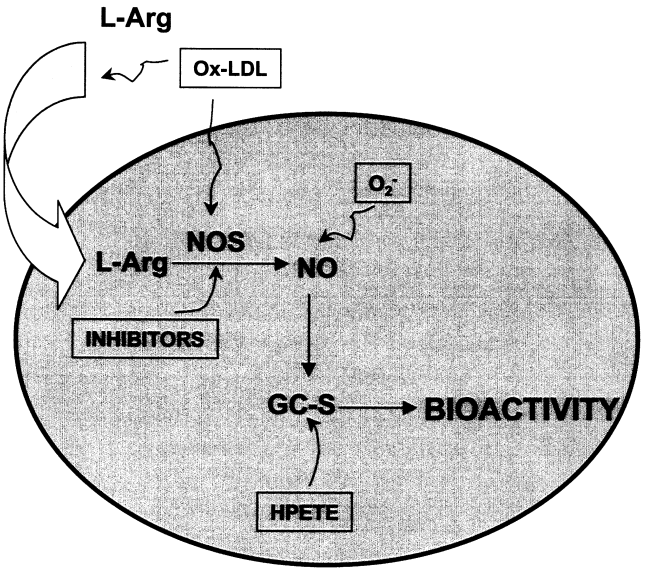


Figure 12 Some pathological factors that affect the bioactivity of NO. Ox-LDL, oxidized low-density lipoproteins; inhibitors, endogenous inhibitors of NOS including dimethylated analogs of L-arginine; HPETE, hydroperoxides of arachidonic acid.

Pathological Disruption of NO Generation and Action, and Its Involvement in the Pathogenesis of Vascular Disorders Associated with Platelet Dysfunction

The vasodilator and platelet-regulatory functions of the endothelium are impaired during the course of vascular disorders, including atherosclerosis, coronary artery disease, essential hypertension, diabetes mellitus, and preeclampsia (DeBelder and Radomski, 1994); however, the reasons underlying this impairment are not clear. The endothelial dysfunction was ascribed to both decreased and enhanced generation of NO. To explain this discrepancy it was proposed that the changes in the generation of NO are often accompanied by reduced bioactivity of NO (Radomski and Salas, 1995) (Fig. 12).

A detrimental effect of superoxide ion generation on the NO-dependent cellular signaling was first demonstrated by Gryglewski *et al.* (1986). In 1990, Beckman and associates found that superoxide reacts under conditions of physiological pH with NO to form peroxynitrite ($ONOO^-$) (for references, see Beckman and Tsai, 1994). Peroxynitrite is a highly reactive oxidant that can oxidize various biomolecules in the cellular microenvironment. In 1994, we found that $ONOO^-$ can decrease the vasodilator and platelet-inhibitory activity of NO and prostacyclin (Moro *et al.,* 1994; Villa *et al.,* 1994). Thiols and glucose (Moro *et al.,* 1994, 1995) attenuated these detrimental effects of $ONOO^-$. The reaction of $ONOO^-$ with thiols in cell membranes and glucose in the extracellular fluid results in the synthesis of NO donor agents that counteract the vasoconstrictor and platelet-aggregatory activities of the parent oxidant (Moro *et al.,* 1994; Brown *et al.,* 1998). Interestingly, there is now evidence that $ONOO^-$ may be generated during aggregation of normal platelets (Naseem and Bruckdorfer, 1997). Indeed, tyrosine nitration, a marker of $ONOO^-$ generation, has been detected in resting platelets. Moreover, there was increased generation of nitrated tyrosine in agonist-stimulated platelets. The nitration of tyrosine residues could influence the tyrosine phosphorylation reactions that play a paramount role in controlling platelet function.

Under physiologic conditions the impact of $ONOO^-$ generation in the platelet microenvironment is likely to be downregulated following its reactions with platelet thiols. These reactions result in the conversion of the oxidant to NO donor agents (Brown *et al.,* 1998). The oxidizing stress could decrease the efficiency of this regulating mechanism and precipitate platelet dysfunction and damage.

ATHEROSCLEROSIS, THROMBOSIS, AND HYPERTENSION

Atherogenesis is associated with profound changes in the oxidative status of the vascular wall. Oxidative modifications of low-density lipoproteins (LDL) play a key role in atherogenesis, and a number of studies (reviewed by Radomski and Salas, 1995) have examined the effects of native and oxidized LDL on NO-mediated vascular functions. In most of these studies lipoproteins decreased the bioactivity of NO (Luscher *et al.,* 1993; Cooke and Tsao, 1992, and references therein). The decreased bioactivity of NO in atherosclerosis could also result from changes in the metabolism of this molecule and generation of $ONOO^-$ from superoxide and inducible NO (Beckman and Tsao, 1994). In addition, LDL may inhibit L-arginine uptake into platelets and through this

mechanism decrease NOS activity and promote thrombosis (Chen and Mehta, 1994). These effects are prevented by the administration of L-arginine in the diet (Tsao *et al.,* 1994). In contrast to LDL, high-density lipoproteins (HDL) decreased platelet activation and thrombosis by increasing NOS activity in platelets (Chen and Mehta, 1994). Moreover, human apolipoprotein E, which mediates hepatic clearance of lipoproteins, exerts a significant inhibitory effect on platelets through stimulation of platelet NOS (Riddell *et al.,* 1997).

Lipid peroxidation also leads to free radical-catalyzed generation of F_2 prostaglandin isomers from peroxidation of arachidonic acid. Interestingly some of these isoprostanes, such as 8-epiprostaglandin $F_{2\alpha}$, reduce the antiadhesive and antiaggregatory activity of NO on platelets (Minuz *et al.,* 1998). Thus, lipid peroxidation contributes to the pathomechanism of impaired bioactivity of NO in the cardiovascular system.

Ischemic heart disease and myocardial infarction are common manifestations of coronary atherosclerosis. The endogenous NO inhibited microthromboembolism in the ischemic heart, protected myocardium against intracoronary thrombosis, and decreased platelet deposition owing to carotid endarterectomy (Komamura *et al.,* 1994; Olsen *et al.,* 1996). Moreover, decreased generation of NO by platelets is predictive of the presence of acute coronary syndromes in patients with coronary atherosclerosis (Freedman *et al.,* 1998). In addition, acetylcholine-induced release of NO is impaired in patients with coronary artery disease, contributing to a reduction in the endothelial capacity to regulate platelet activation (Diodati *et al.,* 1998). The data clearly show that the alterations in the generation and action of NO are important for the pathogenesis of atherogenesis and its ischemic complications.

Interestingly, an impaired NO generation or action may also underlie the pathomechanism of vasospastic and thrombotic changes of essential hypertension (Cadwgan and Benjamin, 1993; Calver *et al.,* 1992).

DIABETES MELLITUS

There are indications that changes in the bioactivity and metabolism of NO are involved in the pathogenesis of vasculopathy in diabetes mellitus. Insulin, at physiological concentrations, inhibits platelet activation via stimulation of platelet NOS (Trovati *et al.,* 1997). This suggests that a reduced generation of NO in insulin-deficient states could contribute to platelet hyperactivity and diabetic angiopathy. We have examined the formation of NO in the vasculature and platelets of JCR-LA-cp rats, which serve as a model of insulin-resistant states associated with obesity and complicated by atherosclerosis. We have found that generation of NO is crucial for the preservation of vascular homeostasis under these conditions (McKendrick *et al.,* 1998).

VASCULAR INJURY CAUSED BY STRESS

Generation of vascular NO in response to stress protects the vasculature from the vasoconstrictor and platelet-activator effects of stress hormones (Leza *et al.,* 1998). However, the capacity of the vasculature to offset the detrimental effects of stress appears to be limited, and longer-lasting stress exposure leads to decreased generation of platelet NO, thus facilitating platelet activation and thrombosis (Leza *et al.,* 1998).

PREECLAMPSIA

Vasoconstriction and increased platelet activation are also characteristic for preeclampsia, a severe disease that may complicate normal pregnancy. We have measured the activity of NOS and GC-S in preeclamptic women and compared it with nonpregnant and healthy pregnant subjects. Paradoxically, increased generation of NO in platelets of preeclamptic women was associated with increased platelet activation (Salas *et al.,* 1997b). However, despite increased formation of NO, the activity of GC-S was reduced in preeclamptic women, suggesting that this NO was not bioactive, that is, it failed to inhibit platelet activation. We hypothesized that enhanced generation of $ONOO^-$ could contribute to platelet dysfunction and damage in preeclampsia.

ENDOTOXIN, SEPTICEMIA, AND EXPRESSION OF iNOS

The invasion of gram-negative bacteria and exposure of cells to bacterial toxins and cytokines (for references, see DeBelder and Radomski, 1994; Radomski, 1995) lead to a life-threatening syndrome often referred to as septic shock. Bacterial toxins and cytokines cause induction of a number of enzymatic systems, including NOS. It is generally accepted that stimulation of the cascade of cytokines leads to upregulation of expression of inducible enzymes, including NOS.

Cytokine-induced expression of iNOS in the vessel wall is very well documented. However, the interactions of endotoxin with platelets are controversial. Platelets have a very limited capacity to synthesize protein *de novo.* Hence, a major part of the effect of endotoxin on platelets may be indirect and may require megakaryocytic iNOS protein to be passed onto platelets (Lelchuk *et al.,* 1992). Interestingly, there is some evidence for the presence of iNOS in normal platelets. As mentioned earlier, this basal expression of iNOS in platelets may reflect the dynamism of thrombopoiesis and megakaryocytopoiesis. The presence of iNOS in normal platelets could potentially explain reports showing acute effects of endotoxin on normal platelets that were mediated via generation of NO by iNOS (Mehta *et al.,* 1995; Sheu *et al.,* 1998). These authors hypothesized that basally expressed iNOS is inactive, owing to its being complexed with a large protein carrier, and that endotoxin has a capacity to remove protein blockade and stimulate iNOS-mediated production of NO.

Considering all these data, the expression of iNOS is likely to have complex repercussions for vascular hemostasis. Inducible NO could attentuate the detrimental effects of bacterial toxins on hemostasis resulting from stimulation of the clotting cascade (disseminated intravascular coagulation). Indeed, inhibition of NO generation by NOS inhibitors potentiated cytokine-stimulated platelet adhesion to cultured human endothelial cells (Radomski *et al.,* 1993), precipitated

renal glomerular thrombosis (Schultz and Raij, 1992), and exacerbated sepsis-induced renal hypoperfusion (Spain *et al.,* 1994) *in vivo.* On the other hand, exposure of endothelial cells to cytokine-induced NO may result in cell toxicity and destruction (Palmer *et al.,* 1992), and it has been reported that inhibition of NOS may be beneficial in the treatment of septic shock (Kilbourn *et al.,* 1990; Wright *et al.,* 1992). A partial explanation for this discrepancy may be that the currently available inhibitors of iNOS are not selective and inhibit the activities of other NOS isoenzymes. Indeed, an intact generation of constitutive NO may be important in maintaining the integrity of the microvasculature during sepsis.

UREMIA

Platelet diathesis associated with bleeding is a classic complication of uremia due to the suppression of platelet function by the disease process. As early as 1970, it was found that L-arginine and some other metabolites of the urea cycle are accumulated in uremia (Horowitz *et al.,* 1970). More recent work showed that uremia leads to an increase in cytokine levels (Noris *et al.,* 1993). Platelets obtained from uremic patients generate more NO than controls, so that increased expression and/or activity of NOS may play a role in the platelet dysfunction observed in this condition.

CANCER

Platelets contribute to the cytotoxic cell effector system controlling neoplasia (for references, see Okada *et al.,* 1996). A part of this cytotoxic mechanism of platelets could be NO dependent (Okada *et al.,* 1996).

Platelets also play a role in the pathogenesis of tumor metastasis by increasing the formation of tumor cell–platelet aggregates, thus facilitating cancer cell arrest in the microvasculature. Tumor cell-induced platelet aggregation *in vitro* is modulated by the ability of tumor cells to generate NO, and this correlates with their propensity for metastasis (Radomski *et al.,* 1991). Indeed, human colon carcinoma cells isolated from metastases exhibited lower NO activity than cells isolated from the primary tumor. Moreover, the expression of iNOS by murine melanoma cells inversely correlated with their ability to form metastases *in vivo* (Dong *et al.,* 1994). These data suggest that a differential synthesis of NO may distinguish between cells of low and high metastatic potential. Interestingly, NOS has been found in some human gynecological malignancies, and the highest NOS activities were detected in poorly differentiated tumors (Thomson *et al.,* 1994). Thus, further work is needed to unravel the biological significance of NO for tumor growth, tumor metastasis, and platelet–tumor cell interactions.

Another interesting aspect of NO action on the metastatic cascade of events is its interactions with matrix metalloproteinases (MMPs). Matrix metalloproteinases represent a family of matrix-degrading enzymes that play an important role in the growth, invasion, and metastasis of cancer cells (Stetler-Stevenson, 1996). We have found that MMP-2 plays a crucial role in tumor cell-induced platelet aggregation (Sawicki *et al.,* 1999). The release of MMP-2 was inhibited by NO donor agents, suggesting that NO may interfere with cancer invasion and spread by reducing the release of MMPs.

Pharmacology of NO Gas and NO Donor Agents on Platelets

L-ARGININE, THE SUBSTRATE FOR ENDOGENOUS GENERATION OF NO

The amounts of endogenous L-arginine in the platelet microenvironment are high (millimolar), suggesting that the availability of substrate is unlikely to constitute a rate-limiting factor for activation of platelet eNOS. Indeed, pharmacological stimulation of resting platelets with L-arginine does not result in the generation of NO (Radomski *et al.,* 1990a). Platelet activation is a potent stimulus for stimulation of NOS, and under these conditions exogenous L-arginine is promptly converted by platelet eNOS to NO (Radomski *et al.,* 1990a; Malinski *et al.,* 1993; Freedman *et al.,* 1997; Bode-Boger *et al.,* 1998). The presence of extracellular calcium is crucial for the activation of platelet eNOS by L-arginine, as the platelet-inhibitory activity of L-arginine in citrated platelet-rich plasma (low calcium levels) is lower than in whole blood anticoagulated with hirudin, which preserves physiological calcium levels (Radomski *et al.,* 1990a; Bode-Boger *et al.,* 1998).

In contrast to eNOS, basal expression of iNOS activity in vascular disorders such as atherosclerosis and preeclampsia (Dube *et al.,* 1998; Salas *et al.,* 1997b) may be sufficient to ensure the enzymatic conversion of L-arginine to NO. Indeed, Cooke and colleagues (Tsao *et al.,* 1994) provided convincing evidence for the platelet-inhibitory actions of L-arginine when administered to animals and humans with atherosclerosis. Moreover, pharmacological administration of L-arginine to patients suffering from coronary artery disease and peripheral arterial obstructive disease alleviated the symptoms of arterial insufficiency (Slawinski *et al.,* 1996; Ceremuzynski *et al.,* 1997). Thus, L-arginine may be a useful pharmacological agent in treatment of vascular disorders associated with platelet activation.

ACTIVATORS OF NOS

Some pharmacological agents inhibit platelet function via stimulation of the activity of platelet NOS. These include relaxin, a uterine hormone (Bani *et al.,* 1995), and trilinolein, a triacylglycerol obtained from the medicinal herb *Panax pseudoginseng* (Shen and Hong, 1995).

ROLE OF INHIBITORS OF NOS AND NO SCAVENGERS

Inhibitors of NOS (Radomski *et al.,* 1990a) oppose the platelet-inhibitory effects of L-arginine. These compounds shorten bleeding time in humans (Simon *et al.,* 1995) and may precipitate thrombosis under conditions of vascular stress exemplified by septicemia (Schultz and Raij, 1992). Interestingly, long-term smoking impairs the activity of

platelet NOS (Ichiki *et al.,* 1996), although the mechanism responsible for this impairment remains to be elucidated. Purified human hemoglobins, cross-linked to prevent renal damage, are currently undergoing clinical trials as an oxygen-carrying agent. In addition to binding oxygen, hemoglobin has a very high affinity to NO. Olsen and colleagues (1996) showed that cross-linked hemoglobin enhances platelet deposition in a rat carotid endarterectomy model, an effect prevented by administration of L-arginine. Thus, cross-linked hemoglobins may stimulate platelet activation through a mechanism involving NO scavenging.

Interestingly, under some conditions the binding of NO to hemoglobin may be reversible. Stamler and colleagues hypothesized that the binding of NO to the cysteinyl residue of globin in hemoglobin results in the generation of a NO donor agent, which is capable of releasing NO and inhibiting platelet function (Pawloski *et al.,* 1998).

Nitric Oxide Gas

Studies using NO gas *in vitro* showed that the molecule was a potent but short-acting (biological half-life < 4 min) inhibitor of platelet adhesion and aggregation and also a stimulator of platelet disaggregation (Radomski *et al.,* 1987a–d). Because of its short-lasting pharmacological effects, inhaled NO is used increasingly as a selective pulmonary vasodilator to treat critically ill adults and infants (for review, see Cheung *et al.,* 1997). Some investigators studied the effects of inhaled NO on platelet function in health and disease. Interestingly, the effects of inhaled NO on platelet function in healthy subjects are not certain (Albert *et al.,* 1996). However, both in adults with adult respiratory distress syndrome (Samama *et al.,* 1995) and in critically ill neonates (Cheung *et al.,* 1998) the NO treatment causes inhibition of platelet function. Under some conditions these effects are unexpectedly long lasting and may enhance the risk of intracranial bleeding. Therefore, caution should be taken during administration of NO gas to critically ill patients.

Interestingly, animal experiments showed that inhaled NO might inhibit the development of coronary thrombosis (Adrie *et al.,* 1996). The pharmacological significance of these findings remains to be investigated.

Nitric Oxide Donor Agents

The pharmacological activity of NO donor agents is related to the release of NO (Katsuki *et al.,* 1977; Feelisch and Noack, 1987). The mechanism of NO release from these compounds is spontaneous or is catalyst and enzyme dependent. In contrast to relatively short-lived pharmacological effects of NO gas, those of NO donor agents are longer lasting.

Organic nitrates are poor spontaneous releasers of NO and require the presence of a thiol cofactor for acceleration of this liberation (Feelisch, 1991). However, the *in vivo* release of NO from organic nitrates is greatly enhanced by thiols and enzyme(s) including plasma glutathione-*S*-transferases (Chen *et al.,* 1996). Whether these or similar enzymes are present in platelets remains controversial (Gerzer *et al.,* 1988; Weber *et al.,* 1996). Nitrate-induced inhibition of platelet aggregation *in vitro* can be greatly potentiated in the presence of thiols or cultured vascular cells (Loscalzo, 1985; Feelisch, 1991; Benjamin *et al.,* 1991). This indicates that the conversion of organic nitrates by the vascular tissue *in vivo* can result in the release of NO in amounts sufficient for inhibition of platelet function. Indeed, in experimental animals, as well as in healthy volunteers, oral and intravenous administration of glyceryl trinitrate and isosorbide mononitrates resulted in inhibition of platelet aggregation *ex vivo* (for review, see Salas *et al.,* 1997a; Plotkine *et al.,* 1991; Werns *et al.,* 1994). The effectiveness of organic nitrates as antithrombotics increases with the extent of vascular injury (Lam *et al.,* 1988). Furthermore, short- and long-lasting administration of nitroglycerin and isosorbide dinitrate to patients suffering from coronary artery disease and acute myocardial infarction resulted in a significant inhibition of platelet adhesion and aggregation (Diodati *et al.,* 1990; Sinzinger *et al.,* 1990).

What is the position of organic nitrates among ''classic'' inhibitors of platelet function? Aspirin is by far the most widely used antiplatelet drug in clinical practice, and its benefits in terms of decreasing mortality due to repeated infarction have been unequivocally demonstrated (ISIS-2, 1988, ISIS-3, 1992; Patrono, 1989), whereas those of organic nitrates have not yet been established. A meta-analysis found significant reduction in mortality when intravenous glyceryl trinitrate or nitroprusside were used during the acute course of myocardial infarction (Yusuf *et al.,* 1988). Moreover, when combined with *N*-acetylcysteine, glyceryl trinitrate substantially reduced myocardial infarction in unstable angina, an effect compatible with an antiplatelet effect of glyceryl trinitrate (Horowitz *et al.,* 1988). Surprisingly, GISSI III (1994) and ISIS-4 (1993) studies failed to show a clinically beneficial effect of organic nitrates on mortality after myocardial infarction. However, further analysis of GISSI III suggests that the apparent additive effect of glyceryl trinitrate and lisinopril could be attributed to antiplatelet effects of this NO donor agent (R. Andrews *et al.,* 1994). In addition, it is possible that nitrates may act by reducing the infarct size in small rather than large infarcts, so that the neutral results of GISSI-3 and ISIS-4 may be explained by the heterogeneity of effect.

Interestingly aspirin, a cyclooxygenase inhibitor, blocks only thromboxane-mediated platelet aggregation (for references, see Patrono, 1989), leaving the remaining pathways of adhesion and aggregation unopposed. In contrast, NO inhibits the activation cascade of mediators generated by all known pathways of platelet aggregation (Salas *et al.,* 1997a), including matrix metalloproteinase-2-dependent platelet aggregation (Sawicki *et al.,* 1997), and some pathways of platelet adhesion to the subendothelium (Shahbazi *et al.,* 1994).

Whether platelets become tolerant to the platelet-inhibitory effects of organic nitrates is again controversial (Weber *et al.,* 1996; Booth *et al.,* 1996). There have been many attempts to synthesize tolerance-free NO donor agents. One of the

more promising groups of drugs is the cysteine-containing nitrates. Incorporation of a cellular thiol cysteine into the structure of organic nitrate resulted in a high effectiveness of these compounds as inhibitors of platelet and leukocyte functions both *in vitro* and *in vivo* (Lefer *et al.,* 1993).

The phenomenon of tolerance is of lesser pharmacological significance for other NO donor compounds including sodium nitroprusside, molsidomine, and 1,3-morpholinosydnonimine hydrochloride (SIN-1). Because of its powerful vasodilator action, sodium nitroprusside is often used to treat vascular emergencies associated with hypertensive crisis. Because this compound shows some antiplatelet activity both *in vitro* and *in vivo* (Levin *et al.,* 1982; Hines and Barash, 1989), its acute clinical effects may also be mediated, in part, through inhibition of platelet function. Sodium nitroprusside has been administered intrapericardially to treat experimentally induced coronary thrombosis in dogs (Willerson *et al.,* 1996). The antiplatelet actions of nitroprusside are short lasting, as the release of cyanide from nitroprusside downregulates the pharmacological generation of NO (Brune and Hanstein, 1998). As this route of administration of sodium nitroprusside produced less vasodilation than the systemic route, localized administration of this drug may offer new therapeutic possibilities for the treatment of coronary thrombosis.

Molsidomine and its active metabolite SIN-1 inhibit experimental thrombosis and platelet aggregation in healthy volunteers and in patients suffering from acute myocardial infarction (Wautier *et al.,* 1989). Interestingly, SIN-1 in addition to NO generates superoxide and $ONOO^-$ (Hogg *et al.,* 1993). Because $ONOO^-$ causes platelet aggregation and counteracts the platelet inhibitory activity of NO (Moro *et al.,* 1994), the formation of this radical may offset the antiplatelet activity of NO released from SIN-1.

Novel NO Donors

The platelet-inhibitory actions of organic nitrates cannot be separated from their effects on the vascular wall. The concept of platelet-selective NO donor agents has arisen from our experiments with *S*-nitrosoglutathione (GSNO) (Radomski *et al.,* 1992). *S*-Nitrosoglutathione is a tripeptide *S*-nitrosothiol that is formed by S-nitrosylation of glutathione, the most abundant intracellular thiol. We have found that intravenous administration of GSNO into conscious rats inhibits platelet aggregation at doses that have only a small effect on blood pressure (Radomski *et al.,* 1992). Moreover, similar platelet/vascular differentiation is detected following intaarterial administration of GSNO into the circulation of human forearm (DeBelder *et al.,* 1994). Finally, we have infused GSNO into patients undergoing balloon angioplasty and found that this NO donor agent effectively protected platelets from activation at the site of angioplastic injury without altering blood pressure (Langford *et al.,* 1994). Interestingly, exposure of human neutrophils to NO led to depletion of glutathione stores, activation of the hexose monophosphate shunt, synthesis of endogenous GSNO, and inhibition of superoxide generation by neutrophils. Synthetic GSNO resulted in similar effects (Clancy *et al.,* 1994). Moreover, the administration of GSNO inhibited leukocyte activation, expression of iNOS, and bypass-induced myocardial lesion in dogs (Mayers *et al.,* 1999).

There is evidence that some of the platelet-regulatory properties of GSNO may also depend on GSNO/glutathione disulfide (GSSG)-stimulated L-arginine transport to platelets (Howard *et al.,* 1998) and may proceed in a cGMP-independent manner (Gordge *et al.,* 1998). These observations show that GSNO is a potent regulator of platelet and neutrophil functions, and it may be a prototype for the development of blood cell-selective NO donor compounds.

Nitro derivatives of aspirin and RGDS peptide have been synthesized in order to capitalize on the synergy of NO with cyclooxygenase inhibitors (aspirin) and the antagonists of the fibrinogen receptor (RGDS) (Wallace *et al.,* 1997; Gurevich *et al.,* 1997). The pharmacological and clinical potential of such hybrid compounds remains to be investigated.

NO-Independent Activators of GC-S

Benzylindazole derivatives including 3-(5'hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) have been synthesized and shown to be effective inhibitors of platelet adhesion and aggregation (Wu *et al.,* 1995). The pharmacological potential of direct stimulators of GC-S as antithrombotics remains to be studied.

Summary and Conclusions

Research over the past two decades has provided unequivocal evidence for the platelet-regulatory activity of NO generated during physiological and pharmacological reactions. Nitric oxide, thus formed, inhibits platelet adhesion, platelet recruitment, and aggregate formation. Moreover, NO can help in dissipating preformed platelet aggregates and can accelerate the process of repair of the vascular wall. The mechanisms of the remarkable properties of NO are both cGMP dependent and cGMP independent.

Pathological derangement in the generation, action, or metabolism of NO has been shown to contribute to the pathogenesis of occlusive vascular disorders. Exploring potential of NO donor compounds as antithrombotics holds a promise for pharmacological development of potent and platelet-selective therapies of vascular disorders associated with platelet activation and damage.

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References

Adams, M. R., Forsyth, C. J., Jessup, W., Robinson, J., and Celermajer, D. S. (1995). Oral L-arginine inhibits platelet aggregation but does not

enhance endothelium-dependent dilation in healthy young men. *J. Am. Coll. Cardiol.* **26,** 1054–1061.

Adrie, C., Bloch, K. D., Moreno, P. R., and Hurford, W. E. (1996). Inhaled nitric oxide increases coronary artery patency after thrombolysis. *Circulation* **94,** 1919–1926.

Aharony, D., Smith, J. B., and Silver, M. J. (1981). Regulation of arachidonate-induced platelet aggregation by the lipoxygenase product, 12-hydroperoxyeicosatetraenoic acid. *Biochim. Biophys. Acta* **718,** 193–200.

Albert, J., Wallen, N. H., Broijersen, A., Frostell, C., and Hjemdahl, P. (1996). Effects of inhaled nitric oxide compared with aspirin on platelet function in vivo in healthy volunteers. *Clin. Sci.* **91,** 225–231.

Alheid, U., Reichwehr, I., and Forstermann, U. (1989). Human endothelial cells inhibit platelet aggregation by separately stimulating platelet cyclic AMP and cyclic GMP. *Eur. J. Pharmacol.* **164,** 103–110.

Andrews, N. P., Dakak, N., Schenke, W. H., and Quyyumi, A. A. (1994). Platelet–endothelium interactions in humans: Changes in platelet cyclic guanosine monophosphate content in patients with endothelial dysfunction. *Circulation* **90,** I–397.

Andrews, R., May, J. A., Vickers, J., and Heptinstall, S. (1994). Inhibition of platelet aggregation by transdermal glyceryl trinitrate. *Br. Heart J.* **72,** 575–579.

Azuma, H., Ishikawa, M., and Sekizaki, S. (1986). Endothelium-dependent inhibition of platelet aggregation. *Br. J. Pharmacol.* **88,** 411–415.

Bani, D., Bigazzi, M., Massini, E., Bani, G., and Sacchi, T. B. (1995). Relaxin depresses platelet aggregation: In vitro studies on isolated rabbit and human platelets. *Lab. Invest.* **73,** 709–716.

Beckman, J., and Tsao, J. H. (1994). Reactions and diffusion of nitric oxide and peroxynitrite. *Biochemist* **16,** 8–10.

Benjamin, N., Dutton, J. A. E., and Ritter, J. M. (1991). Human vascular smooth muscle cells inhibit platelet aggregation when incubated with glyceryl trinitrate: Evidence for generation of nitric oxide. *Br. J. Pharmacol.* **102,** 847–850.

Berkels, R., Stockklauser, K., Rosen, P., and Rosen, R. (1997). Current status of platelet NO synthases. *Thromb. Res.* **87,** 51–55.

Bhardwaj, R., Page, C. P., May, G. R., and Moore, P. K. (1988). Endothelium-derived relaxing factor inhibits platelet aggregation in human whole blood in vitro and in the rat in vivo. *Eur. J. Pharmacol.* **157,** 83–91.

Bode-Boger, S. M., Boger, R. H., Galland, A., and Frolich, J. C. (1998). Differential inhibition of human platelet aggregation and thromboxane A_2 formation by L-arginine *in vivo* and *in vitro*. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **357,** 143–150.

Bodzenta-Lukaszyk, A., Gabryelewicz, A., Lukaszyk, A., Bielawiec, M., Konturek, J. W., and Domschke, W. (1994). Nitric oxide synthase inhibition and platelet function. *Thromb. Res.* **75,** 667–672.

Booth, B. P., Jacob, S., Bauer, J. A., and Fung, H. L. (1996). Sustained antiplatelet properties of nitroglycerin during hemodynamic tolerance in rats. *J. Cardiovasc. Pharmacol.* **28,** 432–438.

Broekman, M. J., Eiroa, A. M., and Marcus, A. J. (1991). Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension: Blockade of aggregation and secretion by an aspirin-insensitive mechanism. *Blood* **78,** 1033–1040.

Brown, A. S., and Martin, J. F. (1994). The megakaryocyte platelet system and vascular disease. *Eur. J. Clin. Invest.* **24**(Suppl. 1), 9–15.

Brown, A. S., Moro, M. A., Masse, J. M., Cramer, E. M., Radomski, M. W., and Darley-Usmar, V. (1998). Nitric oxide-dependent and -independent effects on human platelet treated with peroxynitrite. *Cardiovasc. Res.* **40,** 380–388.

Brune, B., and Hanstein, K. (1998). Rapid reversibility of nitric oxide-induced platelet inhibition. *Thromb. Res.* **90,** 83–91.

Brune, B., and Lapetina, E. G. (1989). Activation of a cytosolic ADP-ribosyltransferase by nitric oxide-generating agents. *J. Biol. Chem.* **264,** 8455–8458.

Buchanan, M. R., and Brister, S. J. (1991). Anithrombotics and the lipoxygenase pathway. *In* "Antithrombotics" (A. G. Herman, ed.), pp. 159–180. Kluwer Academic Publ., Dordrecht, The Netherlands.

Busse, R., and Fleming, I. (1998). Pulsatile stretch and shear stress: Physical stimuli determining the production of endothelium-derived relaxing factors. *J. Vasc. Res.* **35,** 73–84.

Busse, R., Luckhoff, A., and Bassenge, E. (1987). Endothelium-derived relaxing factor inhibits platelet activation. *Naunyn-Schmiedeberg's. Arch. Pharmacol.* **336,** 566–571.

Cadwgan, T. M., and Benjamin, N. (1993). Evidence for altered platelet nitric oxide synthesis in essential hypertension. *J. Hypertens.* **11,** 417–420.

Calver, A., Collier, J., Moncada, S., and Vallance, P. (1992). Effect of local infusion of N^G-monomethyl-L-arginine in patients with hypertension. The nitric oxide dilator mechanism appears abnormal. *J. Hypertens.* **10,** 1025–1031.

Caren, R., and Corbo, L. (1973). Response of plasma lipids and platelet aggregation to intravenous arginine. *Proc. Soc. Exp. Biol. Med.* **143,** 1067–1071.

Ceremuzynski, L., Chamiec, T., and Herbaczynska-Cedro, K. (1997). Effect of supplemental oral L-arginine on exercise capacity in patients with stable angina pectoris. *Am. J. Cardiol.* **80,** 331–333.

Chen, L. Y., and Mehta, J. L. (1994). Inhibitory effect of high-density lipoprotein on platelet function is mediated by increase in nitric oxide synthase activity in platelets. *Life Sci.* **23,** 1815–1821.

Chen, L. Y., and Mehta, J. L. (1996). Further evidence of the presence of constitutive and inducible nitric oxide synthase isoforms in human platelets. *J. Cardiovasc. Pharmacol.* **27,** 154–158.

Chen, L. Y., Mehta, P., and Mehta, J. L. (1996). Platelet inhibitory effect of nitroglycerin in platelet-rich plasma: Relevance of glutathione-*s*-transferase in plasma. *J. Invest. Med.* **44,** 5611–565.

Cheung, P. Y., Salas, E., Schulz, R., and Radomski, M. W. (1997). Nitric oxide and platelet function: Implications for neonatology. *Semin. Perinatol.* **21,** 409–417.

Cheung, P.-Y., Salas, E., Etches, P. C., Phillipos, E., Schulz, R., and Radomski, M. W. (1998). Inhaled nitric oxide and inhibition of platelet aggregation in critically ill neonates. *Lancet* **351,** 1181–1182.

Clancy, R. M., Levartovsky, D., Leszczynska-Piziak, J., Yegudin, J., and Abramson, S. B. (1994). Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrophils: Evidence for *S*-nitrosoglutathione as a bioactive intermediary. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 3680–3684.

Coller, B. S. (1992). Platelets in cardiovascular thrombosis and thrombolysis. *In* "The Heart and the Cardiovascular System Second Edition" (H. A. Fozzard, ed.), pp. 219–273. Raven, New York.

Cooke, J. P., and Tsao, P. (1992). Cellular mechanisms of atherogenesis and the effects of nitric oxide. *Curr. Opin. Cardiol.* **7,** 799–804.

Craven, P. A., and DeRubertis, F. R. (1978). Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and heme proteins. Evidence for involvement of the paramagnetic nitrosyl–heme complex in enzyme activation. *J. Biol. Chem.* **253,** 8433–8443.

DeBelder, A. J., and Radomski, M. W. (1994). Nitric oxide in the clinical arena. *J. Hypertens.* **12,** 617–624.

DeBelder, A. J., MacAllister, R., Radomski, M. W., Moncada, S., and Vallance, P. J. (1994). Effects of *S*-nitrosoglutathione in the human forearm circulation. Evidence for selective inhibition of platelet activation. *Cardiovasc. Res.* **28,** 691–694.

Diodati, J., Theroux, P., Latour, J. G., Lacoste, L., Lam, J. Y., and Waters, D. (1990). Effects of nitroglycerin at therapeutic doses on platelet aggregation in unstable angina pectoris and acute myocardial infarction. *Am. J. Cardiol.* **66,** 683–688.

Diodati, J. G., Dakak, N., Gilligan, D. M., and Quyyumi, A. A. (1998). Effect of atherosclerosis on endothelium-dependent inhibition of platelet activation in humans. *Circulation* **98,** 17–24.

Dong, Z., Staroselsky, A. H., Qi, X., Xie, K., and Fiedler, I. (1994). Inverse correlation between expression of inducible nitric oxide synthase activity and production of metastasis in K-1735 murine melanoma cells. *Cancer Res.* **54,** 789–793.

Dube, G., Salas, E., Kurtz, W., Christie, R., and Radomski, M. W. (1998). Effects of estrogen and an estrogen receptor modulator on atherogenesis in rabbits: Role of NO. *In* "The Biology of Nitric Oxide Part 6"

(S. Moncada, N. Toda, H. Maeda, and E. A. Higgs, eds.), p. 146. Portland Press, London and Miami, Florida.

Feelisch, M. (1991). The action and metabolism of organic nitrates and their similarity with endothelium-derived relaxing factor (EDRF). *In* ''Clinical Relevance of Nitric Oxide in the Cardiovascular System'' (S. Moncada, E. A. Higgs, and J. R. Berrazueta, eds.), pp. 29–43. Edicomplet, Madrid.

Feelisch, M., and Noack, E. A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylyl cyclase. *Eur. J. Pharmacol.* **139,** 19–30.

Fernandez-Patzon, C., Martinez-Cuesta, M., Salas, E., Sawicki, G., Wozniak, M., Radomski, M. W., and Davidge, S. T. (1999). Differential regulation of platelet aggregation by matrix-metalloproteinase -9 and -2. *Thrombosis Hemostasis* **82,** 1730–1735.

Freedman, J. E., Loscalzo, J., Barnard, M. R., Alpert, C., Keaney, J. F., Jr., and Michelson, A. D. (1997). Nitric oxide released from activated platelets inhibits platelet recruitment. *J. Clin. Invest.* **100,** 350–356.

Freedman, J. E., Ting, B., Hankin, B., Loscalzo, J., Keaney, J. F., and Vita, J. A. (1998). Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. *Circulation* **98,** 1481–1486.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Furlong, B., Henderson, A. H., Lewis, M. J., and Smith, J. A. (1987). Endothelium-derived relaxing factor inhibits in vitro platelet aggregation. *Br. J. Pharmacol.* **90,** 687–692.

Geng, Y., and Lotz, M. (1995). Increased intracellular Ca^{2+} selectively suppresses IL-1-induced NO production by reducing iNOS mRNA stability. *J. Cell Biol.* **129,** 1651–1657.

Gerzer, R., Karrenbrock, B., Siess, W., and Heim, J. M. (1988). Direct comparison of the effects of nitroprusside, SIN-1 and various nitrates on platelet aggregation and soluble guanylyl cyclase activity. *Thromb. Res.* **52,** 11–21.

GISSI-3 (GISSI III) study group (1994). Effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction. *Lancet* **343,** 1115–1122.

Goldberg, N. D., Haddox, M. K., Nicol, S. E., Glass, D. B., Sanford, C. H., Kuehl, F. A., Jr., and Estensen, R. (1975). Biological regulation through opposing influences of cyclic GMP and cyclic AMP: The yin yang hypothesis. *Adv. Cyclic Nucleotide Res.* **5,** 307–330.

Golino, P., Capelli-Bigazzi, M., Ambrosio, G., Ragui, M., Russolillo, E., Condorelli, M., and Chiaziello, M. (1992). Endothelium-derived relaxing factor modulates platelet aggregation in an in vivo model of recurrent platelet activation. *Circ. Res.* **71,** 1447–1456.

Gordge, M. P., Hothersall, J. S., and Noronha-Dutra, A. A. (1998). Evidence for a cyclic GMP-independent mechanism in the antiplatelet action of *S*-nitroso-glutathione. *Br. J. Pharmacol.* **124,** 141–148.

Gryglewski, R. J., Palmer, R. M. J., and Moncada, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* **320,** 454–456.

Gurevich, V. S., Lominadze, D. G., Adeagbo, A. S. O., Burov, S. V., Popov, Yu G., Leko, M. V., Miller, F. N., and Schuschke, D. A. (1997). Antithrombotic and vasorelaxant properties of a novel synthetic RGD peptide containing nitric oxide. *Pharmacology* **55,** 1–9.

Haffner, C., Jarchau, T., Reinhard, M., Hoppe, J., Lohmann, S. M., and Walter, U. (1995). Molecular cloning, structural analysis and functional expression of the proline-rich focal adhesion and microfilament-associated protein VASP. *EMBO J.* **14,** 19–27.

Hamberg, M., Svensson, J., and Samuelsson, B. (1974). Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 2994–2998.

Herbaczynska-Cedro, K., Lembowicz, K., and Pytel, B. (1991). N^G-Monomethyl-L-arginine increases platelet deposition on damaged endothelium in vivo. A scanning electron microscopy study. *Thromb. Res.* **64,** 1–9.

Hines, R., and Barash, P. G. (1989). Infusion of sodium nitroprusside induces platelet dysfunction in vitro. *Anesthesiology* **71,** 805–806.

Hogan, J. C., Lewis, M. J., and Henderson, A. H. (1988). In vivo EDRF activity influences platelet function. *Br. J. Pharmacol.* **94,** 1020–1022.

Hogg, N., Darley-Usmar, V. M., Wilson, M. T., and Moncada, S. (1993). Oxidation of alpha-tocopherol in human low density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Lett.* **326,** 199–203.

Horowitz, H. I., Stein, I. M., Cohen, B. D., and White, J. G. (1970). Further studies on the platelet-inhibitory effect of guanidinosuccinic acid and its role in uremic bleeding. *Am. J. Med.* **49,** 336–345.

Horowitz, J. D., Henry, C. A., Syrjanen, M. L., Louis, W. J., Fish, R. D., Smith, T. W., and Antman, E. M. (1988). Combined use of nitroglycerin and N-acetylcysteine in the management of unstable angina pectoris. *Circulation* **77,** 787–794.

Horstrup, K., Jablonka, B., Hönig-Liedl, P., Just, M., Kochsiek, K., and Walter, U. (1994). Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *Eur. J. Biochem.* **225,** 21–27.

Houston, D. S., and Buchanan, M. R. (1994). Influence of endothelium-derived relaxing factor on platelet function and hemostasis in vivo. *Thromb. Res.* **74,** 25–37.

Houston, D. S., Robinson, P., and Gerrard, J. M. (1990). Inhibition of intravascular platelet aggregation by endothelium-derived relaxing factor: Reversal by red blood cells. *Blood* **76,** 953–958.

Howard, C. M., Sexton, D. J., and Mutus, B. (1998). *S*-Nitrosoglutathione/glutathione disulphide/Cu^{2+}-dependent stimulation of L-arginine transport in human platelets. *Thromb. Res.* **91,** 113–120.

Humphries, R. G., Tomlinson, W., O'Connor, S. E., and Leff, P. (1990). Inhibition of collagen- and ADP-induced platelet aggregation by substance P in vivo: Involvement of endothelium-derived relaxing factor. *J. Cardiovasc. Pharmacol.* **16,** 292–297.

Ichiki, K., Ikeda, H., Haramaki, N., Ueno, T., and Imaizumi, T. (1996). Long-term smoking impairs platelet-derived nitric oxide release. *Circulation* **94,** 3109–3114.

ISIS-2 (1988). (Second International Study of Infarct Survival) Collaborative Group: Randomized trial of intravenous streptokinase, oral aspirin, both or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. *Lancet* **2,** 349–360.

ISIS-3 (1992). A randomized comparison of streptokinase vs tissue plasminogen activator vs anistreplase and of aspirin plus heparin vs aspirin alone among 41,299 cases of suspected acute myocardial infarction. *Lancet* **339,** 753–770.

ISIS-4 (1993). ISIS collaborative group, Oxford, U.K.: Randomised study of oral isosorbide mononitrate in over 50,000 patients with suspected acute myocardial infarction. *Circulation* **88,** I–394.

Johansson, J. S., and Haynes, D. H. (1992). Cyclic GMP increases the rate of the calcium extrusion pump in intact platelets but has no direct effect on the dense tubular calcium accumulation system. *Biochim. Biophys. Acta* **1105,** 40–50.

Katsuki, S., Arnold, W., Mittal, C., and Murad, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic Nucleotide Res.* **3,** 23–35.

Kilbourn, R. G., Gross, S. S., Adams, J., Jubran, A., Griffith, O. W., Levi, R., and Lodato, R. F. (1990). N^G-Methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: Implications for the involvement of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 3029–3032.

Komamura, K., Node, K., Kosaka, H., and Inoue, M. (1994). Endogenous nitric oxide inhibits microthromboembolism in the ischemic heart. *Circulation* **90,** I–345.

Lam, J. Y. T., Chesebro, J. H., and Fuster, V. (1988). Platelets, vasoconstriction, and nitroglycerin during arterial wall injury. A new antithrombotic role for an old drug. *Circulation* **78,** 712–716.

Langford, E. J., Brown, A. S., Wainwright, R. J., de Belder, A. J., Thomas, M. R., Smith, R. E., Radomski, M. W., Martin, J. F., and Moncada, S. (1994). Inhibition of platelet activity by *S*-nitrosoglutathione during coronary angioplasty. *Lancet* **344,** 1458–1460.

Lantoine, F., Brunet, A., Bedioui, F., Devynck, J., and Devynck, M. A. (1995). Direct measurement of nitric oxide production in platelets: Re-

lationship with cytosolic Ca^{2+} concentration. *Biochem. Biophys. Res. Commun.* **215,** 842–848.

Launay, J. M., Bondoux, D., Oset-Gasque, M. J., Emami, S., Mutel, V., Haimart, M., and Gespach, C. (1994). Increase of human platelet serotonin uptake by atypical histamine receptors. *Am. J. Physiol.* **266,** R526–R536.

Lefer, D. J., Nakanishi, K., and Vinten-Johansen, J. (1993). Endothelial and myocardial cell protection by a cysteine-containing nitric oxide donor after myocardial ischemia and reperfusion. *J. Cardiovasc. Pharmacol.* **22**(Suppl. 7), S34–S43.

Lelchuk, R., Radomski, M. W., Martin, J. F., and Moncada, S. (1992). Constitutive and inducible nitric oxide synthases in human megakaryoblastic cells. *J. Pharmacol. Exp. Ther.* **262,** 1220–1224.

Levin, R. L., Weksler, B. B., and Jaffe, E. A. (1982). The interaction of sodium nitroprusside with human endothelial cells and platelets: Nitroprusside and prostacyclin synergistically inhibit platelet function. *Circulation* **66,** 1299–1307.

Leza, J. C., Salas, E., Sawicki, G., Russell, J. C. R., and Radomski, M. W. (1998). The effects of stress on homeostasis in JCR-LA-cp rats: The role of nitric oxide. *J. Pharmacol. Exp. Ther.* **286,** 1397–1403.

Liu, Z., Nakatsu, K., Brien, J. F., Beaton, E. D., Marks, G. S., and Maurice, D. H. (1993). Selective sequestration of nitric oxide by subcellular components of vascular smooth muscle and platelets: Relationship to nitric oxide stimulation of the soluble guanylyl cyclase. *Can. J. Physiol. Pharmacol.* **71,** 938–945.

Loscalzo, J. (1985). *N*-Acetylcysteine potentiates inhibition of platelet aggregation nitroglycerin. *J. Clin. Invest.* **76,** 703–738.

Luscher, T. F., Tanner, F. C., Tschudi, M. R., and Noll, G. (1993). Endothelial dysfunction in coronary artery disease. *Annu. Rev. Med.* **44,** 395–418.

Macdonald, P. S., Read, M. A., and Dusting, G. J. (1988). Synergistic inhibition of platelet aggregation by endothelium-derived relaxing factor and prostacyclin *Thromb. Res.* **49,** 437–449.

McKendrick, J. D., Salas, E., Dube, G. P., Murat, J., Russell, J. C., and Radomski, M. W. (1998). Inhibition of nitric oxide generation unmasks vascular dysfunction in insulin-resistant, obese JCR:LA-cp rats. *Br. J. Pharmacol.* **124,** 361–369.

Malinski, T., Radomski, M. W., Taha, Z., and Moncada, S. (1993). Direct electrochemical measurement of nitric oxide released from human platelets *Biochem. Biophys. Res. Commun.* **194,** 960–965.

Martinez-Cuesta, M. A., and Radomski, M. W. (1998). Cyclic GMP mediates the inhibitory effect of nitric oxide on platelet adhesion. *In* ''The Biology of Nitric Oxide Part 6'' (S. Moncada, N. Toda, N. H. Maeda, and E. A. Higgs, eds.), p. 141. Portland Press, London and Miami.

Maurice, D. H., and Haslam, R. J. (1990). Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: Inhibition of cyclic AMP breakdown by cyclic GMP. *Mol. Pharmacol.* **37,** 671–681.

May, G. R., Crook, P., Moore, P. K., and Page, C. P. (1991). The role of nitric oxide as an endogenous regulator of platelet and neutrophil activation within the pulmonary circulation. *Br. J. Pharmacol.* **102,** 759–763.

Mayers, I., Johnson, D., Salas, E., Hurst, T., and Radomski, M. W. (1999). Myocardial activity of nitric oxide synthase is increased following canine cardiopulmonary bypass. *J. Cardiovsc. Thorac. Surg.* **117,** 1009–1016.

Mehta, J. L., Chen, L. Y., Kone, C. B., Mehta, P., and Turner, P. (1995). Identification of constitutive and inducible forms of nitric oxide synthase in human platelets. *J. Lab. Clin. Med.* **125,** 370–377.

Mellion, B. T., Ignarro, L. J., Ohlstein, E. H., *et al.* (1981). Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related nitrovasodilators. *Blood* **57,** 946–955.

Mendelsohn, M. E., O'Neill, S., George, D., and Loscalzo, J. (1990). Inhibition of fibrinogen binding to human platelets by *S*-nitroso-*N*-acetylcysteine. *J. Biol. Chem.* **265,** 19028–19034.

Menshikov, M. Y., Ivanova, K., Schaefer, M., Drummer, C., and Gerzer, R. (1993). Influence of the cGMP analogue 8-PCPT-cGMP on agonist-induced increases in ionized calcium and on aggregation of human platelets. *Eur. J. Pharmacol.* **245,** 281–284.

Michelson, A. D., Benoit, S. E., Furman, M. I., Breckwolt, W. L., Rohrer, M. J., Barnard, M. R., and Loscalzo, J. (1996). Effects of nitric oxide/EDRF on platelet surface glycoproteins. *Am. J. Physiol.* **39,** H1640–H1648.

Minuz, P., Andriolli, G., Degan, M., Gaino, S., Ortolani, R., Tommasoli, R., Zaliani, V., Lechi, A., and Lechi, C. (1998). The F_2-isoprostane 8-epiprostaglandin $F_{2\alpha}$ increases platelet adhesion and reduces the anti-adhesive and antiaggregatory effects of NO. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1248–1256.

Moncada, S. (1982). Biological importance of prostacyclin. *Br. J. Pharmacol.* **76,** 3–31.

Moncada, S., Gryglewski, R. J., Bunting, S., and Vane, J. R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* **263,** 663–665.

Moro, M. A., Darley-Usmar, V. M., Goodwin, D. A., Read, N. G., Zamora-Pino, R., Feelisch, M., Radomski, M. W., and Moncada, S. (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6702–6706.

Moro, M. A., Darley-Usmar, V. M., Lizasoain, I., Su, Y., Knowles, R. G., Radomski, M. W., and Moncada, S. (1995). The formation of nitric oxide donors from peroxynitrite. *Br. J. Pharmacol.* **116,** 1999–2004.

Moro, M. A., Russell, R. J., Cellek, S., Lizasoain, I., Su, Y., Darley-Usmar, V. M., Radomski, M. W., and Moncada, S. (1996). cGMP mediates the vascular and platelet actions of nitric oxide. Confirmation using an inhibitor of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1480–1485.

Murohara, T., Parkinson, S. J., Waldman, S. A., and Lefer, A. M. (1995). Inhibition of nitric oxide biosynthesis provides P-selectin expression in platelets. Role of protein kinase C. *Arterioscler Thromb. Vasc. Biol.* **15,** 2068–2075.

Muruganandam, A., and Mutus, B. (1994). Isolation of nitric oxide synthase from human platelets. *Biochim. Biophys. Acta* **1200,** 1–6.

Nakashima, S., Tohmatsu, T., Hattori, H., Okano, Y., and Nozawa, Y. (1986). Inhibitory action of cyclic GMP on secretion, phosphoinositide hydrolysis and calcium mobilization in thrombin-stimulated human platelets. *Biochem. Biophys. Res. Commun.* **135,** 1099–1104.

Nakatsuka, M., and Osawa, Y. (1994). Selective inhibition of the 12-lipoxygenase pathway of arachidonic acid metabolism by L-arginine or sodium nitroprusside in intact human platelets. *Biochem. Biophys. Res. Commun.* **200,** 1630–1634.

Nassem, K. M., and Bruckdorfer, K. R. (1997). Nitration of platelet cytosolic proteins by peroxynitrite. *Biochem. Soc. Trans.* **25,** 397S.

Noris, M., Benigni, A., Boccardo, P., Aiello, S., Gaspari, F., Todeschini, M., Figliuzzi, M., and Remuzzi, G. (1993). Enhanced nitric oxide synthesis in uremia: Implications for platelet dysfunction and dialysis hypotension. *Kidney Int.* **44,** 445–450.

Nowak, J., Radomski, M., Kaijser, L., and Gryglewski, R. J. (1981). Conversion of exogenous arachidonic acid to prostaglandins in the pulmonary circulation in vivo. A human and animal study. *Acta Physiol. Scand.* **112,** 405–411.

Okada, M., Sagawa, T., Tominaga, A., Kodama, T., and Hitsumoto, Y. (1996). Two mechanisms of platelet-mediated killing of tumour cells: One cyclo-oxygenase dependent and the other nitric oxide dependent. *Immunology* **89,** 158–164.

Olsen, S. B., Tang, D. B., Jackson, M. R., Gomez, E. R., Ayala, B., and Alving, B. M. (1996). Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model. *Circulation* **93,** 327–332.

Palmer, R. M. J., Ashton, D. S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333,** 664–666.

Palmer, R. M. J., Bridge, L., Foxwell, N. A., and Moncada, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.* **105,** 11–12.

Patrono, C. (1989). Aspirin and human platelets: From clinical trials to

acetylation of cyclooxygenase and back. *Trends Pharmacol. Sci.* **10,** 453–458.

Pawloski, J. R., Swaminathan, R. V., and Stamler, J. S. (1998). Cell free and erythrocytic *S*-nitrosohemoglobin inhibits human platelet aggregation. *Circulation* **97,** 263–267.

Plotkine, M., Allix, M., Guillou, J., and Boulu, R. (1991). Oral administration of isosorbide dinitrate inhibits arterial thrombosis in rats. *Eur. J. Pharmacol.* **201,** 115–116.

Pohl, U., and Busse, R. (1989). EDRF increases cyclic GMP in platelets during passage through the coronary vascular bed. *Circ. Res.* **65,** 1798–1803.

Polanowska-Grabowska, R., and Gear, A. R. L. (1994). Role of cyclic nucleotides in rapid platelet adhesion to collagen. *Blood* **83,** 2508–2515.

Radomski, M. W. (1995). Nitric oxide-biological mediator, modulator and effector molecule. *Ann. Med.* **27,** 321–330.

Radomski, M. W., and Moncada, S. (1991). Role of nitric oxide in endothelial cell–platelet interactions. *In* ''Antithrombotics'' (A. G. Herman, ed.), pp. 25–48. Kluwer Academic Publ., Dordrecht, The Netherlands, Boston, and London.

Radomski, M. W., and Radomski, A. S. (1999). Regulation of blood cell function by the endothelial cells. *In* ''Vascular Endothelium in Human Physiology and Pathophysiology'' (P. Vallance and D. Webb, eds.). Kluwer Academic Publ. Dordrecht, The Netherlands.

Radomski, M. W., and Salas, E. (1995). Nitric oxide-biological mediator, modulator and factor of injury: Its role in the pathogenesis of atherosclerosis. *Atherosclerosis* **118**(Suppl.), S69–S80.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987a). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2,** 1057–1058.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987b). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem. Biophys. Res. Commun.* **148,** 1482–1489.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987c). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.* **92,** 181–187.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987d). The anti-aggregating properties of vascular endothelium: Interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.* **92,** 639–646.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1990a). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 5193–5197.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1990b). Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br. J. Pharmacol.* **101,** 325–328.

Radomski, M. W., Jenkins, D. C., Holmes, L., and Moncada, S. (1991). Human colorectal adenocarcinoma cells: Differential nitric oxide synthesis determines their ability to aggregate platelets. *Cancer Res.* **51,** 6073–6078.

Radomski, M. W., Rees, D. D., Dutra, A., and Moncada, S. (1992). *S*-Nitrosoglutathione inhibits platelet activation in vitro and in vivo. *Br. J. Pharmacol.* **107,** 745–749.

Radomski, M. W., Vallance, P., Whitley, G., Foxwell, N., and Moncada, S. (1993). Platelet adhesion to human vascular endothelium is modulated by constitutive and cytokine induced nitric oxide. *Cardiovasc. Res.* **27,** 1380–1382.

Radomski, M. W., Zakar, T., and Salas, E. (1996). Methods of working with nitric oxide in platelets. *Methods Enzymol.* **269,** 88–107.

Read, N. G., Radomski, M. W., Goodwin, D. A., and Moncada, S. (1985). An ultrastructural study of human washed platelets after washing with prostacyclin. *Br. J. Haematol.* **60,** 305–314.

Reinhard, M., Halbrügge, M., Scheer, U., Wergand, C., Jockusch, B. M., and Walter, U. (1992). The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. *EMBO J.* **11,** 2063–2070.

Riddell, D. R., Graham, A., and Owen, J. S. (1997). Apolipoprotein E inhibits platelet aggregation through the L-arginine:nitric oxide pathway. Implications for vascular disease. *J. Biol. Chem.* **272,** 89–95.

Rosenblum, W. I., Nelson, G. H., and Povlishock, J. T. (1987). Laser-induced endothelial damage inhibits endothelium-dependent relaxation in the cerebral microcirculation of the mouse. *Circ. Res.* **60,** 169–176.

Salas, E., Moro, M. A., Askew, S., Hodson, H. F., Butler, A. R., Radomski, M. W., and Moncada, S. (1994). Comparative pharmacology of analogues of *S*-nitroso-*N*-acetyl-DL-penicillamine in platelets. *Br. J. Pharmacol.* **112,** 1071–1076.

Salas, E., Miszta-Lane, H., and Radomski, M. W. (1997a). Regulation of platelet function by nitric oxide and other nitrogen- and oxygen-derived species. *In* ''Handbook of Experimental Pharmacology'' (F. Von Bruchhausen and U. Walter, eds.), Vol. 126, pp. 371–397. Springer-Verlag, Berlin, Heidelberg, and New York.

Salas, E., Busletta, A., and Radomski, M. W. (1997b). Generation of NO, cGMP and platelet function in normal pregnancy and preeclampsia. *Jpn. J. Pharmacol.* **75**(Suppl. I), 116P.

Samama, C. M., Diaby, M., Fellahi, J. L., Molhafar, A., Eyrand, D., Arock, M., Guillosson, J. J., Coriat, P., and Rouby, J. J. (1995). Inhibition of platelet aggregation by inhaled nitric oxide in patients with acute adult respiratory distress syndrome. *Anesthesiology* **83,** 56–65.

Sane, D. C., Bielawska, A., Greenberg, C. S., and Hannun, Y. A. (1989). Cyclic GMP analogs inhibit gamma thrombin-induced arachidonic acid release in human platelets. *Biochem. Biophys. Res. Commun.* **165,** 708–714.

Sase, K., and Michel, T. (1995). Expression of constitutive, endothelial nitric oxide synthase in human blood platelets. *Life Sci.* **57,** 2049–2055.

Sawicki, G., Salas, E., Murat, J., Miszta-Lane, H., and Radomski, M. W. (1997). Release of gelatinase A from human platelets mediates aggregation. *Nature* **386,** 616–619.

Sawicki, G., Jurasz, P., Duszyk, M., Mayers, I., Radomski, M. W. (1999). Matrix metalloproteinases in tumour cell-induced human platelet aggregation: Regulation by NO. *Acta Physiol. Scand.* **167,** 18.

Schultz, P. J., and Raij, L. (1992). Endogenously synthesized nitric oxide prevents endotoxin-induced glomerular thrombosis. *J. Clin. Invest.* **90,** 1718–1725.

Shahbazi, T., Jones, N., Radomski, M. W., Moro, M. A., and Gingell, D. (1994). Nitric oxide donors inhibit platelet spreading on surfaces coated with fibrinogen but not fibronectin. *Thromb. Res.* **75,** 631–642.

Shen, Y. C., and Hong, C. Y. (1995). Effect of trilinolein on cyclic nucleotide formation in human platelets: Relationship with its antiplatelet effect and nitric oxide synthesis. *Br. J. Pharmacol.* **116,** 1644–1648.

Sheu, J. R., Hung, W. C., Kan, Y. C., Lee, Y. M., and Yen, M. H. (1998). Mechanisms involved in the antiplatelet activity of *Escherichia coli* lipopolysaccharide in human platelets. *Br. J. Haematol.* **103,** 29–38.

Simon, D. I., Stamler, J. S., Loh, E., Loscalzo, J., Francis, S. A., and Craeger, M. A. (1995). Effect of nitric oxide synthase inhibition on bleeding time in humans. *J. Cardiovasc. Pharmacol.* **26,** 339–342.

Sinzinger, H., Fitscha, P., O'Grady, J., Rauscha, F., Rogatti, W., and Vane, J. R. (1990). Synergistic effect of prostaglandin E_1 and isosorbide dinitrate in peripheral vascular disease. *Lancet* **335,** 627–628.

Slawinski, M., Grodzinska, L., Kostka-Trabka, E., Goszcz, A., and Gryglewski, R. J. (1996). L-Arginine, substrate for NO synthesis, its beneficial effects in therapy of patients with peripheral arterial disease: Comparison with placebo—preliminary results. *Acta Physiol. Hung.* **84,** 457–458.

Sneddon, J. M., and Vane, J. R. (1988). Endothelium-derived relaxing factor reduces platelet adhesion to bovine endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2800–2804.

Spain, D. A., Wilson, M. A., and Garrison, R. N. (1994). Nitric oxide synthase inhibition exacerbates sepsis-induced renal hypoperfusion. *Surgery* **116,** 322–331.

Stetler-Stevenson, W. G. (1996). Dynamics of matrix turnover during pathologic remodelling of the extracellular matrix. *Am. J. Pathol.* **148,** 1345–1350.

Thomson, L., Lawton, F. G., Knowles, R. G., Beesley, J. E., Riveros-Moreno, V., and Moncada, S. (1994). Nitric oxide synthase activity in human gynecological cancer. *Cancer Res.* **54,** 1352–1354.

Trovati, M. Anfossi, G., Massucco, P., Mattiello, L., Costamagna, C.,

Piretto, V., Mularoni, E., Cavalot, F., Bosia, A., and Ghigo, D. (1997). Insulin stimulates nitric oxide synthesis in human platelets and, through nitric oxide, increases platelet concentrations of both guanosine-3',5'-cyclic monophosphate and adenosine-3',5'-cyclic monophosphate. *Diabetes* **46,** 742–749.

Tsao, P. S., Theilmeier, G., Singer, A. H., Leung, L. L. K., and Cooke, J. P. (1994). L-Arginine attenuates platelet reactivity in hypercholesterolemic rabbits. *Arterioscler. Thromb.* **14,** 1529–1533.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.* **231,** 232–235.

Venturini, C. M., Del Vecchio, P. J., and Kaplan, J. E. (1989). Thrombin-induced platelet adhesion to endothelium is modified by endothelial derived relaxing factor (EDRF). *Biochem. Biophys. Res. Commun.* **159,** 349–354.

Villa, L. M., Salas, E., Darley-Usmar, V. M., Radomski, M. W., and Moncada, S. (1994). Peroxynitrite induces both vasodilation and impaired vascular relaxation in the rat isolated perfused heart. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12383–12387.

Wallace, J. L., McKnight, W., Del Soldato, P., Baydoun, A. R., and Cirino, G. (1997). Anti-thrombotic effects of a nitric oxide-releasing, gastric-sparing aspirin derivative. *J. Clin. Invest.* **96,** 2711–2718.

Wallerath, T., Gath, I., Aulitzky, W. E., Pollock, J. S., Kleinert, H., and Förstermann, U. (1997). Identification of NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets. *Thromb. Haemostas.* **77,** 163–167.

Walter, U. (1989). Physiological role of cGMP and cGMP-dependent protein kinase in the cardiovascular system. *Rev. Physiol. Biochem. Pharmacol.* **113,** 41–88.

Wang, G. R., Zhu, Y., Halushka, P. V., Lincoln, T. M., and Mendelsohn, M. E. (1998). Mechanism of platelet inhibition by nitric oxide: In vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 4888–4893.

Wautier, J. L., Weill, D., Kadeva, H., Maclouf, J., and Soria, C. (1989). Modulation of platelet function by SIN-1A. *J. Cardiovasc. Pharmacol.* **14,** S111–S114.

Weber, A. A., Neuhaus, T., Seul, C., Dusing, R., Schror, K., Sachinidis, A., and Vetter, H. (1996). Biotransformation of glyceryl trinitrate by blood platelets as compared to vascular smooth muscle cells. *Eur. J. Pharmacol.* **309,** 209–213.

Werns, S. W., Rote, W. E., Davis, J. H., Guevara, T., and Lucchesi, B. R. (1994). Nitroglycerin inhibits experimental thrombosis and reocclusion after thrombolysis. *Am. Heart J.* **127,** 727–737.

Willerson, J. T., Igo, S. R., Yao, S. K., Ober, J. C., Macris, M. P., and Ferguson, J. J. (1996). Localised administration of sodium nitroprusside enhances its protection against platelet aggregation in stenosed and injured coronary arteries. *Texas Heart Inst. J.* **23,** 1–8.

Wright, C. E., Rees, D. D., and Moncada, S. (1992). Protective and pathological role of nitric oxide in endotoxin shock. *Cardiovasc. Res.* **26,** 48–57.

Wu, C.-C., Ko, F.-N., Kuo, S.-C., Lee, F.-Y., and Teng, C.-M. (1995). YC-1 inhibited platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br. J. Pharmacol.* **116,** 1973–1978.

Yao, S. K., Ober, J. C., Krishnaswami, A., Ferguson, J. J., Anderson, H. V., Golino, P., Buja, L. M., and Willerson, J. T. (1992). Endogenous nitric oxide protects against platelet aggregation and cyclic flow variations in stenosed and endothelium-injured arteries. *Circulation* **86,** 1302–1309.

Yusuf, S., MacMahon, S., Collins, R., and Peto, R. (1988). Effect of intravenous nitrates on mortality in acute myocardial infarction: An overview of the randomised trials. *Lancet* **1,** 1088–1092.

Zhou, Q., Hellermann, G. R., and Solomonson, L. P. (1995). Nitric oxide release from resting platelets. *Thromb. Res.* **77,** 87–96.

CHAPTER 52

Pathophysiological Roles of Nitric Oxide in Inflammation

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LOCAL AND SYSTEMIC INFLAMMATION IS CHARACTERIZED BY THE OVERPRODUCTION OF NITRIC OXIDE (NO). IN MOST INSTANCES, THE SOURCE OF THIS NO IS THE INDUCIBLE ISOFORM OF NO SYNTHASE (iNOS). THE EXPRESSION OF iNOS, WHICH HAS BEEN DEMONSTRATED IN ACTIVATED MACROPHAGES AND IN A VARIETY OF OTHER CELL TYPES IN INFLAMMATION, SERVES TO FIGHT INVADING MICROORGANISMS. HOWEVER, NO PRODUCED BY iNOS ALSO CAN EXERT A VARIETY OF AUTOCRINE CYTOTOXIC EFFECTS. SELECTIVE INHIBITION OF iNOS HAS BEEN SHOWN TO SUPPRESS INFLAMMATION IN A VARIETY OF INFLAMMATORY STATES, INCLUDING ARTHRITIS, COLITIS, ENCEPHALOMYELITIS, AND SYSTEMIC INFLAMMATORY RESPONSE SYNDROME (CIRCULATORY SHOCK). HIGH LOCAL LEVELS OF NO CAN BE TOXIC AND CAN INHIBIT KEY CELLULAR ENZYMES, INCLUDING ENZYMES OF THE MITOCHONDRIAL RESPIRATORY CHAIN. THESE EFFECTS ARE OFTEN DUE TO S-NITROSYLATION REACTIONS.

MANY FORMS OF INFLAMMATION ARE ASSOCIATED WITH THE OVERPRODUCTION OF SUPEROXIDE ANION AS WELL AS NO. THE TOXICITY OF NO INCREASES WHEN IT COMBINES WITH SUPEROXIDE AND FORMS PEROXYNITRITE ANION, A LABILE REACTIVE OXIDANT SPECIES. PEROXYNITRITE CAN REACT VIA A NUMBER OF MECHANISMS WITH PROTEINS, LIPIDS, AND DNA UNDER CONDITIONS OF INFLAMMATION. INITIATION OF LIPID PEROXIDATION AND DIRECT INHIBITION OF VARIOUS KEY ENZYMES IN THE CELL MEMBRANE, MITOCHONDRIA, AND CYTOPLASM CONTRIBUTE TO THE CYTOTOXIC EFFECT OF PEROXYNITRITE. IN ADDITION, PEROXYNITRITE IS A POTENT TRIGGER OF DNA STRAND BREAKAGE, WITH SUBSEQUENT ACTIVATION OF THE NUCLEAR ENZYME POLY(ADP-RIBOSE) SYNTHETASE (PARS), WITH EVENTUAL NECROTIC-TYPE CELL DEATH. NO, OR PEROXYNITRITE, CAN ALSO ENHANCE THE CATALYTIC ACTIVITY OF CYCLOOXYGENASES, WHICH TRIGGERS AND ENHANCES SOME OF THE INFLAMMATORY RESPONSES.

ALTHOUGH NO, DERIVED FROM iNOS, IS GENERALLY VIEWED AS A TERMINAL EFFECTOR OF INFLAMMATION, UNDER CERTAIN CONDITIONS IT CAN ALSO ACT AS AN AMPLIFIER OF PROINFLAMMATORY PATHWAYS. STUDIES UTILIZING iNOS-DEFICIENT MICE CONFIRMED THE CRUCIAL ROLE OF iNOS IN SOME BUT NOT ALL FORMS OF INFLAMMATION. FUTURE WORK, USING SELECTIVE INHIBITORS OF iNOS, IS REQUIRED TO DETERMINE THE EVENTUAL UTILITY OF iNOS INHIBITION FOR THE EXPERIMENTAL THERAPY OF INFLAMMATORY DISEASES IN HUMANS.

Introduction

The production of nitric oxide (NO) in inflammation is generally considered a defense mechanism of the body against invading microorganisms. Indeed, activated macrophages and neutrophils are able to kill a variety of microorganisms via NO-mediated mechanisms, or via mechanisms that involve a combination of nitrogen- and oxygen-derived

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free radicals and oxidants. In this chapter, we do not discuss the roles of NO and related species in host defense, for these topics are subjects of separate chapters in this book. Similarly, we will not discuss, in detail, the defense mechanisms that microorganisms developed against the toxic effects of NO. In this chapter, we will focus on the following issues: (1) the regulation of NO production in inflammation, with special reference to *in vivo* studies, and (2) the mechanisms of the cytotoxic actions of NO, as they relate to injury to the host (i.e., mammalian or human) organism. We will also avoid detailed discussions related to the role of NO in specific inflammatory conditions [such as arthritis, diabetes, encephalomyelitis, and systemic inflammatory response syndrome (circulatory shock)], as these are subjects of separate chapters in this book.

Production of Nitric Oxide in Inflammation

Nitric Oxide Derived from Inducible NO Synthase

Inducers of iNOS Expression in Inflammation

In inflammation, proinflammatory cytokines and lipid mediators play a key role in triggering the expression of the inducible isoform of NO synthase (iNOS, or NOS-2) in various cell types. This statement is supported by multiple lines of evidence. (1) A number of proinflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), γ-interferon (IFN-γ), and interleukin-2 (IL-2), can induce the expression of iNOS *in vitro* or *in vivo.* This usually occurs in an additive or synergistic fashion (for review, see Szabó and Thiemermann, 1995). Although proinflammatory cytokines are generally considered the prime or most important stimuli of iNOS expression, there is also growing evidence that the lipid mediator platelet-activating factor (PAF) can also induce the expression of iNOS in various cell types. For example, PAF and its various analogs have been shown to induce iNOS and enhance lipopolysaccharide (LPS)-induced expression of iNOS in macrophages (Szabó *et al.,* 1993a; Arthur *et al.,* 1995; De Kimpe *et al.,* 1995; Howard and Erickson, 1996). Kupffer cells (Mustafa *et al.,* 1996), rat astrocytes (Cardile *et al.,* 1996), and cultured vascular smooth muscle cells (Arthur *et al.,* 1995). (2) The just mentioned cytokine and lipid mediators are known to be produced in various forms of inflammation. (3) Finally, neutralization of these mediators, by the use of receptor blockers or neutralizing antibodies, suppresses the expression of iNOS in inflammation (Thiemermann *et al.,* 1993a; Szabó *et al.,* 1993a,b; Cunha *et al.,* 1994; Szabó, 1995; De Kimpe *et al.,* 1995; Mustafa *et al.,* 1996; Arthur *et al.,* 1995; Ruetten and Thiemermann, 1997a; Ter Steege *et al.,* 1998; Perkins *et al.,* 1998) (Fig. 1).

The most extensively studied inflammatory condition, with respect to the mechanism of iNOS expression, is systemic inflammation induced by bacterial lipopolysaccharide (i.e., endotoxic shock). The primary initiator of the inflammatory response in gram-negative septic shock is bacterial LPS (or endotoxin), a component of the bacterial outer membrane. Endotoxins consist of a structurally and antigenically diverse set of series of oligosaccharides that are located in the outermost part of the molecule, including core oligosaccharides, which have a more homogeneous structure, and an innermost lipid part, named lipid A, with a highly conserved structure. According to many reports, lipid A is responsible for most of the immunological and toxic effects of LPS (Freudenberg and Galanos, 1990; Raetz *et al.,* 1991). LPS is a potent stimulator of iNOS expression, which, in many cell types, acts in synergy with IFN-γ (Szabó, 1995; Kilbourn *et al.,* 1997). Lipid A was found to be a weak inducer of iNOS in various cell types (Hattori *et al.,* 1995). However, lipid A was proposed as a good candidate for the elicitation of tolerance against the induction of iNOS by endotoxin (Hattori *et al.,* 1995). Similarly, using a detoxified LPS molecule, one can induce tolerance against the ability of LPS to induce iNOS, without inducing high, potentially toxic levels of iNOS (Gilad *et al.,* 1996).

Although gram-negative bacterial components, chiefly LPS, are the subjects of most investigations focusing on septic shock and systemic inflammation, there is also good evidence that various gram-positive bacterial components can elicit the expression of iNOS *in vitro* and *in vivo* (Freyer *et al.,* 1996; Hirvonen *et al.,* 1997; Orman *et al.,* 1998; Hattori *et al.,* 1997, 1998; Kengatharan *et al.,* 1996, 1998). Importantly, lipoteichoic acid, a component of the membrane of gram-positive bacteria, induces iNOS (Hattori *et al.,* 1997, 1998) in marked synergy with peptidoglycan, another gram-positive membrane component (Kengatharan *et al.,* 1996). Studies have also established that a specific fragment of peptidoglycan, *N*-acetylglucosamine-β(1→4)-*N*-acetylmuramyl-L-alanine-D-isoglutamine, is the key moiety responsible for the synergism with lipoteichoic acid (or IFN-γ) to induce NO formation in rodent cells (Kengatharan *et al.,* 1998).

Whole gram-positive or gram-negative bacteria have also been shown to elicit the expression of iNOS (Orman *et al.,* 1998; Hirvonen *et al.,* 1998; Witthoft *et al.,* 1998; Salzman *et al.,* 1998; Eaves-Pyles *et al.,* 1999). Bacterial adhesion, rather than invasion appears to be a key factor in the process (Salzman *et al.,* 1998; Eaves-Pyles *et al.,* 1999). The cellular mechanism of iNOS expression involves IκB degradation and nuclear factor κB (NF-κB) activation, as demonstrated in human intestinal epithelial cells (Eaves-Pyles *et al.,* 1999).

Cellular Mechanisms of iNOS Expression in Inflammation

There are relatively few studies on the cellular mechanisms of iNOS expression in inflammation *in vivo.* On the basis of *in vitro* studies (Xie and Nathan, 1994; MacMicking *et al.,* 1997a), it can be assumed that the induction of iNOS *in vivo* is also mediated by tyrosine kinase activation, the activation of NF-κB, and interferon-regulatory factor-1 (IRF-1), followed by increased expression of iNOS mRNA (Fig. 1). In fact, inhibitors of tyrosine kinases and of NF-κB activation have been shown to suppress the expression of

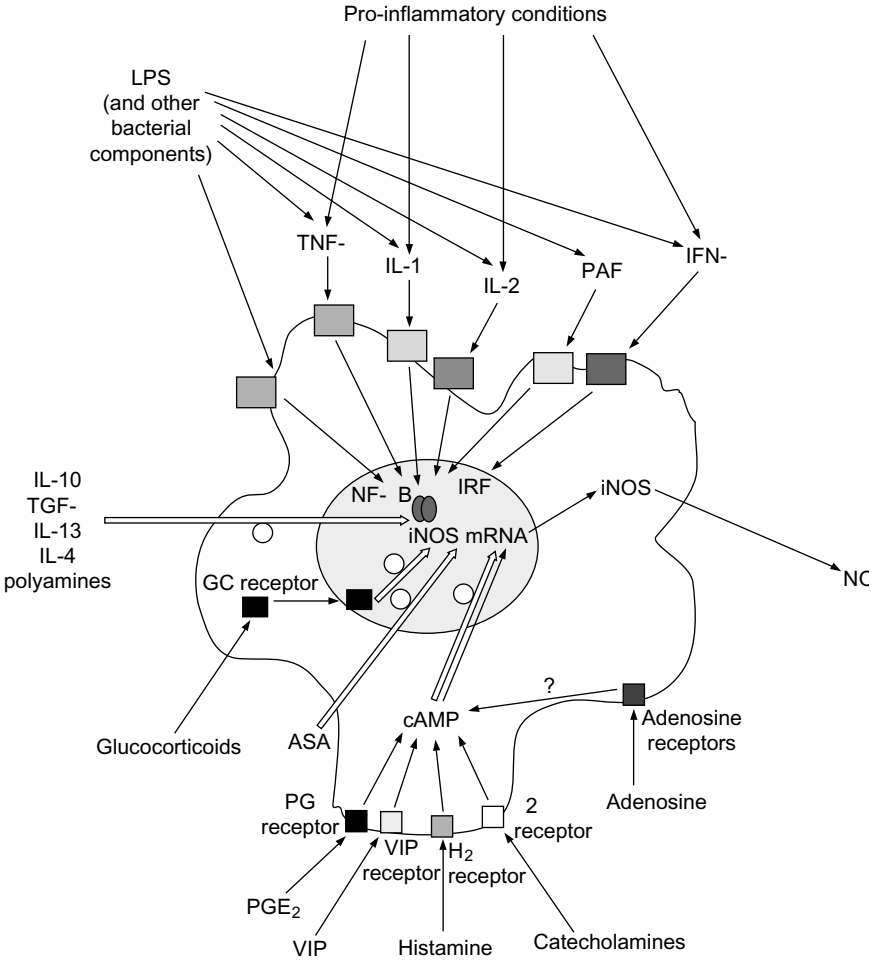


Figure 1 Regulation of iNOS expression in inflammation. Under proinflammatory conditions, proinflammatory cytokines and lipid mediators induce the expression of iNOS in various cell types. Anti-inflammatory mediators can suppress or downregulate the process of iNOS expression. Various mediators that increase intracellular cAMP levels in cells expressing iNOS can increase or decrease iNOS expression, depending on the cell type. The mechanism of iNOS expression, and its regulation, show substantial cell and species differences. Black arrows show activation, open arrows with (−) signs show inhibitory effects. See text for further details.

iNOS in systemic models of inflammation (S. F. Liu *et al.,* 1997; Ruetten and Thiemermann, 1997b,c; Németh *et al.,* 1998; Hong *et al.,* 1998; Kishnani *et al.,* 1999) and also in colitis (Sadowska-Krowicka *et al.,* 1998) and in cardiac allograft rejection (Cooper *et al.,* 1998). Furthermore, studies utilizing IRF-1-deficient mice demonstrated the crucial role of this pathway in the process of iNOS expression in myocarditis (Bachmaier *et al.,* 1997). In accordance with findings derived from *in vitro* studies, iNOS *in vivo* is also regulated at the level of its mRNA stability, which can be exploited to modulate the course of inflammation. For example, treatment of mice with transforming growth factor β (TGF-β) has been used to destabilize iNOS mRNA and suppress the systemic inflammatory response induced by bacterial lipopolysaccharide (Perrella *et al.,* 1996). Mice treated with or overexpressing TGF-β produce less NO in response to a systemic inflammatory stimulus (Pender *et al.,* 1996; Vodovotz *et al.,* 1998), whereas TGF-β-deficient mice have an abnormally high level of iNOS expression (Vodovotz *et al.,* 1996). There are interactions between and TGF-β and α_2-macroglobulin: α_2-macroglobulin binds to TGF-β and prevents it from binding to its receptor. Thus, α_2-macroglobulin can induce iNOS expression, an activity which probably results from the neutralization of autocrine TGF-β activity (Webb *et al.,* 1996). C-reactive protein, which was traditionally considered a marker rather than a mediator of inflammation, has been shown to enhance the expression of iNOS in macrophages (Ratnam and Mookerjea, 1998). On the other hand, the induction of the heat-shock response has been shown to suppress the expression of iNOS (Wong *et al.,* 1995, 1997a,b; Xu *et al.,* 1997; Scarim *et al.,* 1998).

There is some evidence that intracellular cyclic nucleotide levels can also influence the expression of iNOS, although the literature is somewhat conflicting in this respect (Fig. 1). Probably the regulation is dependent on the cell type and possibly the stimulus of iNOS induction. There are a

number of studies, for example, in macrophages, smooth muscle cells, and mesangial cells demonstrating that elevation of intracellular cAMP levels induces iNOS expression or enhances the induction of iNOS (Alonso *et al.,* 1995; Koide *et al.,* 1993; Pang and Hoult, 1997; Eberhardt *et al.,* 1998). In contrast, in studies in hepatocytes, Kupffer cells, microglia, and astrocytes, elevation of cAMP levels has been shown to suppress the induction of iNOS (F. S. Smith *et al.,* 1997; Minghetti *et al.,* 1997; Pahan *et al.,* 1997; Mustafa and Olson, 1998; Haskó *et al.,* 1998a,b). On the basis of *in vivo* studies in animals subjected to LPS-induced systemic inflammation, the latter mechanism appears to be more relevant for the pathophysiology of inflammation (i.e., cAMP is an endogenous suppressor of iNOS expression): increases in cAMP levels elicited by adenosine receptor agonists or adrenergic agonists, or by elevation of intracellular cAMP levels by inhibition of cAMP phosphodiesterase inhibitors (e.g., rolipram), suppress the production of NO *in vivo* (Szabó *et al.,* 1997a; Haskó *et al.,* 1998a,b; Haskó and Szabó, 1998).

Endogenous Inhibitors of iNOS Expression in Inflammation

Glucocorticoids and IL-10 represent endogenous inhibitors of iNOS expression in inflammation. This conclusion is based on experiments demonstrating the enhanced expression of iNOS in response to LPS in adrenalectomized rats (Szabó *et al.,* 1993c) and in IL-10-deficient mice (Haskó *et al.,* 1998b). Polyamines, melatonin, and a large number of other endogenous molecules have also been shown to inhibit the expression of iNOS *in vitro* (Szabó *et al.,* 1995a; Baydoun and Morgan, 1998; Gilad *et al.,* 1998). *In vitro* studies also demonstrated that a variety of cytokines, such as IL-4 and IL-13, are capable of inhibiting the expression of iNOS (Bogdan *et al.,* 1994; Berkman *et al.,* 1996; Kolios *et al.,* 1998). However, whether the endogenous production of molecules other than cAMP, TGF-β, glucocorticoids, and IL-10 can play a role in regulating the expression or stability of iNOS in inflammation *in vivo* is unclear at the moment (Fig. 1). It needs to be pointed out that there are marked cell and species differences in the regulation of iNOS expression.

Time Course and Sites of iNOS Expression in Inflammation

The expression of iNOS, in response to a transient stimulus, is transient. For instance, the expression of iNOS in response to systemic administration of bacterial lipopolysaccharide in a rodent peaks at approximately 6 hours and returns to baseline levels by 24 hours (Knowles *et al.,* 1990; Salter *et al.,* 1991; Szabó *et al.,* 1994a). This response is followed by a period of tolerance for the induction of iNOS by subsequent inflammatory stimuli (Szabó *et al.,* 1994a; Chang *et al.,* 1996; Del Castillo *et al.,* 1997), the mechanism of which involves a reduced ability of the cells to express iNOS mRNA (Chang *et al.,* 1996), in part due to upregulation of endogenous glucocorticoids, which suppress the expression of iNOS (Szabó *et al.,* 1994a). Interestingly, the development of tolerance against iNOS induction appears to be cell type dependent: whereas overall net NO synthesis was found to be downregulated during protracted LPS exposure, aortas and glomeruli maintained most of their ability to express iNOS and produce NO for prolonged periods of time (Del Castillo *et al.,* 1997).

In response to a continuing inflammatory stimulus, there may be a continuing expression of iNOS (albeit probably at lower levels than the initial peak, which develops in response to a sudden single burst of inflammatory mediators). For example, in chronic experimental models of human forms of arthritis or colitis, there is continued increase in the plasma levels of nitrite and nitrate, the breakdown products of NO (Weinberg *et al.,* 1994; Aiko and Grisham, 1995; Stichtenoth *et al.,* 1995; Grabowski *et al.,* 1996; Aiko *et al.,* 1998; Levine *et al.,* 1998; Oudkerk Pool *et al.,* 1995; Pelletier *et al.,* 1998). Similarly, prolonged increases in the levels of nitrite and nitrate can be measured in humans suffering from circulatory shock of various etiologies (Evans *et al.,* 1993; Groeneveld *et al.,* 1996; Krafte-Jacobs *et al.,* 1997).

The induction of iNOS is cell type and organ specific. The most typical cell types that express iNOS in inflammation are macrophages, neutrophils, vascular and nonvascular smooth muscle cells, cardiac myocytes, astrocytes, mesangial cells, epithelial cells, and endothelial cells. These different cell types, depending on the inflammatory condition, can contribute, to a varying extent, to the net NO output of the body (measured as circulating or urinary levels of nitrite/nitrate). For example, in systemic inflammation induced by LPS, the majority of circulating nitrite/nitrate is macrophage derived (Salkowski *et al.,* 1997). This means that measurements of systemic production of NO production do not necessarily reflect the changes in NO production in an individual tissue or organ during inflammation.

Although iNOS is mainly expressed in inflammatory conditions and is not expressed in the absence of inflammatory stimuli in most cell types, there is some evidence for ''constitutive iNOS expression'' in selected sites of the body. One of these sites is the lung, where epithelial cells of clinically disease-free humans express iNOS (Guo *et al.,* 1995; Guo and Erzurum, 1998). This phenomenon is possibly related to a low-level, physiologically relevant inflammatory activation in this site. Also, iNOS expression has been reported in the thymus of normal animals, and NO or reactive nitrogen species (such as peroxynitrite, see later) formed from NO have been implicated in the process of negative selection in this organ (Virág *et al.,* 1998a; Downing *et al.,* 1998a,b).

Pharmacological or Therapeutic Inhibition of iNOS Expression in Inflammation

A number of known anti-inflammatory agents are known to suppress the induction of iNOS. Glucocorticoids are probably the most widely studied from this class of substances. Glucocorticoids are well established as potent inhibitors of the expression of iNOS in rodents *in vitro* and *in vivo* (Radomski *et al.,* 1990; Salter *et al.,* 1991; Szabó *et al.,* 1993d). It is likely that the potent anti-inflammatory action of glucocorticoids in rodents is related to their ability to prevent the

induction of iNOS. However, the human iNOS mRNA expression and proinflammatory cytokine-induced NO production are, according to most studies, largely insensitive to glucocorticoids (Robbins *et al.,* 1994; Salzman *et al.,* 1996; Guthikonda *et al.,* 1998). It is worthwhile to mention that the human chondrocyte may be an exception: at least in some of the *in vitro* studies, iNOS expression in human chondrocytes can be inhibited by steroids (Palmer *et al.,* 1993; Amin *et al.,* 1995a; Cipolletta *et al.,* 1998; de Gendt *et al.,* 1998). This is in agreement with the clinical observations showing that glucocorticoids suppress urinary nitrite/nitrate levels in rheumatic patients (Stichtenoth *et al.,* 1995). With respect to the human iNOS, it is important to point out that its regulation is radically different from the regulation of rodent iNOS. Human iNOS expression (1) is mainly regulated at the level of mRNA stability, rather than at the level of iNOS mRNA expression, and (2) generally requires a costimulation by a mixture of proinflammatory cytokines. Further, the promoter of human iNOS has different regulatory elements, including a distant upstream enhancer element, and also, as mentioned above, iNOS expression is not generally inhibited by glucocorticoids (DeVera *et al.,* 1996; Linn *et al.,* 1997).

Other clinically used well-known anti-inflammatory or immunosuppressive agents that turned out to inhibit the expression of iNOS include cyclosporin A (Shindo *et al.,* 1995; Wu *et al.,* 1998; Trajkovic *et al.,* 1999), methotrexate (Robbins *et al.,* 1998), melatonin (Gilad *et al.,* 1998), tetracyclines (Amin *et al.,* 1996; Trachtman *et al.,* 1996), and the immunosuppressive agent tacrolimus (FK506) (Goto *et al.,* 1997). Nonsteroidal anti-inflammatory agents have also been demonstrated to suppress the expression of iNOS in several cell types. In the first report, it was found that in LPS-stimulated murine macrophages, therapeutic concentrations of aspirin (IC_{50} = 3 m*M*) inhibit the expression of iNOS and production of nitrite (Amin *et al.,* 1995b, Amin *et al.,* 1997). In subsequent studies, indomethacin was found to inhibit NO production in immunostimulated macrophages (Pang and Hoult, 1996); aspirin was effective in transformed pancreatic β cells and rat islets (Kwon *et al.,* 1997), aspirin and salicylate in rat fibroblasts (Farivar *et al.,* 1996), and a number of structurally unrelated cyclooxygenase (COX) inhibitors in immunostimulated microglial cells (Minghetti *et al.,* 1997). Furthermore, ibuprofen has been shown to concentration-dependently decrease iNOS mRNA levels and to inhibit the activity of iNOS in glial cells (Minghetti *et al.,* 1997). However, there was no correlation between a decrease in iNOS activity and reduction in iNOS mRNA levels (Stratman *et al.,* 1997). On the basis of earlier work (Grilli *et al.,* 1996; Yin *et al.,* 1998), it is conceivable that the nonsteroidal anti-inflammatory compounds probably exert their effects on iNOS expression, at least in part, by inhibition of the activation of NK-κB.

Expression of iNOS in Human Inflammatory Conditions

Overall, in humans, the degree of expression of iNOS and the production of NO by iNOS is lower than in many animal species, especially rodents. This is evidenced by lower levels of plasma nitrite/nitrate in inflammatory conditions and a lower output of NO production in immunostimulated human cells, when compared to immunostimulated rodent cells. Importantly, human monocytes/macrophages, in many early studies, were found not to induce iNOS in response to endotoxin and/or proinflammatory cytokine mixtures, whereas rodent macrophages or macrophage-like cell lines were found to be good expressors of iNOS. Now it is clear that this curious behavior of human monocytes/macrophages is most likely a consequence of inappropriate stimulation conditions in the previously mentioned *in vitro* experiments: cross-linking of CD23 by a monoclonal antibody consistently induces iNOS in human monocytes (Dugas *et al.,* 1998). Similarly, *Mycobaterium tuberculosis* is able to induce iNOS in human monocytes *in vitro* (Jagannath *et al.,* 1998). Furthermore, studies in patients suffering from various forms of inflammation have demonstrated that iNOS is expressed *in situ* in macrophages. A predominant example is a study focusing on the lungs of tuberculotic patients, where marked iNOS staining has been demonstrated in resident pulmonary macrophages (MacMicking *et al.,* 1997b; Nicholson *et al.,* 1996). Similarly, enhanced iNOS expression has been demonstrated in neutrophils from patients suffering from urinary tract infection (Wheeler *et al.,* 1997) and septic shock (Tsukahara *et al.,* 1998), in synovial macrophages from arthritic patients (McInnes *et al.,* 1996), in macrophages surrounding aseptic inflammation associated with loosened hip protheses (Watkins *et al.,* 1997; Hukkanen *et al.,* 1997), and in macrophages infiltrating kidneys of glomerulonephrotic patients (Kashem *et al.,* 1996).

Other human cell types are also known to express iNOS and produce large amounts of NO, including hepatocytes, chondrocytes, and intestinal and pulmonary epithelial cells. Human neutrophils can also be induced *in vitro* to generate significant levels of reactive nitrogen species (Evans *et al.,* 1996; Gagnon *et al.,* 1998). In addition to the previously listed examples, the expression of iNOS (in various cell types) in humans has been demonstrated in a wide variety of conditions including Alzheimer's disease, multiple sclerosis, AIDS-associated dementia, viral uveitis, asthma, lung cancer, pulmonary sarcoidosis, bacterial pneumonia, Crohn's disease, ulcerative colitis, renal allografts, aortic aneurysms, psoriasis, alcoholic hepatitis, and systemic inflammatory response syndrome (circulatory shock) (overviewed in MacMicking *et al.,* 1997a; Kilbourn *et al.,* 1997).

Hepatocytes and intestinal epithelial cells have been the subject of extensive investigations; the promoter of the human iNOS has also been cloned and expressed in these cell types. There is some degree of cell specificity in the regulation, although it is clear that the promoter of the human iNOS gene is radically different from the murine promoter. In the human iNOS promoter, a classic enhancer element is present, which is located between 8.7 and 10.7 kb upstream of the transcription initiation site (Linn *et al.,* 1997). The region between the enhancer element and the transcription initiation site is almost completely silent and uninducible in

human intestinal epithelial cells (Linn *et al.,* 1997), whereas it is somewhat responsive to NF-κB elements in human liver and lung epithelial cell lines (De Vera *et al.,* 1996; Taylor *et al.,* 1998). As mentioned previously, it is now also evident that the human iNOS is mainly regulated at a posttranscriptional rather than transcriptional level.

Inducible NO Synthase-Independent Sources of NO and Reactive Nitrogen Species in Inflammation

Although it is generally acknowledged that iNOS is the source of most NO produced in various forms of inflammation, it is also clear that sources other than iNOS can also contribute to the production of NO or reactive nitrogen intermediates under certain specialized conditions.

Constitutive NOS Isoforms

It is now well established, that the ''constitutive'' NOS isoforms ecNOS (constitutive endothelial NOS) and bNOS (brain NOS) can also undergo transcriptional regulation (Förstermann *et al.,* 1995). Examples of upregulation of ecNOS-like proteins in inflammation-related conditions include cyclosporin-treated renal transplant patients (Calo *et al.,* 1998), chronic heart failure (Khadour *et al.,* 1998), hepatic cirrhosis (Martin *et al.,* 1996), iron-deficiency anemia (Ni *et al.,* 1997), and hemorrhagic shock (Zingarelli *et al.,* 1998a). In the latter two conditions, increased levels of both ecNOS as well as iNOS (see below) were found (Ni *et al.,* 1997; Zingarelli *et al.,* 1998a). Estrogen and vascular endothelial growth factor (VEGF) may serve as physiologically or pathophysiologically relevant upregulators of ecNOS (MacRitchie *et al.,* 1997; Hood *et al.,* 1998).

Even in the absence of transcriptional or posttranscriptional regulation of the constitutive isoforms of NOS, physiological levels of NO can be converted to potentially cytotoxic species. All three isoforms of NO synthase, namely, ecNOS, bNOS, and iNOS, can contribute to the production of NO with subsequent formation of reactive nitrogen species, such as peroxynitrite (a labile potent oxidant, produced by the reaction of NO and superoxide; see also later). For instance, superoxide, produced during the reperfusion phase of ischemia–reperfusion injury or in the acute phase of shock, reacts with NO produced from constitutive, presumably endothelial NOS, thereby forming peroxynitrite (Szabó *et al.,* 1995a,b; Wang and Zweier, 1996; Zingarelli *et al.,* 1997a). Under these conditions, formation of superoxide, and not that of NO, is the rate-limiting step in the formation of peroxynitrite. In various forms of neuroinjury, bNOS activation precedes the formation of peroxynitrite (Dawson, 1995; Szabó, 1996; Dawson and Dawson, 1996; Bolanos *et al.,* 1997).

It is important to point out that under certain conditions, all isoforms of NOS are capable of producing both precursors of peroxynitrite (in the form of simultaneous generation of NO and superoxide). Such conditions cannot be found under normal circumstances, but they can occur during cellular L-arginine depletion. Massive activation of NOS can deplete the cellular levels of its precursor L-arginine (despite an activation of an enzyme system, which recycles the end product L-citrulline back to arginine, and upregulation of membrane L-arginine transport mechanisms). Under low cellular arginine concentrations, NOS produces both NO and superoxide, and the resulting generation of peroxynitrite can contribute to cytotoxicity. This mechanism has been confirmed in neuronal cultures, and in macrophages expressing iNOS (Xia *et al.,* 1996; Xia and Zweier, 1997).

Nonenzymatic Pathways

In addition to enzymatic sources, it is possible that the reduction of nitrite to NO under acidic conditions (Zweier *et al.,* 1995) can eventually lead to generation of peroxynitrite in certain forms of inflammation. Moreover, a recently described, nonenzymatic pathway generating NO from L-arginine and hydrogen peroxide may also be considered (Nagase *et al.,* 1997). We include these two nonenzymatic processes of NO generation for the purpose of completeness. Further investigations are required to elucidate whether these latter processes can significantly contribute to NO or peroxynitrite generation in physiology or pathophysiology.

Myeloperoxidase-Catalyzed Toxic Processes Involving Nitrite

Tyrosine nitration is considered one of the key mechanisms through which peroxynitrite can induce inflammatory posttranscriptional modification of proteins (see later). Van Der Vliet *et al.* (1997) have established that nitrite, a major end product of NO metabolism, can also promote tyrosine nitration through formation of nitryl chloride (NO_2Cl) and nitrogen dioxide by reaction with the inflammatory mediators hypochlorous acid (HOCl) or myeloperoxidase. Activated human polymorphonuclear neutrophils convert nitrite NO_2Cl and nitrogen dioxide through myeloperoxidase-dependent pathways. Polymorphonuclear neutrophil-mediated nitration and chlorination of tyrosine residues or 4-hydroxyphenylacetic acid is enhanced by addition of nitrite. The physiological significance of these reaction pathways to cellular dysfunction has been established by studies demonstrating that polymorphonuclear neutrophil-mediated inactivation of endothelial cell angiotensin-converting enzyme is exacerbated by nitrite (Eiserich *et al.,* 1996, 1998).

Another myeloperoxidase-mediated process involving nitrite and hydrogen peroxide is related to the covalent cross-linking of immune complexes. This process is known to be catalyzed by activated neutrophils, and it exclusively requires nitrite (and not NO) (Uesugi *et al.,* 1998). As oxygen radicals and NO are likely to interact at the cartilage surface during inflammatory arthritis, the resulting immune complex cross-linking may have important pathophysiological consequences. It is important to note that nitrite, which previously was considered an innocuous degradation product of NO), is now also recognized as a species capable of regulating inflammatory processes through various myeloperoxidase-mediated oxidative mechanisms. From the standpoint of therapeutic intervention, the presence of nitrite-dependent

cytotoxic pathways may indicate that appropriate strategies which prevent the overproduction of NO in inflammation may be more desirable than the use of agents which react with NO or peroxynitrite and catalyze their decomposition to nitrite and nitrate.

Mechanisms of the Detrimental Actions of Nitric Oxide in Inflammation

Nitric Oxide-Mediated Effects

Large concentrations of locally produced NO are historically considered to have large cytotoxic potential. It is becoming increasingly clear, however, that the intrinsic oxidant capacity of ''pure'' NO is low. In other words, although many studies concluded that NO itself is able to induce cytotoxic or cytostatic effects (such as the suppression of the mitochondrial respiratory chain, or induction of cell necrosis and apoptosis), these studies should be viewed with some caution: the formation of superoxide is present in many experimental systems, and the production of peroxynitrite (from the extremely rapid reaction of NO and superoxide) may ultimately mediate many of the toxic or pro-oxidant actions of NO.

CYCLIC GMP-RELATED EFFECTS OF NO IN INFLAMMATION

Pure NO or nitrosothiols are generally responsible for physiological processes, such as vasodilation, via a mechanism that involves activation of guanylyl cyclase in the vascular smooth muscle cells (Ignarro, 1991). Large concentrations of NO, produced by iNOS, can cause autocrine or paracrine actions that can be considered as an ''overactivation'' of the same physiological process that is normally regulated by NO produced from ecNOS. A classic example of this process is the toxic, therapy-resistant, systemic vasodilation associated with systemic inflammatory response syndrome (circulatory shock). In this case, a normal, physiological cGMP-dependent vasodilatory effect is pathophysiologically enhanced by the higher local levels of iNOS-derived NO (Szabó, 1995; Kilbourn *et al.,* 1997). Another example of a cGMP-mediated process is the inhibition of neutrophil or platelet adhesion to the endothelial surface, which is a process normally related to NO production from endothelial NOS (Radomski *et al.,* 1987; Kubes *et al.,* 1991). However, during inflammation, iNOS-derived NO can exert similar effects, which, in this case, can be considered beneficial (Shultz and Raij, 1992; Yan *et al.,* 1996; Hickey *et al.,* 1997; Binion *et al.,* 1998).

S-NITROSYLATION-RELATED EFFECTS OF NO IN INFLAMMATION

The chemical reactions of NO are largely dictated by its redox state. Increasing evidence suggests that the various redox states of the NO group exist endogenously in biological tissues. In the case of nitrosonium cation (NO^+) equivalents, the mechanism of reaction often involves S-nitrosylation (transfer of the NO group to a cysteine sulfhydryl to form an RS-NO), and further oxidation of critical thiols can possibly form disulfide bonds. Some of the first physiological and chemical evidence for the potential regulation of pathophysiological processes by NO-mediated S-nitrosylation was related to the regulation of the activity of the *N*-methyl-D-aspartate (NMDA) receptor, a key receptor in various forms of neuroinflammatory conditions. Now it is clear that NMDA receptor activity can be modulated by S-nitrosylation, resulting in a decrease in channel opening (Lipton *et al.,* 1993, 1998). However, other data also suggest that NO, probably in the singlet (or high-energy) state, can also react with critical sulfhydryl group(s) of the NMDA receptor to downregulate its activity, whereas in the triplet (lower-energy) state nitroxyl anion (NO^-) may oxidize these NMDA receptor sulfhydryl groups by the formation of an intermediate such as peroxynitrite (Lipton *et al.,* 1993, 1998). Inhibition of NMDA receptor activation can be considered as a beneficial (anti-inflammatory) function of NO in neuroinflammatory conditions, whereas activation of this receptor is detrimental, because it enhances neuronal calcium overload and NO and oxyradical production.

S-Nitrosylation by NO of key proteins of various signal transduction pathways has been reported in a number of experimental systems; the enzymes affected include protein kinase C (Gopalakrishna *et al.,* 1993; Akesson and Lundquist, 1998), c-Jun N-terminal kinase 2 (So *et al.,* 1998), tyrosine kinase (Estrada *et al.,* 1997), and various intracellular calcium release channels (Campbell *et al.,* 1996; Favre *et al.,* 1998; Xu *et al.,* 1998). The S-nitrosylation of the transcription factor NF-κB p50 has been shown to lead to a significant decrease in DNA binding (DeLaTorre *et al.,* 1998). NO, probably via S-nitrosylation, has also been shown to reduce the DNA binding of the transcription factor c-Myb (Brendeford *et al.,* 1998). Similarly p21(ras) and activator protein 1 (AP-1) have been shown to be regulated by S-nitrosylation (Lander *et al.,* 1997; Tabuchi *et al.,* 1994). These S-nitrosative processes, if they also occur *in vivo,* may result in an enhancement of the dysregulation of cellular signal transduction processes, which may exacerbate the course of inflammation.

S-Nitrosylation and subsequent inactivation of enzymes involved in biosynthetic processes, for example, methionine adenosyltransferase (Ruiz *et al.,* 1998) and glyceraldehyde-3-phosphate dehydrogenase (Padgett and Whorton, 1997), have also been reported. These effects are most likely detrimental in inflammation.

S-Nitrosylation of mitochondrial complex I (Clementi *et al.,* 1998) would also be expected to have deleterious effects on cellular energetics. Low (nanomolar) levels of NO reversibly inhibit mitochondrial respiration by binding to the oxygen binding site of cytochrome oxidase in competition with oxygen. This raises the apparent K_m for oxygen of mitochondrial respiration into the physiological range, potentially making respiration sensitive to the oxygen level. The NO inhibition of oxygen consumption was seen in isolated cytochrome oxidase, mitochondria, brain nerve terminals, and cultured cells. These observations suggest that any cell

producing high levels of NO will inhibit its own respiration and that of surrounding cells, which makes the respiration rate sensitive to the oxygen level. This NO-related inhibition of energy metabolism may contribute to cytotoxicity or cytostasis in some pathophysiological conditions (Brown, 1997) (Fig. 2). Endogenously produced, low levels of NO regulate tissue oxygen extraction under resting conditions (Wolin *et al.,* 1999). NO and peroxynitrite also inhibit the activity of

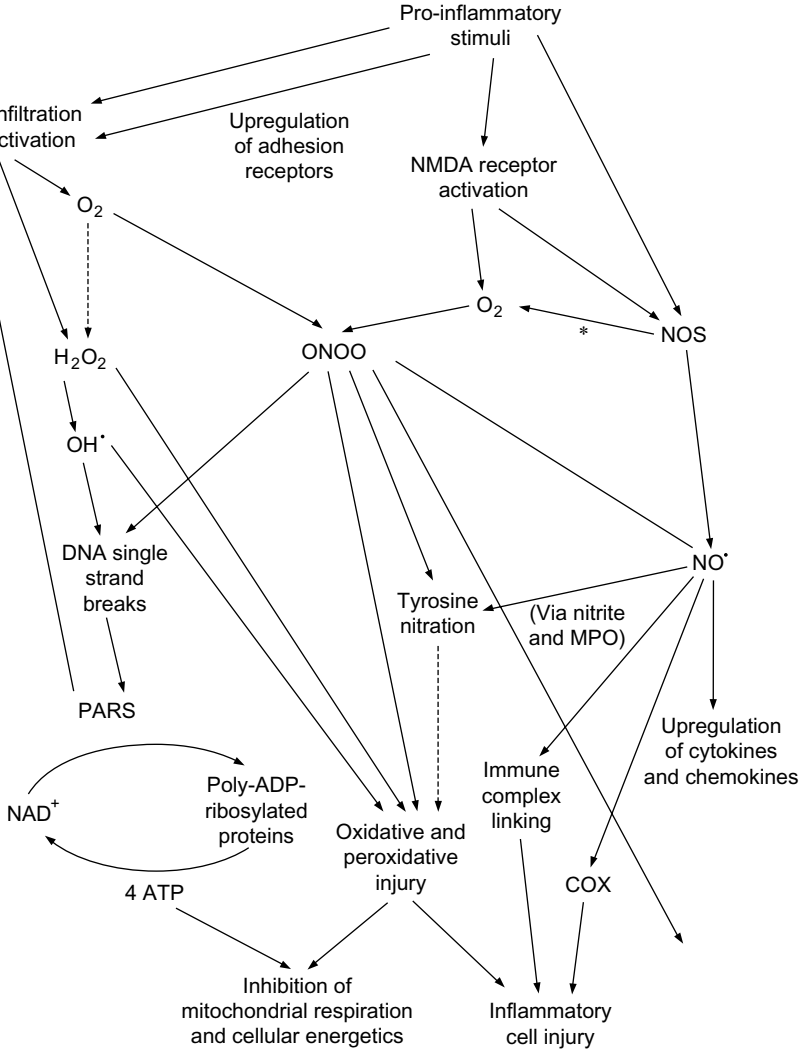


Figure 2 Proposed scheme of PARS-dependent and PARS-independent cytotoxic pathways involving nitric oxide (NO·), hydroxyl radical (OH·), and peroxynitrite ($ONOO^-$) in inflammation. Proinflammatory mediators induce the expression of the inducible NO synthase (iNOS), whereas NMDA receptor ligands activate the constitutive neuronal NOS (bNOS). NO, in turn, combines with superoxide to yield peroxynitrite. Hydroxyl radical (produced from superoxide via the iron-catalyzed Haber–Weiss reaction) and peroxynitrite or peroxynitrous acid induce the development of DNA single-strand breakage, with consequent activation of PARS. Depletion of the cellular NAD^+ leads to inhibition of cellular ATP-generating pathways, leading to cellular dysfunction. NO alone does not induce DNA single-strand breakage, but it may combine with superoxide (produced from the mitochondrial chain or from other cellular sources) to yield peroxynitrite. Under conditions of low cellular L-arginine, NOS may produce both superoxide (*) and NO, which then can combine to form peroxynitrite. There are PARS-independent, parallel pathways of cellular metabolic inhibition, and these pathways can be activated by NO, hydroxyl radical, superoxide, and peroxynitrite (alone or in combination or synergy). For instance, peroxynitrite can induce cell injury via protein tyrosine nitration. Tyrosine nitration can also be induced by an alternative pathway utilizing myeloperoxidase (MPO) and nitrite. A similar pathway can also result in immune complex cross-linking. In inflammation, activation of cyclooxygenase (COX) by NO or peroxynitrite may also amplify the inflammatory response. PARS activation promotes neutrophil (PMN) recruitment, thereby triggering a positive feedback cycle. NO, in addition to being a terminal effector of inflammation, also acts as an amplifier of the inflammatory response. See text for further details.

aconitase in various cell types (Castro *et al.,* 1998; Gardner *et al.,* 1997, 1998a; Hausladen and Fridovich, 1994; Castro *et al.,* 1994). It appears that most of the detrimental effects of NO on mitochondrial respiration and cellular energetics occur via peroxynitrite-mediated processes (see later).

Several independent groups of investigators have reported that caspases (key proapoptotic effector enzymes) become S-nitrosylated and inactivated by NO. This reaction leads to a subsequent suppression of apoptotic DNA fragmentation (Melino *et al.,* 1997; Li *et al.,* 1997; Kim *et al.,* 1997, 1998a; Ceneviva *et al.,* 1998). The suppression of apoptosis by NO may be beneficial or detrimental, depending on the particular cell type involved in apoptosis and the specific form of inflammatory condition. Another protein that may be important in the process of apoptosis and that has been shown to be regulated by NO and S-nitrosylation is transglutaminase (Melino and Piacentini, 1998).

Another example of S-nitrosylation with potential implications for inflammation is related to the affinity of the glucocorticoid receptor. This receptor is known to have critical cysteine residues for steroid binding in its hormone-binding and DNA-binding domains. It has been demonstrated that NO, most likely by S-nitrosylation, reduces the ability of ligands to bind to the glucocorticoid receptor (Galigniana *et al.,* 1999). This mechanism, if also operative *in vivo,* may have implications for the regulation by glucocorticoids of iNOS expression and for the steroid regulation of many other pathways of inflammation.

NO can also polynitrosylate proteins, such as albumin (Stamler *et al.,* 1992; Simon *et al.,* 1996), and can react with hemoglobin to form nitrosohemoglobin (Stamler *et al.,* 1997). These reactions in inflammation may be beneficial, because the proteins may act as ''sponges'' to remove NO from sites where it is produced in high local levels. These proteins may also transport the NO to other parts of the body, where it may be released without significant cytotoxic effects.

Peroxynitrite-Mediated Effects

Evidence for the Production of Peroxynitrite in Inflammation

Although the investigations into the effects of exogenously administered peroxynitrite in cells and tissues are relatively straightforward from a technical point of view, there are a number of problems related to the detection of endogenously produced peroxynitrite. Similarly, there are theoretical and experimental difficulties related to the delineation of the actual role of peroxynitrite in pathophysiological conditions. Some of these limitations are discussed in this section.

Theoretical considerations strongly favor the production of peroxynitrite when NO and superoxide are produced simultaneously. The reaction of these two species is nearly diffusion controlled. Furthermore, the reaction of superoxide with NO is the only reaction that outcompetes the reaction of superoxide with superoxide dismutase (Beckman *et al.,* 1990; Pryor and Squadrito, 1995; Beckman and Koppenol, 1996). Although chemical considerations favor the production of peroxynitrite, the actual demonstration of the presence or production of peroxynitrite in pathophysiological conditions is far from straightforward. Peroxynitrite rapidly oxidizes the fluorescent probe dihydrorhodamine 123 to rhodamine 123 *in vitro* (Kooy *et al.,* 1995a). The production of peroxynitrite can be evidenced as increased oxidation of dihydrorhodamine 123 to rhodamine 123 in the plasma (Szabó *et al.,* 1995b). Caution should be exercised with this method, however: oxidation of dihydrorhodamine can be triggered by oxidants other than peroxynitrite (e.g., hydroxyl radical). A NOS inhibitor-inhibitable component of an increased oxidation of dihydrorhodamine can be taken as a relatively specific evidence of an effect of peroxynitrite (Szabó *et al.,* 1995b; Cuzzocrea *et al.,* 1997).

Nitrotyrosine formation, and its detection by immunostaining, was initially proposed as a relatively specific means for detection of the ''footprint'' of peroxynitrite (Ischiropoulos *et al.,* 1992a). On the other hand, as discussed earlier, more recent evidence indicates that certain other reactions can also induce tyrosine nitration: for example, the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase (and certain other peroxidases) with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich *et al.,* 1996, 1998; Van Der Vliet *et al.,* 1997). The physiological or pathophysiological relevance of this reaction remains to be further clarified. Increased nitrotyrosine staining was considered an indication of ''increased nitrosative stress,'' rather than a specific marker of peroxynitrite (Halliwell, 1997). Nitrated tyrosine residues have been demonstrated in a wide variety of inflammatory and shock conditions, including arthritis, colitis, uveitis, allergic encephalomyelitis, and systemic inflammation or circulatory shock (e.g., Kaur and Halliwell, 1994; Wizemann *et al.,* 1994; Szabó *et al.,* 1995a,b; Abe *et al.,* 1995, 1997; Miller *et al.,* 1995; Akaike *et al.,* 1996; Singer *et al.,* 1996; Kooy *et al.,* 1995b, 1997; Brujin *et al.,* 1997; Van Der Veen *et al.,* 1997; Saleh *et al.,* 1997; Zingarelli *et al.,* 1997a, 1998a; overviewed in Szabó, 1996).

Specific peroxynitrite scavengers, which could help in delineating the specific role of peroxynitrite in inflammation or shock or in any other pathophysiological condition, are not generally available. Agents that scavenge peroxynitrite often neutralize other oxidants as well (e.g., glutathione, melatonin; see earlier). Uric acid, a putative scavenger of peroxynitrite, is sometimes used as a probe for peroxynitrite (Kooy *et al.,* 1995a; Szabó and Salzman, 1995; Szabó *et al.,* 1996a; Xie and Wolin, 1996; Johnson and Ferro, 1996; Hooper *et al.,* 1997; Xia and Zweier, 1997). However, uric acid can interfere with a number of other oxidants (Ames *et al.,* 1981; Frei *et al.,* 1989), and therefore caution should be applied when interpreting the results obtained with uric acid. A novel class of peroxynitrite decomposition catalysts has been identified; these compounds, indeed, exert potent anti-inflammatory actions (Salvemini *et al.,* 1998).

Taken together, the evidence implicating the role of peroxynitrite in a given inflammatory condition can only be indirect. A simultaneous protective effect of superoxide

neutralizing strategies and NO synthesis inhibition, coupled with the demonstration of the production of peroxynitrite (via one or more of the previously listed indirect methods) in the particular inflammatory condition, can be taken as strong indication for the role of peroxynitrite. However, it is likely that additional interactions of oxygen- and nitrogen-derived free radicals also contribute to the inflammatory cell injury, possibly in concert or in synergy.

OXIDATION, NITRATION, AND NITROSATION INDUCED BY PEROXYNITRITE

In vitro experiments have demonstrated that peroxynitrite is highly reactive (Fig. 2). The oxidant reactivity of peroxynitrite is mediated by an intermediate with the biological activity of hydroxyl radical, which is not hydroxyl radical per se, but rather peroxynitrous acid or its activated isomer (Pryor and Squadrito, 1995; Pou *et al.,* 1995; Beckman and Koppenol, 1996: Goldstein *et al.,* 1996). Peroxynitrite induces the oxidation of sulfhydryl groups and thioethers, and it induces nitration and hydroxylation of aromatic compounds, such as tyrosine, tryptophan, and guanine (Ischiropoulos *et al.,* 1992a; Salman-Tabcheh *et al.,* 1995; Alvarez *et al.,* 1996; Beckman, 1996; Radi, 1996; Kato *et al.,* 1997). These reactions, when occurring during the reaction of peroxynitrite with various enzymes of the cell, can markedly suppress the catalytic activity of the affected enzymes. For instance, peroxynitrite has been shown to inhibit manganese superoxide dismutase, tyrosine hydroxylase, membrane Na^+,K^+-ATPase, membrane sodium channels, glyceraldehyde-3-phosphate dehydrogenase, mitochondrial and cytosolic aconitase, and a number of critical enzymes in the mitochondrial respiratory chain (Bauer *et al.,* 1992; Ischiropoulos *et al.,* 1995a; Hausladen and Fridovich, 1994; Castro *et al.,* 1994; Mohr *et al.,* 1994; Hu *et al.,* 1994; Radi *et al.,* 1994; Rubbo *et al.,* 1994; Crow and Beckman, 1995; Guzman *et al.,* 1995; Bolanos *et al.,* 1995; Selden *et al.,* 1995; MacMillan-Crow *et al.,* 1996; Cassina and Radi, 1996; Lizasoain *et al.,* 1996; Sato *et al.,* 1997). Tyrosine nitration in neutrophils has been shown to enhance the responsiveness of the cells to express and produce inflammatory mediators in response to subsequent proinflammatory stimuli (Rohn *et al.,* 1999). Peroxynitrite also exerts a feedback inhibitory effect on NO synthase (Pasquet *et al.,* 1996; Huhmer *et al.,* 1997).

Nitrotyrosine serves as a relatively specific marker of peroxynitrite production *in vivo,* although, as mentioned earlier, other pathways such as the reaction of nitrite and myeloperoxidase can also result in tyrosine nitration (Van Der Vliet *et al.,* 1997; Eiserich *et al.,* 1998). Until very recently, it was presumed that the nitration of tyrosine, and thus the nature of the inhibition of the affected enzymes by peroxynitrite, was irreversible. However, in a pioneering line of work, Murad and colleagues identified an LPS-inducible putative enzyme from the supernatant of spleen homogenates. This enzymatic activity catalyzes the loss of the nitrotyrosine epitope without protein degradation. Although the product and possible cofactors for this reaction have not yet been identified, this activity, which may be a ''nitrotyrosine denitrase'' that reverses protein nitration, would be expected to act as an anti-inflammatory enzyme that serves to decrease or reverse peroxynitrite toxicity (Kamisaki *et al.,* 1998).

In addition to the interactions of peroxynitrite with proteins, an important interaction of peroxynitrite occurs with nucleic acids (for review, see Szabó and Ohshima, 1997). Two main types of reactions have been described: DNA base modifications and DNA single-strand breakage. The reported base modifications include the formation of 8-nitroguanine, 8-oxoguanine, and 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2'-deoxyguanosine (nox-dG), and the formation of oxidized and deaminated base products, such as 5-hydroxyhydantoin, 5-(hydroxymethyl)uracil, thymine glycol, 4,6-diamino-5-formamidepyrimidine (FAPy-adenine), 2,6-diamino-5-formamidepyrimidine (FAPy-guanine), 8-oxo-adenine, 8-oxoguanine, hypoxanthine, and xanthine (Inoue and Kawanishi, 1995; Yermilov *et al.,* 1995, 1996; Spencer *et al.,* 1996; Uppu *et al.,* 1996; Kennedy *et al.,* 1997). The peroxynitrite-induced DNA single-strand breakage (King *et al.,* 1992; Salgo *et al.,* 1995a; Szabó *et al.,* 1996a,b) is probably related to abstraction of hydrogen atoms from the ribose of the DNA moiety, thereby opening the sugar ring. Single-strand breaks of the DNA induce the activation of the nuclear enzyme poly(ADP-ribose) synthetase, with concomitant cellular energy depletion and cell necrosis (see later).

Some of the biological actions of peroxynitrite are summarized in Table I. These effects can manifest in many ways in intact cells. For instance, in pulmonary type II cells, inhibition by peroxynitrite of membrane Na^+,K^+-ATPase activity and sodium uptake has been reported (Hu *et al.,* 1994). In neurons, glial cells (Bolanos *et al.,* 1995), cultured macrophages (Zingarelli *et al.,* 1996; Szabó *et al.,* 1996b), cultured rat aortic smooth muscle cells (Szabó *et al.,* 1996a), endothelial cells (Szabó *et al.,* 1997b), epithelial cells (Szabó *et al.,* 1997c; Kennedy *et al.,* 1998), as well as a number of other cell types, profound inhibition by peroxynitrite of mitochondrial respiration has been observed, with inhibition of NADH–coenzyme Q1 reductase, succinate–cytochrome *c* reductase, and cytochrome *c* oxidase activities. In macrophages, smooth muscle cells, epithelial cells, and endothelial cells, peroxynitrite induces a marked reduction of intracellular NAD^+ and ATP levels (Szabó *et al.,* 1996a,b, 1997c; Kennedy *et al.,* 1998).

Mechanistically, peroxynitrite or NO generation from exogenous or endogenous sources can ultimately lead to necrotic or apoptotic cell death. Whereas higher levels of sudden oxidant bursts tend to induce necrosis, lower levels with longer times of exposure tend to lead to cell death via the apoptotic pathway (Bonfoco *et al.,* 1995; Lin *et al.,* 1995; Estevez *et al.,* 1995; Salgo *et al.,* 1995a,b; Sandoval *et al.,* 1996). Several independent groups have reported that caspase-3, a mediator of apoptosis, but not caspase-1, becomes activated in cells exposed to peroxynitrite: its pharmacological inhibition suppresses peroxynitrite-induced apoptotic DNA fragmentation (Virág *et al.,* 1998a,b; Lin *et al.,* 1998). The activation of caspases may be related to the release of cytochrome *c* from the mitochondria induced by peroxynitrite.

Table I Selected Detrimental Actions of NO or Peroxynitrite in Inflammation

Effect	Mechanism of action
Toxic vasodilation	cGMP, energetic failure
Toxic myocardial effects	cGMP, energetic failure
Inhibition of cellular respiration	Multiple (S-nitrosylation, peroxynitrite)
Cell necrosis	Peroxynitrite, PARS
Apoptosis	Multiple: peroxynitrite, NO, caspase activation, transglutaminase
Inhibition of biosynthetic pathways	Multiple
Signal transduction disturbances	Multiple (S-nitrosylation, peroxynitrite, interaction with sulfhydryl groups, tyrosine nitration
Endothelial dysfunction	Multiple (peroxynitrite, PARS)
NMDA receptor activation	Peroxynitrite
Inhibition of glucocorticoid receptor affinity	S-Nitrosylation
Activation of cyclooxygenases	Peroxynitrite
Inflammatory pain development	Multiple, in part cGMP-dependent
Inflammatory plasma extravasation	Multiple, in part cGMP-dependent
Priming of neutrophil activation	Peroxynitrite, tyrosine nitration
Amplification of signal transduction pathways	Multiple, peroxynitrite dependent
Inhibition and inactivation of antioxidant systems	Multiple, peroxynitrite (in part)
Damage to lipids	Peroxynitrite-mediated peroxidation
DNA nicking, DNA strand breakage	Peroxynitrite-mediated oxidation
Inhibition of DOPA biosynthesis	Tyrosine nitration of tyrosine hydroxylase
Activation of PARS	DNA single-strand breakage.

It is noteworthy that, although the predominant actions of peroxynitrite are cytotoxic, small amounts of peroxynitrite are produced under basal, physiological conditions (since, in many cell types, NO from the constitutive NOS isoforms and superoxide from mitochondria and other cellular sources are always produced) (Kelm *et al.,* 1997). It is probable that the endogenous antioxidant systems are sufficient to neutralize such low-level peroxynitrite production (and, possibly, convert it to NO donor agents; see later). Therefore, low levels of peroxynitrite are not cytotoxic, or they even may be cytoprotective. For example, low levels of peroxynitrite have been shown to inhibit neutrophil adhesion (Lefer *et al.,* 1997). Under these conditions, peroxynitrite is likely to form NO adducts with glucose, thiols, and other species (Moro *et al.,* 1994, 1995), which, in turn, can act as NO donor compounds, which can exert cytoprotective effects by activating guanylyl cyclase (Tarpey *et al.,* 1995; Mayer *et al.,* 1995, Davidson *et al.,* 1997) or by other mechanisms.

THE PEROXYNITRITE–POLY(ADP-RIBOSE) SYNTHETASE PATHWAY

In addition to direct cytotoxic effects, an indirect pathway of peroxynitrite-induced cellular injury has been proposed. The generation of peroxynitrite, either intracellularly or extracellularly, has been shown to trigger DNA single-strand breakage and the activation of the nuclear enzyme poly(ADP-ribose) synthetase [PARS, also termed poly(ADP-ribose) polymerase or PARP]. The importance of this pathway is reviewed in detail elsewhere (Cochrane, 1991; Szabó and Dawson, 1998; Szabó *et al.,* 1998). Briefly, DNA single-strand breakage is the obligatory trigger of activation of PARS. When activated, PARS catalyzes the cleavage of NAD^+ to ADP-ribose and nicotinamide. PARS covalently attaches ADP-ribose to various nuclear proteins, such as histones and PARS itself. Activation of PARS can rapidly deplete NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation, resulting in cell dysfunction. These processes can lead to necrotic cell death (Heller *et al.,* 1995; Eliasson *et al.,* 1997; Virág *et al.,* 1998a–c).

Pharmacological inhibition of PARS activity protects against cell damage in response to exogenously or endogenously produced peroxynitrite (Szabó *et al.,* 1996b, 1997b–d; Kennedy *et al.,* 1998, Virág *et al.,* 1998a–c). *In vivo,* inhibition or genetic inactivation of PARS has been shown to protect against inflammatory cell injury and disease development in arthritis (Miesel *et al.,* 1994, 1996; Kroger *et al.,* 1996; Szabó *et al.,* 1998), colitis (Kennedy *et al.,* 1998; Zingarelli *et al.,* 1999), allergic encephalomyelitis (Scott *et al.,* 1998), endotoxic and hemorrhagic shock (Szabó *et al.,* 1996a; 1997d–f), and diabetes (Heller *et al.,* 1995; Burkart *et al.,* 1999; Masutani *et al.,* 1999). $PARS^{-/-}$ animals were also found resistant against zymosan-induced inflammation and multiple organ failure when compared to the response of wild-type mice (Szabó *et al.,* 1997d), and they were also resistant against colitis (Zingarelli *et al.,* 1999) and reperfusion injury of the brain and heart (Endres *et al.,* 1997; Eliasson *et al.,* 1997; Zingarelli *et al.,* 1998b). The role of PARS in various inflammatory conditions is overviewed elsewhere (Szabó, 1998).

Peroxynitrite is probably the best candidate for triggering the DNA single-strand breakage and subsequent activation of PARS in inflammation. Another potential trigger is hydroxyl radical, which is known to contribute to the development of many inflammatory responses and is also capable of DNA single-strand breakage and poly(ADP-ribose) synthetase activation (Berger, 1985; Szabó, 1998). However, peroxynitrite (in contrast to hydroxyl radical) can travel significant distances from producer cell to target cell. Furthermore, NO can also travel significant distances and subsequently react with superoxide in the vicinity of cells, which then become subjected to peroxynitrite-mediated oxidant or nitrosative attack. Currently, in addition to peroxynitrite and hydroxyl radical, the only other trigger of DNA single-strand breakage that has been identified is NO^- (Ohshima *et al.,* 1998). Very little is known about the reactivity and production of this latter species *in vivo* (in

general) or in inflammation (in specific). Nevertheless, nitroxyl anion can be formed by the reaction of *S*-nitrosothiols with thiols (Wong *et al.,* 1998) or by the reaction of NO with superoxide dismutase (Murphy and Sies, 1991). Nitroxyl anion can also exert cytotoxic effects *in vitro* (Wink *et al.,* 1998). Nitroxyl anion induces cell necrosis, in some part, via PARS activation *in vitro* (C. Szabó, unpublished observation, 1999). Further work will be needed to determine whether NO^- is a new candidate capable of mediating DNA injury and PARS activation in inflammation.

PARS activation is important in peroxynitrite-induced cell necrosis, but it does not play a role as an effector pathway in NO^- or peroxynitrite-induced apoptosis. Inhibition of PARS does not prevent but, in some instances, increases peroxynitrite-induced apoptosis (Leist *et al.,* 1997; O'Connor *et al.,* 1997; Szabó *et al.,* 1997b; Virág *et al.,* 1998a). The mechanism of this enhanced oxidant-induced apoptosis in response to PARS inhibition is likely to be a consequence of the increased intracellular energetic stores (apoptosis being an energy-dependent process) (Virág *et al.,* 1998a–c).

Peroxynitrite-Induced Interference with Signal Transduction

As previously discussed, NO, via S-nitrosylation reactions, can interrupt some signal transduction processes. Peroxynitrite-induced toxicity may also be related to the disruption of membrane signal transduction pathways. Multiple mechanisms of such interactions have been characterized (Gow *et al.,* 1996; Berlett *et al.,* 1996; Kong *et al.,* 1996; Elliott, 1996; Darley-Usmar and White, 1997). For example, phosphatidylinositol 3-kinase, a key enzyme involved in signal transduction cascades, has been shown to be nitrated by peroxynitrite, which results in an inhibition of its activity (Hellberg *et al.,* 1998). Peroxynitrite inhibits bradykinin-stimulated Ca^{2+} signaling in endothelial cells (Elliott, 1996). Peroxynitrite induced the nitration of both tyrosines of lymphocyte-specific tyrosine kinase, resulting in a complete inhibition of the catalytic activity of the enzyme (Kong *et al.,* 1996). In a more recent series of studies, exposure to peroxynitrite inhibited phosphoinositide hydrolysis activated by muscarinic or glutamatergic metabotropic receptors in cerebral cortical slices and in astrocytoma cells (De Sarno and Jope, 1998; Li *et al.,* 1998).

Membrane receptors can also be modified by peroxynitrite. An example is the epidermal growth factor receptor, which undergoes dimerization by peroxynitrite, probably involving intermolecular dityrosine cross-linking (Van der Vliet *et al.,* 1998a). In a study in erythrocytes, peroxynitrite induced a variety of alterations including (1) cross-linking of membrane proteins, (2) inhibition of band 3 tyrosine phosphorylation, (3) nitration of tyrosines in the 22K cytoplasmic domain of band 3, (4) binding of hemoglobin to the membrane, (5) irreversible inhibition of phosphotyrosine kinase activity, (6) massive methemoglobin production, and (7) irreversible inhibition of lactate production (Mallozzi *et al.,* 1997). Most of the evidence derives from reductionist experimental models. In addition, it is unclear whether the concentrations or fluxes of peroxynitrite required for the induction of signal transduction disturbances are achieved during inflammation. Nevertheless, it is conceivable that peroxynitrite production *in vivo,* during inflammation, disturbs a variety of signal transduction mechanisms, which contributes to the pathophysiological actions of NO or peroxynitrite in inflammation.

Peroxynitrite-Mediated Activation of Cyclooxygenase

Following the original observation of Salvemini and colleagues in 1993, it was generally accepted that NO acts as an enhancer of the catalytic activity of cyclooxygenase (Salvemini *et al.,* 1993; Molina-Holgado *et al.,* 1995; Salvemini and Masferrer, 1996; Kanematsu *et al.,* 1997; Mollace *et al.,* 1998). This mechanism was found to serve both physiological or beneficial functions, as demonstrated in relation to the NO-mediated inhibition of platelet aggregation (Salvemini *et al.,* 1996a), and it has also been proposed to be of pathophysiological importance, as shown in models of renal inflammation (Salvemini *et al.,* 1994) and in systemic inflammation induced by LPS (Salvemini *et al.,* 1995). More recent studies in cultured, immunostimulated macrophages indicate that peroxynitrite, rather than NO, is responsible for the activation of cyclooxygenase (Landino *et al.,* 1996). It is probable that enhanced NO and/or peroxynitrite generation in inflammation can lead to increased prostaglandin formation, with all the usual consequences of prostaglandin formation in inflammation (pain, fever, swelling, etc.). The NO- or peroxynitrite-mediated activation of COX has been confirmed not only in prototypical reductionist animal models of inflammation (see later), but also in other pathophysiological conditions that are not considered inflammatory per se but have a significant inflammatory component, such as cerebral ischemia and reperfusion (Nogawa *et al.,* 1998).

It is noteworthy that, in addition to regulating the activity of cyclooxygenases, NO has been implicated in the transcriptional regulation of the expression of the inducible cyclooxygenase (COX-2). Inhibition of NOS in immunostimulated macrophages has been reported to suppress the expression of COX-2 (Habib *et al.,* 1997). It remains to be seen whether NOS inhibitors also exert an inhibitory effect on COX-2 expression in various forms of inflammation *in vivo.*

Pathophysiological Roles of Nitric Oxide in Inflammation

Toxic Vasodilation

Local overproduction of NO in the vicinity of blood vessels leads to local vasodilation in inflammation. Similarly, systemic overproduction of NO by the blood vessels in systemic inflammation (or shock) leads to extensive systemic vasodilation. The mechanism of this vasodilation involves a number of distinct mechanisms, which act in an additive or synergistic fashion. Excessive vasodilation (such as that

which occurs in various forms of systemic inflammation and shock) can be extremely detrimental, and it has been a target of many experimental therapeutic approaches involving various NOS inhibitor and NO scavenger compounds (Kilbourn *et al.,* 1997).

cGMP-Dependent Mechanisms of NO-Mediated Vasodilation

As described earlier, many of the physiological actions of NO are mediated through the guanylyl cyclase/cGMP system. In an earlier line of research, Murad and colleagues studied the mechanism by which nitroglycerin and related nitrovasodilators elicit vascular smooth muscle relaxation. By 1977, it had been established that the mechanism by which these agents cause dilations involved NO-mediated activation of the soluble form of guanylyl cyclase, with a subsequent conversion of GTP to cyclic GMP (Katsuki *et al.,* 1977). The guanylyl cyclase–cGMP system turned out to be of central importance for mediating most of the physiological and some of the pathophysiological vascular actions of NO (Ignarro, 1991; Schmidt *et al.,* 1993). In this pathway, NO diffuses to adjacent cells and activates soluble guanylyl cyclase by binding to the iron on its heme component, thereby moving the iron out of the plane of the porphyrin ring. An increase in cytoplasmic cGMP, and the subsequent reduction of intracellular calcium concentration due to enhanced calcium extrusion and sequestration into intracellular stores (Twart and Van Breemen, 1988), was proposed to mediate the relaxation of vascular and nonvascular smooth muscle.

''Classic'' inhibitors of guanylyl cyclase, such as methylene blue and hemoglobin, have been shown to improve vascular contractility in shock, and they cause an increase in the blood pressure of rodents subjected to endotoxin (Paya *et al.,* 1993). There were, however, some controversial issues regarding the specificity or nonspecificity of certain guanylyl cyclase inhibitors. For example, methylene blue is known to have additional pharmacological actions, including the generation of oxyradicals and direct inhibition of NOS (Marczin *et al.,* 1996). In more recent lines of investigation, a more specific and potent guanylyl cyclase inhibitor, which does not appear to have additional pharmacological actions, has been used (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, or ODQ). This agent, too, inhibits the vasodilation induced by NO in intact vascular preparations, both when dilations are elicited by endothelium-dependent vasorelaxants and also when blood vessels are incubated with LPS for several hours in order to induce iNOS within the vasculature (Moro *et al.,* 1996).

cGMP-Independent Mechanisms of Vasodilation

As mentioned earlier, it is now well established that NO (or a related species, such as peroxynitrite, see later) also has a number of cGMP-independent actions. The ability of NO to directly inhibit mitochondrial respiration within vascular smooth muscle cells is well established (Geng *et al.,* 1992). Moreover, NO generation has been shown to be associated with DNA single-strand breakage in the vascular smooth muscle, which then activates PARS. Activation of this enzyme, in turn, leads to an ineffective DNA repair cycle, which depletes smooth muscle cells of their ATP and NAD stores and suppresses contractility during endotoxic shock (Szabó, 1998).

Part of the oxidant and cytotoxic effects of NO are due to reaction of NO with superoxide anion to form peroxynitrite, with subsequent formation of nitrogen dioxide and hydroxyl radical. The production of peroxynitrite is well established in various blood vessels and organs of animals subjected to endotoxic shock (Szabó *et al.,* 1995a; Szabó, 1996). Peroxynitrite can induce a number of other oxidant and cytotoxic processes, such as inhibition of the activity of membrane sodium–potassium antiport and inhibition of calcium pumps in the sarcoplasmatic membrane (Viner *et al.,* 1996). It is conceivable that such effects would also impair the contractility of the vascular smooth muscle cells.

cGMP-independent activation by NO or peroxynitrite of other enzymes, such as COX, has also been described in various cell types (see earlier). Such an effect would be expected to increase the production of prostaglandins within the vascular smooth muscle. The finding that the cyclooxygenase inhibitor indomethacin further impaired rather than improved the contractility of the thoracic aortic rings obtained from endotoxin-treated rats (Wu *et al.,* 1994) suggests that, at least in this particular setting, the prostaglandins produced—possibly by NO-induced enhancement of the activity of COX—are predominantly vasoconstrictors. However, the vasoactive effects of the prostaglandins produced may well differ, depending on the species and the vascular bed.

Taken together, and contrary to the original suggestion that the NO-mediated progressive vasodilation is solely mediated by activation of guanylyl cyclase in the smooth muscle, it appears that there are multiple components of the NO-mediated vasodilation, especially under the conditions of local or systemic inflammation. These mechanisms may include metabolic mechanisms (inhibition of cellular energetics via multiple pathways), indirect actions of NO (via COX), and possibly also cGMP-independent activation of calcium-dependent potassium channels (Bolotina *et al.,* 1994; Pataricza *et al.,* 1995; Hall *et al.,* 1996) and ATP-sensitive potassium channels in the smooth muscle cell membrane (Murphy and Brayden, 1995). The relative importance of these pathways in the various stages and forms of inflammation and shock, and the interaction between these pathways, should be the subject of further investigations.

Role of Nitric Oxide or Peroxynitrite in the Development of Endothelial Dysfunction in Inflammation

There is a multitude of data demonstrating the impairment of the biosynthesis of NO from L-arginine by ecNOS in the vascular endothelium in various forms of shock and inflammation (Szabó *et al.,* 1992a,b; Wang *et al.,* 1995). This

phenomenon is sometimes referred to as ''isoform switch,'' whereby the inducible isoform of NOS is expressed while the constitutive isoform of NOS is downregulated at the same time. Reduced production of NO from the endothelium would, in turn, enhance the adhesion and aggregation of platelets and the adhesion and activation of neutrophil granulocytes to the intima, thereby increasing the risk of multiple organ failure. Increased initial neutrophil adherence would, in turn, lead to further development of endothelial injury (via an oxyradical- or peroxynitrite-dependent mechanism) and would further exacerbate tissue injury. Interestingly, accumulating data suggest that, paradoxically, the development of endothelial dysfunction is dependent on NO. In a LPS-induced model of renal inflammation and dysfunction, selective inhibition of iNOS prevents the development of endothelial dysfunction (Swartz *et al.,* 1997). Similarly, in endotoxic shock, inhibition of iNOS protects against the development of endothelial dysfunction (Szabó *et al.,* 1996c; Fatehi-Hassanabad *et al.,* 1996). The mechanism of the NO-mediated endothelial dysfunction may be related to the generation of peroxynitrite in the vicinity of the endothelial cells and the activation of poly(ADP-ribose) synthetase in the vascular endothelium (Zingarelli *et al.,* 1997b; Szabó *et al.,* 1997b).

Role of Nitric Oxide in Inflammatory Pain Development

Pain development is a prototypical constituent of most inflammatory conditions. The mechanisms of pain development and sensation can be classified as peripheral and central mechanisms. The peripheral mechanism of hyperalgesia is considered to be the result of nociceptor sensitization. As potential agents involved in nociceptor sensitization, bradykinin, histamine, prostaglandins, protons, and nerve growth factor have been implicated (Mizumura, 1997; Mogil and Grisel, 1998). There is some evidence implicating a permissive role of basal NO production in prostaglandin E_2 (PGE)-induced hyperalgesia (Aley *et al.,* 1998). It appears that low levels of NO facilitate cAMP-dependent PGE_2-induced hyperalgesia, whereas higher levels of NO produce a cGMP-dependent hyperalgesia (Aley *et al.,* 1998). In another set of studies, the antinociceptive effects of peripherally applied κ- and δ-opioid agonists were found to be potentiated by the local action of NO, mainly via the cGMP pathway (Nozaki-Taguchi and Yamamoto, 1998). In animal models of arthritis, intraarticular injection of a non-isoform-selective NOS inhibitor resulted in a complete reversal of heat hyperalgesia (Lawand *et al.,* 1997). On the basis of these data, it is conceivable that inhibition of NO-mediated peripheral hyperalgesia and pain development may be an additional anti-inflammatory effect of NOS inhibitors. There is also some evidence for the involvement of NO in central pain sensing (Semos and Headley, 1994; Roche *et al.,* 1996). Interestingly, the antinociceptive effects of some drugs (e.g., ketorolac) can be suppressed by pharmacological inhibition of NOS (Ferreira, 1993; Granados-Soto *et al.,* 1995).

Role of Nitric Oxide in Inflammatory Plasma Extravasation

There is accumulating evidence for the involvement of NO, or a related species, in plasma extravasation associated with various forms of inflammation. Direct studies demonstrated that intradermally injected peroxynitrite acts, in a dose-dependent manner, to cause a marked increase in plasma extravasation (Greenacre *et al.,* 1997). Inhibition of NOS has been shown to suppress LPS-induced plasma leakage in the rat skin (Iuvone *et al.,* 1998). In addition, inhibition of iNOS activity with an aminoguanidine in the late phase of endotoxic shock attenuated plasma extravasation (Filep *et al.,* 1997). Furthermore, aminoguanidine abolished this LPS-induced enhancement of plasma leakage to substance P in the airways (Ohuchi *et al.,* 1998), and mercaptoethylguanidine attenuated the plasma extravasation in ligation-induced periodontitis (Lohinai *et al.,* 1998). The IL-2 induced pulmonary edema and pleural effusion is also partially iNOS dependent (Orucevic *et al.,* 1997). It is conceivable that inhibition of NO- or peroxynitrite-mediated plasma extravasation may be an additional anti-inflammatory mode of action of NOS inhibitors.

Regulation of Gene Expression by Nitric Oxide or Peroxynitrite: Relevance to Inflammation

Studies using various NOS inhibitors have been undertaken during the 1990s to unveil the consequences of NO production in inflammation. As described in previous sections, the sum of these observations appear to indicate that a basal level of NO, most likely provided by ecNOS, has important organ-protective and anti-inflammatory effects. A second conclusion from these studies is that enhanced NO production during various forms of shock and inflammation frequently has detrimental consequences. In this scheme, iNOS is considered a terminal, final effector molecule of shock and inflammation. However, more recent work suggests that NO and peroxynitrite can also have signaling functions that might regulate gene expression and related cellular responses in shock and inflammation. In a model of hemorrhagic shock (a systemic inflammatory condition characterized by the production of proinflammatory cytokine and chemokine cascades, leukocyte adhesion molecule expression, and neutrophil infiltration into tissues), the lack of iNOS (selective pharmacological inhibition or genetic inactivation, i.e., with iNOS knockout mice) was associated with a marked reduction in the activation of NFκB in the liver and lung as well as reductions in mRNA levels for IL-6 and granulocyte-colony stimulating factor (G-CSF) (Hierholzer *et al.,* 1998). Both IL-6 and G-CSF activate the transcription factor signal transducer and activator of transcription 3 (STAT3). Accordingly, STAT3 activation was reduced in the absence of iNOS activity. Occurring with these reductions in transcriptional factor activation and cytokine expression were a dramatic reduction in neutrophil infiltration into the lungs as well as reduced liver and lung injury measured by

plasma transaminase levels and wet-to-dry ratios, respectively (Hierholzer *et al.,* 1998). These studies indicated that NO can perform key signaling functions in hemorrhagic shock. With the many known targets for NO and peroxynitrite, this finding is perhaps not surprising. It is currently unclear whether this phenomenon is of general importance in inflammation and shock or, alternatively, it is specific to hemorrhagic shock. A relatively unique aspect of NO-mediated signaling in hemorrhagic shock is the associated redox stress. It is possible under conditions of reduced antioxidant capacity that NO or a reaction product such as peroxynitrite activates intracellular redox-sensitive signaling pathways.

A more general role of iNOS-derived NO (or peroxynitrite) as an amplifier of the inflammatory response is now also supported by the following observations: *in vitro,* inhibition of NOS can suppress the production of IL-1 (Vallette *et al.,* 1997), and peroxynitrite can stimulate the production of IL-8 in human whole blood via a mechanism that probably involves the activation of NF-κB (Filep *et al.,* 1998; Cuthbertson *et al.,* 1998). Importantly, NO has been shown to mediate the cytokine-induced expression of matrix metalloproteinases (Sasaki *et al.,* 1998). In addition, peroxynitrite has been reported to directly enhance the activity of the neutrophil collagenase (Okamoto *et al.,* 1997). Both of these mechanisms may have important implications for the mechanism of the protective action of NOS inhibitors in arthritis. *In vivo,* inhibition of iNOS suppresses TNF production in allergic encephalomyelitis (Brenner *et al.,* 1997); inhibition of iNOS suppresses IL-1, collagenase, and stromelysin production in arthritis (Pelletier *et al.,* 1998; Brahn *et al.,* 1998); inhibition of iNOS suppresses IFN-γ production in a murine model of leishmaniasis (Diefenbach *et al.,* 1998); and the expression of a number of chemokines is suppressed in the absence of iNOS in zymosan-induced peritonitis (Ajuebor *et al.,* 1999). Taken together, this evidence paints an evolving picture of a pathway in which NO contributes to tissue damage both directly (in part, via the formation of peroxynitrite) and indirectly (i.e., through the amplification of the inflammatory response).

Mammalian Defense Mechanisms against the Actions of Nitric Oxide and Peroxynitrite in Inflammation

Both the microorganisms (which can be the target of iNOS-related NO or peroxynitrite production) and cells of the host organism (which, acting as ''innocent bystanders,'' can be damaged in the inflammatory response) have developed multiple mechanisms for protection against NO- or peroxynitrite-related toxicities. Bacterial resistance to NO is not a subject of this chapter. Therefore, we only briefly mention here that in *Escherichia coli,* the SoxRS regulon was found to be required for resistance to redox-cycling agents that elevate cytosolic superoxide levels as well as for resistance to NO-dependent macrophage killing (Nunoshiba *et al.,* 1993, 1995). In contrast, in *Salmonella typhimurium,* SoxS is also required for enhanced expression of Mn-superoxide dismutase and resistance to paraquat, but not for resistance to NO donor compounds, resistance to macrophage killing, or virulence in mice (Fang *et al.,* 1997). Additional studies demonstrated that bacterial resistance can be dependent on the inducible upregulation of the transcription factor oxy-R (Hausladen *et al.,* 1996) and NO dioxygenase (NOD), a microbial flavohemoglobin which oxidizes NO via a peroxynitrite-like intermediate, as demonstrated in *E. coli* (Gardner *et al.,* 1998b; Hausladen *et al.,* 1998). Periplasmic superoxide dismutase has also been shown to protect *Salmonella* from NO-mediated cytotoxicity (De Groote *et al.,* 1997). An additional defense mechanism in bacteria includes the subunit C of alkyl hydroperoxide reductase, an enzyme that reduces organic peroxides and that protects cells from reactive nitrogen intermediates, as demonstrated in *Mycobacterium tuberculosis* and *S. typhimurium* (Chen *et al.,* 1998).

The host organisms are defended against NO- or peroxynitrite-related toxicities by two main types of mechanisms: (1) scavengers/antioxidants and (2) regulated detoxifying pathways. There are a number of antioxidants in the cellular environment that can attenuate peroxynitrite-induced oxidative injury. In plasma, peroxynitrite oxidizes ascorbic acid, uric acid, tyrosine, and -SH groups of plasma proteins (Van Der Vliet *et al.,* 1994; Watts *et al.,* 1995). The reaction of peroxynitrite with thiols, which yields nitrosothiols and which may be the most important physiological detoxification mechanism of peroxynitrite, has been characterized only relatively recently. It appears to involve a direct nucleophilic nitrosation mechanism with elimination of hydroperoxyl anion (Van der Vliet *et al.,* 1998b). *In vitro* studies have established that endogenous scavengers of peroxynitrite include cysteine, glutathione, ascorbic acid, vitamin E, β-carotene, uric acid, and melatonin (Radi *et al.,* 1991; De Groot *et al.,* 1993; Van der Vliet *et al.,* 1994; Shi *et al.,* 1994; Hogg *et al.,* 1994; Pryor and Squadrito, 1995; Karoui *et al.,* 1996; Vatassery, 1996; Gilad *et al.,* 1997; Cuzzocrea *et al.,* 1997; Christen *et al.,* 1997). A marked depletion by exogenous or endogenous peroxynitrite of cellular antioxidants including glutathione has been reported in plasma as well as in various cell types, such as endothelial cells and smooth muscle cells (Van der Vliet *et al.,* 1994; Vatassery, 1996; Phelps *et al.,* 1995; Szabó *et al.,* 1996a). In the presence of plasma, proteins, glucose, or glutathione, peroxynitrite can form intermediates that act as NO donor compounds, as a form of a peroxynitrite-detoxifying mechanism (Moro *et al.,* 1994, 1995). There may be a delicate balance between peroxynitrite-mediated oxidant processes and endogenous antioxidant pathways that limit the reactivity of peroxynitrite.

The importance of endogenous glutathione in modulating peroxynitrite-related cytotoxicity is underlined by experiments using L-buthionine-(*S,R*)-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, which inhibits a key enzyme in the synthesis of glutathione, and thereby depletes intracellular glutathione. *In vitro* studies in neurons (Barker *et al.,* 1996), endothelial cells (Szabó *et al.,* 1997b; Cuzzocrea *et al.,* 1998a), and smooth muscle cells (Cuzzocrea *et al.,* 1998a) demonstrated a significant enhancement

of the peroxynitrite-induced suppression of mitochondrial respiration, DNA injury, tyrosine nitration, and protein oxidation by BSO pretreatment. On the other hand, exogenous glutathione and glutathione ethyl ester elicited protective effects against peroxynitrite-induced cytotoxicity (Barker *et al.,* 1996; Cuzzocrea *et al.,* 1998a). Similarly, in immunostimulated smooth muscle cells, depletion of glutathione enhances the degree of peroxynitrite-induced suppression of mitochondrial respiration, without affecting the amount of NO produced (Cuzzocrea *et al.,* 1998a). There is also a marked enhancement of protein oxidation in immunostimulated or peroxynitrite-challenged cells in the absence of glutathione (Cuzzocrea *et al.,* 1998a). The suppression of mitochondrial respiration in response to immunostimulation can be prevented by the NOS inhibitor N^G-methyl-L-arginine (L-NMA), confirming the role of NO or a related species, such as peroxynitrite, in the process (Cuzzocrea *et al.,* 1998a). These observations are of importance with respect to various forms of inflammation, shock, and ischemia–reperfusion injury. In several studies, depletion of endogenous glutathione pools has been shown to increase organ injury (Keller *et al.,* 1985; Nemeth and Boda, 1989; Stein *et al.,* 1990; P. Liu *et al.,* 1993). In shock, inflammation, and reperfusion injury, superoxide, hydroxyl radical, hydrogen peroxide, and peroxynitrite probably all contribute to the depletion of the endogenous antioxidant pool.

The relationship between antioxidant defenses and NO is complicated by the fact that NO and peroxynitrite have been shown to inactivate some of the antioxidant defenses of the mammalian cells. For example, glutathione reductase and glutathione-*S*-transferases have been shown to be inhibited by the NO carriers *S*-nitrosoglutathione and dinitrosyl–iron-complexed thiols (Asahi *et al.,* 1995, 1997; Keese *et al.,* 1997). Furthermore, peroxynitrite has been shown to inhibit superoxide dismutase (MacMillan-Crow *et al.,* 1996). Thus generation of toxic concentrations of NO or peroxynitrite in the cell can lead to attenuated defense systems or to even more oxidant formation via positive feedback cycles. For instance, exposure of mitochondria to peroxynitrite can lead to enhanced superoxide generation (Packer *et al.,* 1996), and exposure of thymocytes to an early, single burst of peroxynitrite induces a time-dependent reactive oxygen species generation (Virág *et al.,* 1998c). Neutralization of these secondary oxygen species with scavengers, in fact, can attenuate peroxynitrite-induced cell death (Virág *et al.,* 1998c).

With respect to transcriptionally regulated (inducible) eukaryotic defense mechanisms against NO, the available information is limited. Metallothionein, a major protein thiol induced in cells exposed to cytokines and bacterial products, has been shown to reduce the sensitivity of mammalian cells to NO donor compounds (Schwarz *et al.,* 1995). Heat-shock proteins have also been reported to be induced in response to exposure of mammalian cells to NO (Malyshev *et al.,* 1996; Hirvonen *et al.,* 1996; Byrne and Hanson, 1998). Furthermore, heat-shock protein expression or overexpression protects mammalian cells against NO- or peroxynitrite-mediated toxicity (Bellmann *et al.,* 1996; Szabó *et al.,* 1996d; Wong *et al.,* 1997a,b). It is unclear whether the mammalian cells, similar to the bacteria, can also express NOD-like or hemoglobin-like molecules, which would, in turn, protect against NO- or peroxynitrite-mediated toxicities. It is likely that inducible, multiple, regulated NO-detoxifying pathways exist in mammalian cells, and studies in this direction are expected to be a fruitful area of future research.

The Double-Edged Sword Dilemma: Nitric Oxide as Cytoprotector versus Cytotoxin

Under certain conditions, locally produced NO can exert marked antioxidant or cytoprotective actions, whereas in many other conditions, NO was found cytotoxic. This double-edged-sword-like role of NO has intrigued many investigators, but many details of the dual behavior of NO have yet to be clarified. Nevertheless, it is clear that, depending on the oxidative status of the cell and on a number of other conditions, NO can act as an antioxidant, neutralizing the cytotoxic actions of superoxide, whereas in other cases, NO enhances the cytotoxic potential of superoxide. The ratio of NO and superoxide has been implicated as a determinant of the apparent reactivity of peroxynitrite: excess NO reduces the oxidation elicited by peroxynitrite (Rubbo *et al.,* 1994; Villa *et al.,* 1994; Szabó *et al.,* 1995b; Miles *et al.,* 1996). Other cytoprotective mechanisms of NO are related to its reaction with caspases (via-S-nitrosylation) and thereby inhibition of the apoptotic machinery (see earlier). The concentrations of NO required for this latter effect are relatively low, probably in the physiological range.

When considering the multifaceted roles of NO, it is not surprising that the pharmacological inhibition of the production of NO in inflammation may have either detrimental or protective effects. In general, NO produced by the constitutive endothelial NOS is considered protective: its inhibition exacerbates a number of forms of inflammation, mainly due to worsened tissue perfusion and increased neutrophil infiltration, platelet deposition, and microthrombus formation. This early NO production is essential in maintaining vascular patency in shock: its inhibition frequently worsens the outcome, whereas administration of NOS inhibitors in the late stage of shock is mostly protective (overviewed in Szabó, 1995; Kilbourn *et al.,* 1997). Nevertheless, ecNOS-derived NO can also act as a cytotoxic species, for example, via its reaction with superoxide, in the early phase of reperfusion (see Szabó, 1996, for review).

The expression of iNOS can have beneficial effects, or deleterious effects, depending on the inflammatory condition in question. Factors that appear to dictate the consequences of iNOS expression include the type of insult, the tissue type, the level and duration of iNOS expression, and probably the redox status of the tissue. Overall, more attention has focused on the toxicity of iNOS. For example, induction of iNOS in endothelial cells produces endothelial injury (Palmer *et al.,* 1992). Induction of iNOS has been shown to inhibit cellular respiration in macrophages and vascular smooth muscle cells: these processes can lead to cell dys-

function and cell death (e.g., Ischiropoulos *et al.,* 1992b; Szabó *et al.,* 1996a,b,e, 1997e; Iwashina *et al.,* 1998). Such processes, when occurring within vascular smooth muscle cells, play a key role in the pathogenesis of the vascular hyporeactivity and progressive vascular decompensation associated with various forms of circulatory shock (see earlier). NO produced by iNOS also plays a role in the vasodilatory response associated with local inflammation (e.g., in the skin) (Iuvone *et al.,* 1998). In clear contrast, expression of iNOS in liver cells can suppress endotoxin and TNF-α-induced toxicity, by inhibition of caspase activation (Ou *et al.,* 1997; Kim *et al.,* 1998b). Overexpression of iNOS by gene transfer also limits LPS-induced toxicity in endothelial cells (Ceneviva *et al.,* 1998).

The role of iNOS may also well be dependent on the stage of the disease. For example, studies in experimental allergic encephalomyelitis have demonstrated that inhibition of iNOS expression in the early (induction) phase of the disease exacerbates the severity of the condition, whereas inhibition of iNOS in the late (inflammatory) phase of the condition has been shown to exert protective effects (Cross *et al.,* 1995; Brenner *et al.,* 1997; Okuda *et al.,* 1997; Sahrbacher *et al.,* 1998; Fenyk-Melody *et al.,* 1998).

Role of Nitric Oxide in Specific Inflammatory Conditions

Induction of iNOS and enhanced formation of NO have been demonstrated in almost all forms of inflammation, including arthritis (overviewed in Clancy *et al.,* 1998; Amin and Abramson, 1998; Stichtenoch and Frolich, 1998; Jang and Murrell, 1998), diabetes (Kolb-Bachofen, 1996; McDaniel *et al.,* 1996; Rothe and Kolb, 1999), systemic inflammatory response syndrome or circulatory shock (overviewed in Szabó, 1995; Kilbourn *et al.,* 1997), encephalomyelitis and other inflammatory conditions of the central nervous system (as overviewed in Kolb and Kolb-Bachofen, 1998; Hirsch *et al.,* 1998; Brosnan *et al.,* 1997; Parkinson *et al.,* 1997), colitis (Salzman, 1995; Alican and Kubes, 1996; Whittle, 1997; Guslandi, 1998), pancreatitis (Alhan *et al.,* 1998), gingivitis and periodontitis (Lohinai *et al.,* 1998), uveitis (Becquet *et al.,* 1997), transplant rejection (Cannon *et al.,* 1998; Dusting and MacDonald, 1995), pneumonia (Akaike *et al.,* 1996), and myocarditis (Ishiyama *et al.,* 1997) among many others. The expression of iNOS has also been shown in many pathophysiological conditions that do not qualify as inflammation per se but do have a significant inflammatory component (e.g., atherosclerosis or the late phase of reperfusion injury) (e.g., Wildhirt *et al.,* 1995, 1996, 1997; Dusting, 1996). In many of these conditions, the overproduction of NO, the expression of iNOS, the generation of peroxynitrite, and the protective effects of iNOS inhibition have been demonstrated.

As many of the inflammatory conditions listed are subjects of separate chapters in this book, detailed discussion on the role of NO in the previously mentioned specific inflammatory conditions was not included in this chapter. However, we will discuss the findings related to NO in the pleurisy and paw edema models, because they are generally considered widely applicable, generic model systems for local inflammation. Furthermore, because of the general implications of the subject, we briefly contrast the results obtained with pharmacological inhibitors with the findings obtained with iNOS-deficient mice in various models of inflammation.

Pleurisy and Paw Edema Models

Carrageenan- and zymosan-induced paw edema and pleurisy models are widely considered (by pharmacologists working both in academia and in industry) convenient and rapid methods for the investigation of inflammatory responses. The cellular and molecular mechanisms of these inflammatory responses are well characterized, and these models of inflammation serve as standard models of screening for anti-inflammatory activity of various experimental compounds. The early phase of the inflammation is related to the production of histamine, leukotrienes, PAF, and possibly cyclooxygenase products, whereas the delayed phase of the inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radical, as well as to the release of other neutrophil-derived mediators (Di Rosa *et al.,* 1971; Oh-Ishi *et al.,* 1989; Dawson *et al.,* 1991; Peskar *et al.,* 1991). These inflammatory conditions are associated with the expression of iNOS and overproduction of NO, which, after days, tend to return toward baseline or undetectable levels (Salvemini *et al.,* 1996b,c; Cuzzocrea *et al.,* 1997). There is also good evidence demonstrating that inhibition of NOS affects the course of the inflammation in these models. Both isoform-nonselective and iNOS-selective inhibitors have been shown to be effective, although, as expected, the iNOS-selective agents only attenuate the delayed phase of the inflammation (Ialenti *et al.,* 1992; Fracasso *et al.,* 1996; Cuzzocrea *et al.,* 1998b). The protective effect of isoform-nonselective agents may be due to local vasoconstriction, which, in this model, exerts anti-inflammatory effects (Medeiros *et al.,* 1995). Therefore, this model is in stark contrast with many of the pathophysiologically more relevant models of inflammation and shock, where ecNOS has distinct protective actions, where inhibition of ecNOS is crucial to preserve organ blood flow and function, especially in the early phase of the inflammation (see earlier), and where inhibition of ecNOS usually exacerbates rather than reduces the development of inflammation.

Several of the cellular mechanisms of NO-mediated cytotoxicity outlined in the previous section have been identified in the pleurisy and paw edema models, including (1) a mechanism involving NO- or peroxynitrite-mediated activation of cyclooxygenase, (2) a role of peroxynitrite, and (3) the involvement of the PARS pathway. Regarding the role of NO-related activation of COX, endogenous NO has been

shown to modulate carrageenan-induced paw edema by increasing prostaglandin biosynthesis at the inflammatory site (Sautebin *et al.,* 1995; Demello *et al.,* 1997). However, in an air pouch model inflamed with zymosan, no evidence for the activation of COX by NO was found (Paya *et al.,* 1997).

Regarding peroxynitrite generation, a series of studies have demonstrated the production of peroxynitrite in these local models of inflammation (Salvemini *et al.,* 1996b,c; Cuzzocrea *et al.,* 1997, 1998b). Peroxynitrite has been directly shown to be able to cause an increase in both plasma extravasation and blood flow in the hind paw (Ridger *et al.,* 1997). In addition, using NOS inhibitors, superoxide dismutase mimetics, peroxynitrite decomposition catalysts, and other antioxidants, a connection between peroxynitrite generation and the inflammatory response has been put forward (Salvemini *et al.,* 1996b,c; Cuzzocrea *et al.,* 1997; Salvemini *et al.,* 1998).

Regarding PARS activation, studies have clearly demonstrated its role in various forms of local inflammation induced by the prototypical inflammatory stimuli zymosan and carrageenan. For example, in carrageenan-induced paw edema, inhibition of PARS with 3-aminobenzamide reduced paw swelling and inhibited the infiltration of neutrophils into the inflamed paw (Szabó *et al.,* 1997d). Furthermore, in a model of acute local inflammation (carrageenan-induced pleurisy) the poly(ADP-ribose) synthetase inhibitor 3-aminobenzamide inhibited the inflammatory response (pleural exudate formation, mononuclear cell infiltration, histological injury) (Cuzzocrea *et al.,* 1998b). Inhibition of PARS also reduced the formation of nitrotyrosine, an indicator of the formation of peroxynitrite, in the inflamed tissues (Szabó *et al.,* 1997d; Cuzzocrea *et al.,* 1998b). This finding is unexpected, because PARS activation is considered a process distal from the generation of oxidants. The explanation for this finding is likely related to the fact that the $PARS^{-/-}$ phenotype or pharmacological inhibition of PARS reduces the infiltration of neutrophils into inflammatory sites (Szabó *et al.,* 1997d; Cuzzocrea *et al.,* 1998b). Thus, the reduction in tissue injury by PARS inhibitors may result from a decreased inflammatory infiltrate, which would be associated with a reduction in both oxygen- and nitrogen-centered free radical production. The basis for PARS-inhibitable neutrophil infiltration is not yet defined, but it may relate to the effect of PARS activation on the expression of intercellular adhesion molecules and/or may be due to modulation by PARS of a postadhesion event (Szabó *et al.,* 1997d; Zingarelli *et al.,* 1998b). Other mechanisms, such as a PARS-related effect on endothelial integrity, are also possible (Szabó *et al.,* 1997b).

Studies Using Inducible NO Synthase-Deficient Animals

As overviewed by Nathan (1997), in the few years following the generation of three lines of iNOS-deficient mice, a great number of pathophysiological studies were conducted using these animals as tools to provide a definitive answer to the question of the role of iNOS in inflammation. Often, the findings using iNOS knockout animals were, however, found to be in conflict with some of the pharmacological data, and therefore the role of iNOS is still unclear (or, more likely, it is more complex than initially thought) in many forms of inflammation.

In the first set of studies, it was addressed whether the iNOS-deficient mice are resistant against LPS-induced hypotension, inflammation, shock, and lethality. Although anesthetized iNOS-deficient mice were protected against the LPS-induced hypotension and death (MacMicking *et al.,* 1995), no protection (or exacerbation) of the organ injury induced by *Corynebacterium parvum*/LPS shock was seen (MacMicking *et al.,* 1995). The next set of studies, in which the iNOS-deficient mice were completely resistant against LPS-induced death as opposed to wild types (50% death over 4 days) (Wei *et al.,* 1995), is frequently dismissed because of potential problems with genetic background differences between the wild-type and the iNOS-deficient groups of animals. In the study using the largest number of animals to date, no significant differences were found in survival rates in response to high doses of intraperitoneally injected LPS when wild types, heterozygotes, or iNOS knockout homozygotes were compared (Laubach *et al.,* 1995). Thus, the survival benefit that was expected on the basis of the studies with pharmacological inhibitors of NOS in murine models of shock could not be clearly demonstrated in the iNOS-deficient mice. It was, therefore, hypothesized that the pharmacological inhibitors of NOS may have had independent pharmacological effects in addition to NOS or iNOS inhibition, which may have affected the survival studies. Although this is a clear possibility, and it is known that guanidines, isothioureas, and even L-arginine analogs have pharmacological actions independent of NOS inhibition (Szabo *et al.,* 1994; Southan and Szabó, 1996), other considerations should also be kept in mind. For example, in many of the survival studies the NOS inhibitors were administered after the administration of the inducer of shock, whereas all iNOS-deficient studies are equivalent with a pharmacological prolonged ''pretreatment'' paradigm. Many investigators also assume that the iNOS-deficient animals, during development, may have expressed additional parallel cytotoxic pathways or mechanisms, which substitute for the absence of iNOS. In a study using iNOS-deficient mice, no parallel or alternative pathway was identified that would substitute for macrophage-mediated tumor cell lysis (Zingarelli *et al.,* 1998c). Nevertheless, a substantial degree of nitrotyrosine staining was identified in the iNOS-deficient animals after LPS injection, and thus evidence for significant, peroxynitrite-like oxidative processes was found in the absence of functional iNOS (Zingarelli *et al.,* 1998c). Thus, it is possible that in the absence of iNOS, NO produced by other NOS isoforms can combine with superoxide to result in the generation of peroxynitrite-like nitrosative species. More recent studies found that iNOS-deficient mice are resistant against LPS-induced pulmonary damage (Kristof *et al.,* 1998) and vascular hyporeactivity (Gunnett *et al.,* 1998), confirming, in this respect, a multitude of prior studies using pharmaco-

logical inhibitors. In contrast to endotoxic shock, clear data have been presented demonstrating an improvement in the outcome of hemorrhagic shock in iNOS-deficient mice (Hierholzer *et al.,* 1998), in agreement with earlier studies using pharmacological inhibitors of NOS or agents that prevent the induction of iNOS (Thiemermann *et al.,* 1993b).

In allergic encephalomyelitis, iNOS plays opposing roles in the induction and the delayed phase of the disease: inhibition or inactivation of iNOS in the induction phase leads to the enhancement of the disease, whereas inhibition or inactivation of iNOS in the late phase has been found to be protective (Cross *et al.,* 1995; Zielasek *et al.,* 1995; Brenner *et al.,* 1997; Okuda *et al.,* 1997; Sahrbacher *et al.,* 1998; Fenyk-Melody *et al.,* 1998). Although iNOS appears to play opposing roles in the different stages of the disease, the degree of correlation between the pharmacological studies and the studies using iNOS-deficient animals appears to be fairly good.

The situation became more confusing when the studies on arthritis, uveitis, and glomerulonephritis with iNOS-deficient mice were completed. In a study using iNOS-deficient mice bred to the MRL-*lpr/lpr* mice (which, on normal background, develop autoimmune inflammatory pathologies associated with and apparently—based on pharmacological studies—due to iNOS expression), no protection against arthritis or glomerulonephritis was observed (although the vasculitis was reduced to some extent) (Gilkeson *et al.,* 1997). In a septic arthritis model even a significant exacerbation of the disease was found in the iNOS-deficient mice (McInnes *et al.,* 1998). These studies are in sharp contrast to the earlier pharmacological studies, where the development of arthritis has been shown to be ameliorated by various, nonisoform-selective inhibitors of NO synthase in various animal models of adjuvant- or collagen-induced arthritis (Ialenti *et al.,* 1992; McCartney-Francis *et al.,* 1994; Stefanovic-Racic *et al.,* 1993, 1994, 1995; Evans *et al.,* 1995; Weinberg *et al.,* 1994; Connor *et al.,* 1995; Fletcher *et al.,* 1998; Brahn *et al.,* 1998; Pelletier *et al.,* 1998). In one study, however, zymosan-induced gonarthritis has been shown to be reduced in the iNOS-deficient mice (Van de Loo *et al.,* 1998). It remains to be determined whether (similar to the case of allergic encephalomyelitis) iNOS plays different roles in the early and delayed phase of arthritis and in other forms of inflammation.

No protection against uveitis can be found in the iNOS-deficient mice (F. R. Smith *et al.,* 1998). Again, this is in sharp contrast to many previous pharmacological studies (Goureau *et al.,* 1994; Parks *et al.,* 1994).

Emerging studies using iNOS-deficient mice have clarified that the expression of iNOS appears to have diverse roles in the various stages of transplant rejection: NO from iNOS appears to prevents acute rejection but promotes chronic rejection (Koglin *et al.,* 1998a). Transplant-induced atherosclerosis is enhanced in the absence of iNOS (Koglin *et al.,* 1998b). iNOS has also been linked to ischemia–reperfusion mediated injury: renal proximal tubules are resistant against hypoxia-induced injury in the absence of functional iNOS gene (Ling *et al.,* 1998), and the iNOS-deficient mice perform better in the delayed phase of stroke (Iadecola *et al.,* 1997).

Conclusions

The pathophysiological roles of NO in inflammation are far from being simple, uniform, or well understood. NO produced from iNOS may play different roles in various pathophysiological conditions. Moreover, NO plays different, sometime opposing roles in different stages of the same disease. In many cases, for example, in conditions of viral infection and inflammation, the anti-inflammatory effect of NOS inhibition is masked by attenuation of the host defense mechanisms (Karupiah *et al.,* 1993, 1998). Currently, no clinical data are available with selective inhibitors of iNOS. The only clinical study using a NOS inhibitor (N^G-methylarginine in sepsis) failed in Phase III, most likely due to unacceptable side effects related to inhibition of constitutive NO production. Clinical studies with appropriate iNOS inhibitors, in carefully selected inflammatory conditions, will clarify the ultimate pathogenetic role of iNOS in the human inflammatory conditions.

References

Abe, K., Pan, L. H., Watanabe, M., Kato, T., and Itoyama, Y. (1995). Induction of nitrotyrosine-like immunoreactivity in the lower motor neuron of amyotrophic lateral sclerosis. *Neurosci. Lett.* **199,** 152–154.

Abe, K., Pan, L. H., Watanabe, M., Konno, H., Kato, T., and Itoyama, Y. (1997). Upregulation of protein-tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis. *Neurol. Res.* **19,** 124–128.

Aiko, S., and Grisham, M. B. (1995). Spontaneous intestinal inflammation and nitric oxide metabolism in HLA-B27 transgenic rats. *Gastroenterology* **109,** 142–150.

Aiko, S., Fuseler, J., and Grisham, M. B. (1998). Effects of nitric oxide synthase inhibition or sulfasalazine on the spontaneous colitis observed in HLA-B27 transgenic rats. *J. Pharmacol. Exp. Ther.* **284,** 722–727.

Ajuebor, M. N., Virág, L., Flower, R. J., Perretti, M., and Szabó, C. (1999). Role of inducible nitric oxide synthase in the regulation of neutrophil migration in symosan-induced inflammation: Relationship to IL-10 and chemokine production. *Immunology* **95,** 625–630.

Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y. M., Dietzschold, B., and Maeda, H. (1996). Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2448–2453.

Akesson, B., and Lundquist, I. (1998). Evidence for nitric oxide mediated effects on islet hormone secretory phospholipase C signal transduction mechanisms. *Biosci. Rep.* **18,** 199–213.

Aley, K. O., McCarter, G., and Levine, J. D. (1998). Nitric oxide signaling in pain and nociceptor sensitization in the rat. *J. Neurosci.* **18,** 7008–7014.

Alhan, E., Kucutulu, U., and Ercin, C. (1998). The effects of nitric oxide synthase inhibitors on acute necrotising pancreatitis in rats. *Eur. J. Surg.* **164,** 697–702.

Alican, I., and Kubes, P. (1996). A critical role for nitric oxide in intestinal barrier function and dysfunction. *Am. J. Physiol.* **270,** G225–G237.

Alonso, A., Carvalho, J., Alonso-Torre, S. R., Nunez, L., Bosca, L., and Sanchez Crespo, M. (1995). Nitric oxide synthesis in rat peritoneal macrophages is induced by IgE/DNP complexes and cyclic AMP

analogues. Evidence in favor of a common signaling mechanism. *J. Immunol.* **154,** 6475–6483.

Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B. A., and Radi, R. (1996). Peroxynitrite-dependent tryptophan nitration. *Chem. Res. Toxicol.* **9,** 390–396.

Ames, B. N., Cathcart, R., Schwiers, E., and Hochstein, P. (1981). Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6858–6862.

Amin, A. R., and Abramson, S. B. (1998). The role of nitric oxide in articular cartilage breakdown in osteoarthritis. *Curr. Opin. Rheumatol.* **10,** 263–268.

Amin, A. R., Di Cesare, P. E., Vyas, P., Attur, M., Tzeng, E., Billiar, T. R., Stuchin, S. A., and Abramson, S. B. (1995a). The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: Evidence for up-regulated neuronal nitric oxide synthase. *J. Exp. Med.* **182,** 2097–2102.

Amin, A. R., Vyas, P., Attur, M., Leszczynska-Piziak, J., Patel, I. R., Weissmann, G., and Abramson, S. B. (1995b). The mode of action of aspirin-like drugs: Effect on inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7926–7930.

Amin, A. R., Attur, M. G., Thakker, G. D., Patel, P. D., Vyas, P. R., Patel, R. N., Patel, I. R., and Abramson, S. B. (1996). A novel mechanism of action of tetracyclines: Effects on nitric oxide synthases. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14014–14019.

Amin, A. R., Attur, M., Patel, R. N., Thakker, G. D., Marshall, P. J., Rediske, J., Stuchin, S. A., Patel, I. R., and Abramson, S. B. (1997). Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J. Clin. Invest.* **99,** 1231–1237.

Arthur, J. F., Shahin, S., and Dusting, G. J. (1995). Paf antagonists block induction of nitric oxide synthase in cultured macrophages and vascular smooth muscle cells. *Clin. Exp. Pharmacol. Physiol.* **22,** 452–454.

Asahi, M., Fujii, J., Suzuki, K., Seo, H. G., Kuzuya, T., Hori, M., Tada, M., Fujii, S., and Taniguchi, N. (1995). Inactivation of glutathione peroxidase by nitric oxide. Implication for cytotoxicity. *J. Biol. Chem.* **270,** 21035–21039.

Asahi, M., Fujii, J., Takao, T., Kuzuya, T., Hori, M., Shimonishi, Y., and Taniguchi, N. (1997). The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor. *J. Biol. Chem.* **272,** 19152–19157.

Bachmaier, K., Neu, N., Pummerer, C., Duncan, G. S., Mak, T. W., Matsuyama, T., and Penninger, J. M. (1997). iNOS expression and nitrotyrosine formation in the myocardium in response to inflammation is controlled by the interferon regulatory transcription factor 1. *Circulation* **96,** 585–591.

Barker, J. E., Bolanos, J. P., Land, J. M., Clark, J. B., and Heales, S. J. (1996). Glutathione protects astrocytes from peroxynitrite-mediated mitochondrial damage: Implications for neuronal/astrocytic trafficking and neurodegeneration. *Dev. Neurosci.* **18,** 391–396.

Bauer, M. L., Beckman, J. S., Bridges, R. J., Fuller, C. M., and Matalon, S. (1992). Peroxynitrite inhibits sodium uptake in rat colonic membrane vesicles. *Biochim. Biophys. Acta* **1104,** 87–94.

Baydoun, A. R., and Morgan, D. M. (1998). Inhibition of ornithine decarboxylase potentiates nitric oxide production in LPS-activated J774 cells. *Br. J. Pharmacol.* **125,** 1511–1516.

Beckman, J. S. (1996). Oxidative damage and tyrosine nitration from peroxynitrite. *Chem. Res. Toxicol.* **9,** 836–844.

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am. J. Physiol.* **271,** C1424–C1437.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Becquet, F., Courtois, Y., and Goureau, O. (1997). Nitric oxide in the eye: Multifaceted roles and diverse outcomes. *Surv. Ophthalmol.* **42,** 71–82.

Bellmann, K., Jaattela, M., Wissing, D., Burkart, V., and Kolb, H. (1996). Heat shock protein hsp70 overexpression confers resistance against nitric oxide. *FEBS Lett.* **391,** 185–188.

Berger, N. A. (1985). Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat. Res.* **101,** 4–15.

Berkman, N., Robichaud, A., Robbins, R. A., Roesems, G., Haddad, E. B., Barnes, P. J., and Chung, K. F. (1996). Inhibition of inducible nitric oxide synthase expression by interleukin-4 and interleukin-13 in human lung epithelial cells. *Immunology* **89,** 363–367.

Berlett, B. S., Friguet, B., Yim, M. B., Chock, P. B., and Stadtman, E. R. (1996). Peroxynitrite-mediated nitration of tyrosine residues in *Escherichia coli* glutamine synthetase mimics adenylylation: Relevance to signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1776–1780.

Binion, D. G., Fu, S., Ramanujam, K. S., Chai, Y. C., Dweik, R. A., Drazba, J. A., Wade, J. G., Ziats, N. P., Erzurum, S. C., and Wilson, K. T. (1998). iNOS expression in human intestinal microvascular endothelial cells inhibits leukocyte adhesion. *Am. J. Physiol.* **275,** G592–G603.

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q. W., and Nathan, C. (1994). Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukocyte Biol.* **55,** 227–233.

Bolanos, J. P., Heales, S. J., Land, J. M., and Clark, J. B. (1995). Effect of peroxynitrite on the mitochondrial respiratory chain: Differential susceptibility of neurones and astrocytes in primary culture. *J. Neurochem.* **64,** 1965–1972.

Bolanos, J. P., Almeida, A., Stewart, V., Peuchen, S., Land, J. M., and Clark, J. B. (1997). Nitric oxide-mediated mitochondrial damage in the brain: Mechanisms and implications for neurodegenerative diseases. *J. Neurochem.* **68,** 2227–2240.

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368,** 850–883.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., and Lipton, S. (1995). Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with NMDA or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7162–7166.

Brahn, E., Banquerigo, M. L., Firestein, G. S., Boyle, D. L., Salzman, A. L., and Szabó, C. (1998). Collagen-induced arthritis: Reversal by mercaptoethylguanidine, a novel anti-inflammatory agent with a combined mechanism of action. *J. Rheumatol.* **25,** 1785–1793.

Brendeford, E. M., Andersson, K. B., and Gabrielsen, O. S. (1998). Nitric oxide (NO) disrupts specific DNA binding of the transcription factor c-Myb in vitro. *FEBS Lett.* **425,** 52–56.

Brenner, T., Brocke, S., Szafer, F., Sobel, R. A., Parkinson, J. F., Perez, D. H., and Steinman, L. (1997). Inhibition of nitric oxide synthase for treatment of experimental autoimmune encephalomyelitis. *J. Immunol.* **158,** 2940–2946.

Brosnan, C. F., Lee, S. C., and Liu, J. (1997). Regulation of inducible nitric oxide synthase expression in human glia: Implications for inflammatory central nervous system diseases. *Biochem. Soc. Trans.* **25,** 679–683.

Brown, G. C. (1997). Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: Implications for inflammatory, neurodegenerative and ischaemic pathologies. *Mol. Cell. Biochem.* **174,** 189–192.

Brujin, L. I., Beal, M. F., Becher, M. W., Schulz, J. B., Womg, P. C., Proce, D. L., and Cleveland, D. W. (1997). Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout ALS-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked SOD-1 mutant. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 7606–7611.

Burkart, V., Wang, Z. Q., Radons, J., Heller, B., Herceg, Z., Stingl, L., Wanger, E., and Kolb, H. (1999). Mice lacking the poly (ADP-ribose) polymerase gene are resistant to pancreatic β-cell destruction and diabetes development induced by streptozotocin. *Nat. Med.* **5,** 314–319.

Byrne, C. R., and Hanson, P. J. (1998). Induction of heat shock protein 72 by a nitric oxide donor in guinea-pig gastric mucosal cells. *Eur. J. Pharmacol.* **353,** 117–122.

Calo, L., Davis, P. A., Rigotti, P., Milani, M., Cantaro, S., Marchini, F., D'Angelo, A., and Antonello, A. (1998). ecNOS overexpression in CsA-treated renal transplant patients: Implications for CsA-induced hypertension. *Transplant. Proc.* **30,** 2012–2013.

Campbell, D. L., Stamler, J. S., and Strauss, H. C. (1996). Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and *S*-nitrosothiols. *J. Gen. Physiol.* **108,** 277–293.

Cannon, P., Yang, X., Szabolcs, M. J., Ravalli, S., Sciacca, R. R., and Michler, R. E. (1998). The role of inducible nitric oxide synthase in cardiac allograft rejection. *Cardiovasc. Res.* **38,** 6–15.

Cassina, A., and Radi, R. (1996). Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch. Biochem. Biophys.* **328,** 309–316.

Castro, L., Rodriguez, M., and Radi, R. (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.* **269,** 29409–29415.

Castro, L. A., Robalinho, R. L., Cayota, A., Meneghini, R., and Radi, R. (1998). Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. *Arch. Biochem. Biophys.* **359,** 215–224.

Ceneviva, G. D., Tzeng, E., Hoyt, D. G., Yee, E., Gallagher, A., Engelhardt, J. F., Kim, Y. M., Billiar, T. R., Watkins, S. A., and Pitt, B. R. (1998). Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells. *Am. J. Physiol.* **275,** L717–L728.

Chang, C. C., McCormick, C. C., Lin, A. W., Dietert, R. R., and Sung, Y. J. (1996). Inhibition of nitric oxide synthase gene expression in vivo and in vitro by repeated doses of endotoxin. *Am. J. Physiol.* **271,** G539–G548.

Chen, L., Xie, Q. W., and Nathan, C. (1998). Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol. Cell.* **1,** 795–805.

Christen, S., Woodall, A. A., Shigenaga, M. K., Southwell-Keely, P. T., Duncan, M. W., and Ames, B. N. (1997). γ-Tocopherol traps mutagenic electrophiles such as NO(X) and complements α-tocopherol: Physiological implications. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3217–3222.

Cipolletta, C., Jouzeau, J. Y., Gegout-Pottie, P., Presle, N., Bordji, K., and Netter, P. B. (1998). Modulation of IL-1-induced cartilage injury by NO synthase inhibitors: A comparative study with rat chondrocytes and cartilage entities. *Br. J. Pharmacol.* **124,** 1719–1727.

Clancy, R. M., Amin, A. R., and Abramson, S. B. (1998). The role of nitric oxide in inflammation and immunity. *Arthritis Rheum.* **41,** 1141–1151.

Clementi, E., Brown, G. C., Feelisch, M., and Moncada, S. (1998). Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7631–7636.

Cochrane, C. G. (1991). Mechanisms of oxidant injury of cells. *Mol. Aspects Med.* **12,** 137–147.

Connor, J. R., Manning, P. T., Settle, S. L., Moore, W. M., Jerome, G. M., Webber, R. K., Tjoeng, F. S., and Currie, M. G. (1995). Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur. J. Pharmacol.* **273,** 15–24.

Cooper, M., Lindholm, P., Pieper, G., Seibel, R., Moore, G., Nakanishi, A., Dembny, K., Komorowski, R., Johnson, C., Adams, M., and Roza, A. (1998). Myocardial nuclear factor-κ activity and nitric oxide production in rejecting cardiac allografts. *Transplantation* **66,** 838–844.

Cross, A. H., Misko, T. P., Lin, R. F., Hickey, W. F., Trotter, J. L., and Tilton, R. G. (1995). Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J. Clin. Invest.* **93,** 2684–2690.

Crow, J. P., and Beckman, J. S. (1995). The role of peroxynitrite in nitric oxide-mediated toxicity. *Curr. Top. Microbiol. Immunol.* **196,** 57–73.

Cunha, F. Q., Assreuy, J., Moss, D. W., Rees, D., Leal, L. M., Moncada, S., Carrier, M., O'Donnell, C. A., and Liew, F. Y. (1994). Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: Role of TNF-α and IL-1β. *Immunology* **81,** 211–215.

Cuthbertson, B. H., Galley, H. F., and Webster, N. R. (1998). The effects of nitric oxide and peroxynitrite on interleukin-8 and elastase from lipopolysaccharide-stimulated whole blood. *Anesth. Analg.* **86,** 427–431.

Cuzzocrea, S., Zingarelli, B., Costantino, G., Szabó, A., Salzman, A. L., Caputi, A. P., and Szabó, C. (1997). Beneficial effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase in a rat model of splanchnic artery occlusion and reperfusion. *Br. J. Pharmacol.* **121,** 1065–1074.

Cuzzocrea, S., Zingarelli, B., O'Connor, M., Salzman, A. L., and Szabó, C. (1998a). Effect of L-buthionine-(*S,R*)-sulfoximine, an inhibitor of γ-glutamylcysteine synthetase on the peroxynitrite- and endotoxic shock-induced vascular failure. *Br. J. Pharmacol.* **123,** 525–537.

Cuzzocrea, S., Zingarelli, B., Gilad, E., Hake, P., Salzman, A. L., and Szabó, C. (1998b). Protective effects of 3-aminobenzamide, an inhibitor of poly(ADP-ribose) synthase in a carrageenan-induced model of local inflammation. *Eur. J. Pharmacol.* **342,** 67–76.

Darley-Usmar, V., and White, R. (1997). Disruption of vascular signalling by the reaction of nitric oxide with superoxide: Implications for cardiovascular disease. *Exp. Physiol.* **82,** 305–316.

Davidson, C. A., Kaminski, P. M., and Wolin, M. S. (1997). NO elicits prolonged relaxation of bovine pulmonary arteries via endogenous peroxynitrite generation. *Am. J. Physiol.* **273,** L437–L444.

Dawson, J., Sedgwick, A. D., Edwards, J. C., and Lees, P. (1991). A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React.* **13,** 171–185.

Dawson, V. L. (1995). Nitric oxide: Role in neurotoxicity. *Clin. Exp. Pharmacol. Physiol.* **22,** 305–308.

Dawson, V. L., and Dawson, T. M. (1996). Nitric oxide actions in neurochemistry. *Neurochem. Int.* **29,** 97–110.

De Gendt, C. M., De Clerck, L. S., Bridts, C. H., Verbruggen, A., and Stevens, W. J. (1998). Influence of antirheumatic drugs on nitric oxide and interleukin 8 production in human articular chondrocytes. *J. Rheumatol.* **25,** 536–538.

De Groot, H., Hegi, U., and Sies, H. (1993). Loss of alpha-tocopherol upon exposure to nitric oxide or the sydnonimine SIN-1. *FEBS Lett.* **315,** 139–142.

De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y., and Fang, F. C. (1997). Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 13997–14001.

DelaTorre, A., Schroeder, R. A., Bartlett, S. T., and Kuo, P. C. (1998). Differential effects of nitric oxide-mediated S-nitrosylation on p50 and c-jun DNA binding. *Surgery* **124,** 137–141.

Del Castillo, D., Agarwal, A., Jaimes, E. A., and Raij, L. (1997). Glomerular and vascular tissues do not down-regulate nitric oxide synthesis during protracted endotoxemia. *Kidney Int.* **52,** 460–467.

Demello, S. V. B., Novaes, G. S., Laurindo, I. M. M., Muscara, M. N., Maciel, F. M. D., and Cossermelli, W. (1997). Nitric oxide synthase inhibitor influences prostaglandin and interleukin-1 production in experimental arthritis joints. *Inflamm. Res.* **46,** 72–77.

De Sarno, P., and Jope, R. S. (1998). Phosphoinositide hydrolysis activated by muscarinic or glutamatergic, but not adrenergic, receptors is impaired in ApoE-deficient mice and by hydrogen peroxide and peroxynitrite. *Exp. Neurol.* **152,** 123–128.

DeVera, M. E., Shapiro, R. A., Nussler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., and Billiar, T. R. (1996). Transcriptional regulation of human inducible nitric oxide synthase gene by cytokines: Initial analysis of human NOS2 promoter. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1054–1059.

Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Rollinghoff, M., Gresser, I., and Bogdan, C. (1998). Type 1 interferon (IFNα/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* **8,** 77–87.

Di Rosa, M., and Willoughby, D. A. (1971). Screens for anti-inflammatory drugs. *J. Pharm. Pharmacol.* **23,** 297–300.

Downing, J. E., Virag, L., and Perry, M. E. (1998a). Nitrergic mechanism of DC-mediated T-cell elimination. *Immunol. Today* **19,** 190–191.

Downing, J. E. G., Virág, L., Salzman, A. L., and Szabó, C. (1998b). Deficiency of Lewis rat thymus for nitrergic cells. *FASEB J.* **12,** A601.

Dugas, N., Palacios-Calender, M., Dugas, B., Riveros-Moreno, V., Delfraissy, J. F., Kolb, J. P., and Moncada, S. (1998). Regulation by endogenous interleukin-10 of the expression of nitric oxide synthase induced after ligation of CD23 in human macrophages. *Cytokine* **10,** 680–689.

Dusting, G. J. (1996). Nitric oxide in coronary artery disease: Roles in atherosclerosis, myocardial reperfusion and heart failure. *EXS* **76,** 33–55.

Dusting, G. J., and MacDonald, P. S. (1995). Endogenous nitric oxide in cardiovascular disease and transplantation. *Ann. Med.* **27,** 395–406.

Eaves-Pyles, T., Szabo, C., and Salzman, A. L. (1999). Bacterial invasion is not required for activation of NF-κB in enterocytes. *Infect. Immun.* **67,** 800–804.

Eberhardt, W., Pluss, C., Hummel, R., and Pfeilschifter, J. (1998). Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J. Immunol.* **160,** 4961–4969.

Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and Van der Vliet, A. (1996). Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. *J. Biol. Chem.* **271,** 19199–19208.

Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and Van der Vliet, A. (1998). Formation of nitric oxide derivatives catalysed by myeloperoxidase in neutrophils. *Nature* **391,** 393–397.

Eliasson, M. J. L., Sampei, K., Mandir, A. S., Hurn, P. D., Traystman, R. J., Bao, J., Pieper, A., Wang, Z. Q., Sdawson, T. M., Snyder, S. H., and Dawson, V. L. (1997). Poly (ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med.* **3,** 1089–1095.

Elliott, S. J. (1996). Peroxynitrite modulates receptor-activated Ca^{2+} signaling in vascular endothelial cells. *Am. J. Physiol.* **270,** L954–L961.

Endres, M., Wang, Z. Q., Namura, S., Waeber, C., and Moskowitz, M. A. (1997). Ischemic brain injury is mediated by the activation of poly(ADP-ribose) synthetase. *J. Cerebral Blood Flow Metab.* **17,** 1143–1151.

Estevez, A. G., Radi, R., Barbeito, L., Shin, J. T., Thompson, J. A., and Beckman, J. S. (1995). Peroxynitrite-induced cytotoxicity in PC12 cells: Evidence for an apoptotic mechanism differentially modulated by neurotropic factors. *J. Neurochem.* **65,** 1543–1550.

Estrada, C., Gomez, C., Martin-Nieto, J., De Frutos, T., Jimenez, A., and Villalobo, A. (1997). Nitric oxide reversibly inhibits the epidermal growth factor receptor tyrosine kinase. *Biochem. J.* **326,** 369–376.

Evans, C. H., Stefanovic-Racic, M., and Lancaster, J. (1995). Nitric oxide and its role in orthopaedic disease. *Clin. Orthopaed. Related Res.* **312,** 275–294.

Evans, T., Carpenter, A., Kinderman, H., and Cohen, J. (1993). Evidence of increased nitric oxide production in patients with the sepsis syndrome. *Circ. Shock* **41,** 77–81.

Evans, T. J., Buttery, L. D., Carpenter, A., Springall, D. R., Polak, J. M., and Cohen, J. (1996). Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 9553–9558.

Fang, F. C., Vazquez-Torres, A., and Xu, Y. (1997). The transcriptional regulator SoxS is required for resistance of *Salmonella typhimurium* to paraquat but not for virulence in mice. *Infect. Immun.* **65,** 5371–5375.

Farivar, R. S., Chobanian, A. V., and Brecher, P. (1996). Salicylate or aspirin inhibits the induction of the inducible nitric oxide synthase in rat cardiac fibroblasts. *Circ. Res.* **78,** 759–768.

Fatehi-Hassanabad, Z., Burns, H., Aughey, E. A., Paul, A., Plevin, R., Parratt, J. R., and Furman, B. L. (1996). Effects of L-canavenine, an inhibitor of inducible nitric oxide synthase on endotoxin mediated shock in rats. *Shock* **6,** 194–200.

Favre, C. J., Ufret-Vincenty, C. A., Stone, M. R., Ma, H. T., and Gill, D. L. (1998). Ca^{2+} pool emptying stimulates Ca^{2+} entry activated by S-nitrosylation. *J. Biol. Chem.* **273,** 30855–30858.

Fenyk-Melody, J. E., Garrison, A. E., Brunnert, S. R., Weidner, J. R., Shen, F., Shelton, B. A., and Mudgett, J. S. (1998). Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J. Immunol.* **160,** 2940–2946.

Ferreira, S. H. (1993). The role of interleukins and nitric oxide in the mediation of inflammatory pain and its control by peripheral analgesics. *Drugs* **46**(Suppl. 1), 1–9.

Filep, J. G., Delalandre, A., and Beauchamp, M. (1997). Dual role for nitric oxide in the regulation of plasma volume and albumin escape during endotoxin shock in conscious rats. *Circ. Res.* **81,** 840–847.

Filep, J. G., Beauchamp, M., Baron, C., and Paquette, Y. (1998). Peroxynitrite mediates IL-8 gene expression and production in lipopolysaccharide-stimulated human whole blood. *J. Immunol.* **161,** 5656–5662.

Fletcher, D. S., Widmer, W. R., Luell, S., Christen, A., Orevillo, C., Shah, S., and Visco, D. (1998). Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats. *J. Pharmacol. Exp. Ther.* **284,** 714–721.

Förstermann, U., Gath, I., Schwarz, P., Closs, E. I., and Kleinert, H. (1995). Isoforms of nitric oxide synthase. Properties, cellular distribution and expressional control. *Biochem. Pharmacol.* **50,** 1321–1332.

Fracasso, J. F., Nunes-de-Souza, R. L., Teixeira, C. E., Castro, R. C., Lepera, E. Z., and Silva, R. F. (1996). Effect of dipyrone, L-NAME and L-arginine on endotoxin-induced rat paw edema. *Braz. J. Med. Biol. Res.* **29,** 1543–1548.

Frei, B., England, L., and Ames, B. N. (1989). Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6377–6381.

Freudenberg, M. A., and Galanos, C. (1990). Bacterial lipopolysaccharides: Structure, metabolism, and mechanism of acton. *Int. Rev. Immunol.* **6,** 207–221.

Freyer, D., Weih, M., Weber, J. R., Burger, W., Scholz, P., Manz, R., Ziegenhorn, A., Angestwurm, K., and Dirnagl, U. (1996). Pneumococcal cell wall components induce nitric oxide synthase and TNF-α in astroglial-enriched cultures. *Glia* **16,** 1–6.

Gagnon, C., Leblond, F. A., and Filep, J. G. (1998). Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide. *FEBS Lett.* **431,** 107–110.

Galigniana, M. D., Piwien-Pilipuk, G., and Assreuy, J. (1999). Inhibition of glucocorticoid receptor binding by nitric oxide. *Mol. Pharmacol.* **55,** 317–323.

Gardner, P. R., Costantino, G., Szabo, C., and Salzman, A. L. (1997). Nitric oxide sensitivity of the aconitases. *J. Biol. Chem.* **272,** 25071–25076.

Gardner, P. R., Costantino, G., and Salzman, A. L. (1998a). Constitutive and adaptive detoxification of nitric oxide in *Escherichia coli.* Role of nitric-oxide dioxygenase in the protection of aconitase. *J. Biol. Chem.* **273,** 26528–26533.

Gardner, P. R., Gardner, A. M., Martin, L. A., and Salzman, A. L. (1998b). Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10378–10383.

Geng, Y., Hansson, G. K., and Holme, E. (1992). Interferon-γ and tumor necrosis factor synergize to induce nitric oxide production and inhibit mitochondrial respiration in vascular smooth muscle cells. *Circ. Res.* **71,** 1268–1276.

Gilad, E., Zingarelli, B., O'Connor, M., Salzman, A. L., Bertók, L., and Szabó, C. (1996). Effects of radiodetoxified endotoxin on nitric oxide production in J774 macrophages and in endotoxin shock. *J. Endotoxin Res.* **3,** 513–516.

Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A. L., and Szabó, C. (1997). Melatonin is a scavenger of peroxynitrite. *Life Sci.* **60,** PL169–PL174.

Gilad, E., Wong, H. R., Zingarelli, B., Virag, L., O'Connor, M., Salzman, A. L., and Szabo, C. (1998). Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: Role of inhibition of NF-κB activation. *FASEB J.* **12,** 685–693.

Gilkeson, G. S., Mudgett, J. S., Seldin, M. F., Ruiz, P., Alexander, A. A., Misukonis, M. A., Pisetsky, D. S., and Weinberg, J. B. (1997). Clinical and serologic manifestations of autoimmune disease in MRL-*lpr/lpr* mice lacking nitric oxide synthase type 2. *J. Exp. Med.* **186,** 365–373.

Goldstein, S., Squadrito, G. L., Pryor, W. A., and Czapski, G. (1996). Direct and indirect oxidations by peroxynitrite, neither involving the hydroxyl radical. *Free Radicals Biol. Med.* **21,** 965–974.

Gopalakrishna, R., Chen, Z. H., and Gundimeda, U. (1993). Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *J. Biol. Chem.* **268,** 27180–27185.

Goto, M., Yamaguchi, Y., Ichiguchi, O., Miyanani, N., Akizula, E., Matsumura, F., Matsuda, T., Mori, K., and Ogawa, M. (1997). Phenotype and localization of macrophages expressing iNOS in rat hepatic allograft rejection. *Transplantation* **69,** 303–310.

Goureau, O., Bellot, J., Thillaye, B., Courtois, Y., and Kozak, Y. (1994). Increased nitric oxide production in endotoxin-induced uveitis. Reduction of uveitis by an inhibitor of nitric oxide synthase. *J. Immunol.* **15,** 6518–6523.

Gow, A. J., Duran, D., Malcolm, S., and Ischiropoulos, H. (1996). Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett.* **385,** 63–66.

Grabowski, P. S., England, A. J., Dykhuizen, R., Copland, M., Benjamin, N., Reid, D. M., and Ralston, S. H. (1996). Elevated nitric oxide production in rheumatoid arthritis. Detection using the fasting urinary nitrate:creatinine ratio. *Arthritis Rheum.* **39,** 643–647.

Granados-Soto, V., Rutino, M. O., Gomes-Lopez, L. D., and Ferreira, S. H. (1997). Evidence for the involvement of the NO-cGMP pathway in the anti-nociception of morphine in the formalin test. *Enr. J. Pharm.* **340,** 177–180.

Greenacre, S., Ridger, V., Wilsoncroft, P., and Brain, S. D. (1997). Peroxynitrite: A mediator of increased microvascular permeability? *Clin. Exp. Pharmacol. Physiol.* **24,** 880–882.

Grilli, M., Pizzi, M., Memo, M., and Spano, P. (1996). Neuroprotection by aspirin and sodium salicylate through blockade of NF-κB activation. *Science* **274,** 1383–1385.

Groeneveld, P. H., Kwappenberg, K. M., Langermans, J. A., Nibbering, P. H., and Curtis, L. (1996). Nitric oxide (NO) production correlates with renal insufficiency and multiple organ dysfunction syndrome in severe sepsis. *Intensive Care Med.* **22,** 1197–1202.

Gunnett, C. A., Chu, Y., Heistad, D. D., Loihl, A., and Faraci, F. M. (1998). Vascular effects of LPS in mice deficient in expression of the gene for inducible nitric oxide synthase. *Am. J. Physiol.* **275,** H416–H421.

Guo, F. H., and Erzurum, S. C. (1998). Characterization of inducible nitric oxide synthase expression in human airway epithelium. *Environ. Health Perspect.* **106,** 1119–1124.

Guo, F. H., De Raeve, H. R., Rice, T. W., Stuehr, D. J., Thunnissen, F. B., and Erzurum, S. C. (1995). Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7809–7813.

Guslandi, M. (1998). Nitric oxide and inflammatory bowel diseases. *Eur. J. Clin. Invest.* **28,** 904–907.

Guthikonda, P., Baker, J., and Mattson, D. H. (1998). Interferon-β-1-b (IFN-B) decreases induced nitric oxide (NO) production by a human astrocytoma cell line. *J. Neuroimmunol.* **82,** 133–139.

Guzman, N. J., Fang, M. Z., Tang, S. S., Ingelfinger, J. R., and Garg, L. C. (1995). Autocrine inhibition of Na^+/K^+-ATPase by nitric oxide in mouse proximal tubule epithelial cells. *J. Clin. Invest.* **95,** 2083–2088.

Habib, A., Bernard, C., Lebret, M., Creminon, C., Esposito, B., Tedgui, A., and Maclouf, J. (1997). Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J. Immunol.* **158,** 3845–3851.

Hall, S., Turcato, S., and Clapp, L. (1996). Abnormal activation of K^+ channels underlies relaxation to bacterial lipopolysaccharide in rat aorta. *Biochem. Biophys. Res. Commun.* **224,** 184–190.

Halliwell, B. (1997). What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? *FEBS Lett.* **411,** 157–160.

Haskó, G., and Szabó, C. (1998). Modulation of cytokine and chemokine production by transmitters and co-transmitters of the autonomic nervous system. *Biochem. Pharmacol.* **56,** 1079–1087.

Haskó, G., Nemeth, Z. H., Szabó, C., Zsilla, G., Salzman, A. L., and Vizi, E. S. (1998a). Isoproterenol inhibits Il-10, TNF-α, and nitric oxide production in RAW 264.7 macrophages. *Brain Res. Bull.* **45,** 183–187.

Haskó, G., Szabó, C., Nemeth, Z. H., Salzman, A. L., and Vizi, E. S. (1998b). Suppression of IL-12 production by phosphodiesterase inhibition in murine endotoxemia is IL-10 independent. *Eur. J. Immunol.* **28,** 468–472.

Hattori, Y., Szabó, C., Mitchell, J. A., Gross, S. S., Thiemermann, C., and Vane, J. R. (1995). ONO-4007, a lipid A analogue with antitumor activity induces nitric oxide synthase; comparison with lipopolysaccharide. *Eur. J. Pharmacol.* **291,** 83–90.

Hattori, Y., Kasai, K., Akimoto, K., and Thiemermann, C. (1997). Induction of NO synthesis by lipoteichoic acid from *Staphylococcus aureus* in J774 macrophages: Involvement of a CD14-dependent pathway. *Biochem. Biophys. Res. Commun.* **233,** 375–379.

Hattori, Y., Kasai, K., Nakanishi, N., Gross, S. S., and Thiemermann, C. (1998). Induction of nitric oxide and tetrahydrobiopterin synthesis by lipoteichoic acid from *Staphylococcus aureus* in vascular smooth muscle cells. *J. Vasc. Res.* **35,** 104–108.

Hausladen, A., and Fridovich, I. (1994). Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.* **269,** 29405–29408.

Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J., and Stamler, J. S. (1996). Nitrosative stress: Activation of the transcription factor OxyR. *Cell* **86,** 719–729.

Hausladen, A., Gow, A. J., and Stamler, J. S. (1998). Nitrosative stress: Metabolic pathway involving the flavohemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14100–14105.

Hellberg, C. B., Boggs, S. E., and Lapetina, E. G. (1998). Phosphatidylinositol 3-kinase is a target for protein tyrosine nitration. *Biochem. Biophys. Res. Commun.* **252,** 313–317.

Heller, B., Wang, Z. Q., Wagner, E. F., Radons, J., Burkle, A., Fehsel, K., Burkart, V., and Kolb, H. (1995). Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J. Biol. Chem.* **270,** 11176–11180.

Hickey, M. J., Sharkey, K. A., Sihota, E. G., Reinhardt, P. H., Macmicking, J. D., Nathan, C., and Kubes, P. (1997). Inducible nitric oxide synthase-deficient mice have enhanced leukocyte–endothelium interactions in endotoxemia. *FASEB J.* **11,** 955–964.

Hierholzer, C., Harbrecht, B., Menezes, J. M., Kane, J., MacMicking, J., Nathan, C. F., Peitzman, A. B., Billiar, T. R., and Tweardy, D. J. (1998). Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J. Exp. Med.* **187,** 917–928.

Hirsch, E. C., Hunot, S., Damier, P., and Faucheux, B. (1998). Glial cells and inflammation in Parkinson's disease: A role in neurodegeneration? *Ann. Neurol.* **44,** S115–S120.

Hirvonen, M. R., Brune, B., and Lapetina, E. G. (1996). Heat shock proteins and macrophage resistance to the toxic effects of nitric oxide. *Biochem. J.* **315,** 845–849.

Hirvonen, M. R., Ruotsalainen, M., Savolainen, K., and Nevalainen, A. (1997). Effect of viability of actinomycete spores on their ability to stimulate production of nitric oxide and reactive oxygen species in RAW264.7 macrophages. *Toxicology* **124,** 105–114.

Hogg, N., Joseph, J., and Kalyanaraman, B. (1994). The oxidation of α-tocopherol and trolox by peroxynitrite. *Arch. Biochem. Biophys.* **314,** 153–158.

Hong, H. J., Wu, C. C., and Yen, M. H. (1998). Pyrrolidine dithiocarbamate improves the septic shock syndromes in spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* **25,** 600–606.

Hood, J. D., Meininger, C. J., Ziche, M., and Granger, H. J. (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am. J. Physiol.* **274,** H1054–H1058.

Hooper, D. C., Bagasra, O., Marini, J. C., Zborek, A., Ohnishi, S. T., Kean, R., Champion, J. M., Sarker, A. B., Bobroski, L., Farber, J. L., Akaike, T., and Maeda, H. (1997). Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: Implications for the treatment of multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2528–2533.

Howard, A. D., and Erickson, K. L. (1996). Alteration of macrophage responsiveness to platelet-activating factor by interferon-γ and lipopolysaccharide. *Cell. Immunol.* **174,** 155–164.

Hu, P., Ischiropoulos, H., Beckman, J. S., and Matalon, S. (1994). Peroxynitrite inhibition of oxygen consumption and sodium transport in alveolar type II cells. *Am. J. Physiol.* **266,** L628–L634.

Huhmer, A. F., Nishida, C. R., Ortiz de Montellano, P. R., and Schoneich, C. (1997). Inactivation of the inducible nitric oxide synthase by peroxynitrite. *Chem. Res. Toxicol.* **10,** 618–626.

Hukkanen, M., Corbett, S. A., Batten, J., Konttinen, Y. T., McCarthy, I. D., Maclouf, J., Santavirta, S., Hughes, S. P., and Polak, J. M. (1997). Aseptic loosening of total hip replacement. Macrophage expression of inducible nitric oxide synthase and cyclooxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure. *J. Bone Joint Surg. Br.* **79,** 467–474.

Iadecola, C., Zhang, F., Casey, R., Nagayama, M., and Ross, M. E. (1997). Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J. Neurosci.* **17,** 9157–9164.

Ialenti, A., Ianaro, A., Moncada, S., and Di Rosa, M. (1992). Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.* **211,** 177–182.

Ignarro, L. J. (1991). Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.* **41,** 485–490.

Inoue, S., and Kawanishi, S. (1995). Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Lett.* **371,** 86–88.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D., Beckman, J. S. (1992a). Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298,** 431–437.

Ischiropoulos, H., Zhu, L., and Beckman, J. S. (1992b). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* **298,** 446–451.

Ishiyama, S., Hiroe, M., Nishikawa, T., Abe, S., Shimojo, T., Ito, H., Ozasa, S., Yamakawa, K., Matsuzaki, M., Mohammed, M. U., Nakazawa, H., Kasajima, T., and Marumo, F. (1997). Nitric oxide contributes to the progression of myocardial damage in experimental autoimmune myocarditis in rats. *Circulation* **95,** 489–496.

Iuvone, T., D'Acquisto, F., Van Osselaer, N., Di Rosa, M., Carnuccio, R., and Herman, A. G. (1998). Evidence that inducible nitric oxide synthase is involved in LPS-induced plasma leakage in rat skin through the activation of nuclear factor-κB. *Br. J. Pharmacol.* **123,** 1325–1330.

Iwashina, M., Shichiri, M., Marumo, F., and Hirata, Y. (1998). Transfection of inducible nitric oxide synthase gene causes apoptosis in vascular smooth muscle cells. *Circulation* **98,** 1212–1218.

Jagannath, C., Actor, J. K., and Hunter, R. L., Jr. (1998). Induction of nitric oxide in human monocytes and monocyte cell lines by *Mycobacterium tuberculosis. Nitric Oxide* **2,** 174–186.

Jang, D., and Murrell, G. A. (1998). Nitric oxide in arthritis. *Free Radicals Biol. Med.* **24,** 1511–1519.

Johnson, A., and Ferro, T. J. (1996). Nitrovasodilator repletion increases TNF-α-induced pulmonary edema. *J. Appl. Physiol.* **80,** 2151–2155.

Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 11584–11589.

Kanematsu, M., Ikeda, K., and Yamada, Y. (1997). Interaction between nitric oxide synthase and cyclooxygenase pathways in osteoblastic MC3T3-E1 cells. *J. Bone Miner. Res.* **12,** 1789–1796.

Karoui, H., Hogg, N., Frejaville, C., Tordo, P., and Kalyanaraman, B. (1996). Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. ESR-spin trapping and oxygen uptake studies. *J. Biol. Chem.* **271,** 6000–6009.

Karupiah, G., Xie, Q. W., Buller, R. M., Nathan, C., Duarte, C., and MacMicking, J. D. (1993). Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science* **261,** 1445–1448.

Karupiah, G., Chen, J. H., Mahalingam, S., Nathan, C. F., and MacMicking, J. D. (1998). Rapid interferon γ-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J. Exp. Med.* **188,** 1541–1546.

Kashem, A., Endoh, M., Yano, N., Yamauchi, F., Nomoto, Y., and Sakai, H. (1996). Expression of inducible-NOS in human glomerulonephritis: The possible source is infiltrating monocytes/macrophages. *Kidney Int.* **50,** 392–399.

Kato, Y., Kawakishi, S., Aoki, T., Itakura, K., and Osawa, T. (1997). Oxidative modification of tryptophan residues exposed to peroxynitrite. *Biochem. Biophys. Res. Commun.* **234,** 82–84.

Katsuki, S., Arnold, W., Mittal, C., and Murad, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic Nucleotide Res.* **3,** 23–25.

Kaur, H., and Halliwell, B. (1994). Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Lett.* **350,** 9–12.

Keese, M. A., Bose, M., Mulsch, A., Schirmer, R. H., and Becker, K. (1997). Dinitrosyl–dithiol–iron complexes, nitric oxide (NO) carriers in vivo, as potent inhibitors of human glutathione reductase and glutathione-*S*-transferase. *Biochem. Pharmacol.* **54,** 1307–1313.

Keller, G. A., Barke, R., Harty, J. T., Humphrey, E., and Simmons, R. L. (1985). Decreased hepatic glutathione levels in septic shock. Predisposition of hepatocytes to oxidative stress: An experimental approach. *Arch. Surg.* **120,** 941–945.

Kelm, M., Dahmann, R., Wink, D., and Feelisch, M. (1997). The nitric oxide/superoxide assay. Insights into the biological chemistry of the NO/O_2^- interaction. *J. Biol. Chem.* **272,** 9922–9932.

Kengatharan, K. M., De Kimpe, S. J., and Thiemermann, C. (1996). Role of nitric oxide in the circulatory failure and organ injury in a rodent model of gram-positive shock. *Br. J. Pharmacol.* **119,** 1411–1421.

Kengatharan, K. M., De Kimpe, S., Robson, C., Foster, S. J., and Thiemermann, C. (1998). Mechanism of gram-positive shock: Identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. *J. Exp. Med.* **188,** 305–315.

Kennedy, L. J., Moore, K., Jr., Caulfield, J. L., Tannenbaum, S. R., and Dedon, P. C. (1997). Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents. *Chem. Res. Toxicol.* **10,** 386–392.

Kennedy, M., Szabó, C., and Salzman, A. L. (1998). Activation of poly(ADP-ribose) synthetase (PARS) mediates cytotoxicity induced by peroxynitrite in human intestinal epithelial cells. *Gastroenterology* **114,** 510–518.

Khadour, F. H., O'Brien, D. W., Fu, Y., Armstrong, P. W., and Schulz, R. (1998). Endothelial nitric oxide synthase increases in left atria of dogs with pacing-induced heart failure. *Am. J. Physiol.* **275,** H1971–H1978.

Kilbourn, R. G., Szabó, C., and Traber, D. (1997). Beneficial versus detrimental effects of nitric oxide synthase inhibitors in circulatory shock: Lessons learned from experimental and clinical studies. *Shock* **7,** 235–246.

Kim, Y. M., Talanian, R. V., and Billiar, T. R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272,** 31138–31148.

Kim, Y. M., Kim, T. H., Seol, D. W., Talanian, R. V., and Billiar, T. R. (1998a). Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome *c* release. *J. Biol. Chem.* **273,** 31437–31441.

Kim, Y. M., Talanian, R. V., Li, J., and Billiar, T. R. (1998b). Nitric oxide prevents IL-1β and IFN-γ-inducing factor (IL-18) release from macrophages by inhibiting caspase-1 (IL-β-converting enzyme). *J. Immunol.* **161,** 4122–4128.

King, P. A., Anderson, V. E., Edwards, J. O., Gustav, G., Plumb, R. C., and Suggs, J. W. (1992). A stable solid that generates hydroxyl radical dissolutions in aqueous solutions: Reaction with proteins and nucleic acid. *J. Am. Chem. Soc.* **114,** 5430–5432.

Kishnani, N. S., Tabrizi-Fard, M. A., and Fung, H. L. (1999). Diethylthiocarbamate prolongs survival in mice in a lipopolysaccharide-induced

endotoxic shock model: Evidence for multiple mechanisms. *Shock* **11,** 264–268.

Knowles, R. G., Merrett, M., Salter, M., and Moncada, S. (1990). Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.* **270,** 833–836.

Koglin, J., Glysing-Jensen, T., Mudgett, J. S., and Russell, M. E. (1998a). NOS2 mediates opposing effects in models of acute and chronic cardiac rejection: insights from NOS2-knockout mice. *Am. J. Pathol.* **153,** 1371–1376.

Koglin, J., Glysing-Jensen, T., Mudgett, J. S., and Russell, M. E. (1998b). Exacerbated transplant arteriosclerosis in inducible nitric oxide-deficient mice. *Circulation* **97,** 2059–2065.

Koide, M., Kawahara, Y., Nakayama, I., Tsuda, T., and Yokoyama, M. (1993). Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells. Synergism with the induction elicited by inflammatory cytokines. *J. Biol. Chem.* **268,** 24959–24966.

Kolb, H., and Kolb-Bachofen, V. (1998). Nitric oxide in autoimmune disease: Cytotoxic or regulatory mediator? *Immunol. Today* **19,** 556–561.

Kolb-Bachofen, V. (1996). Intraislet expression of inducible nitric oxide synthase and islet cell death. *Biochem. Soc. Trans.* **24,** 233–234.

Kolios, G., Rooney, N., Murphy, C. T., Robertson, D. A., and Westwick, J. (1998). Expression of inducible nitric oxide synthase activity in human colon epithelial cells: Modulation by T lymphocyte derived cytokines. *Gut* **43,** 56–63.

Kong, S. K., Yim, M. B., Stadtman, E. R., and Chock, P. B. (1996). Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: Lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6–20)NH_2 peptide. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 3377–3382.

Kooy, N., Royall, J., Ischiropoulos, H., and Beckman, J. (1995a). Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radicals Biol. Med.* **16,** 149–155.

Kooy, N. W., Royall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1995b). Evidence for in vivo peroxynitrite production in human acute lung injury. *Am. J. Respir. Crit. Care Med.* **151,** 1250–1254.

Kooy, N. W., Lewis, S. J., Royall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1997). Extensive tyrosine nitration in human myocardial inflammation: Evidence for the presence of peroxynitrite. *Crit. Care Med.* **25,** 812–819.

Krafte-Jacobs, B., Brilli, R., Szabo, C., Denenberg, A., Moore, L., and Salzman, A. L. (1997). Circulating methemoglobin and nitrite/nitrate concentrations as indicators of nitric oxide overproduction in critically ill children with septic shock. *Crit. Care Med.* **25,** 1588–1593.

Kristof, A. S., Goldberg, P., Laubach, V., and Hussain, S. N. (1998). Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am. J. Respir. Crit. Care Med.* **158,** 1883–1889.

Kroger, H., Miesel, R., Diethrich, A., Ohde, M., Rajnavolgyi, E., and Ockenfels, H. (1996). Synergistic effects of thalidomide and poly(ADP-ribose) polymerase inhibition of type II collagen-induced arthritis in mice. *Inflammation* **20,** 203–215.

Kubes, P., Suzuki, M., and Granger, D. N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4651–4655.

Kwon, G., Hill, J. R., Corbett, J. A., and McDaniel, M. L. (1997). Effects of aspirin on nitric oxide formation and de novo protein synthesis by RINm5F cells and rat islets. *Mol. Pharmacol.* **52,** 398–405.

Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., and Quilliam, L. A. (1997). A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J. Biol. Chem.* **272,** 4323–4326.

Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996). Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15069–15074.

Laubach, V. E., Shesely, E. G., Smithies, O., and Sherman, P. A. (1995). Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10688–10692.

Lawand, N. B., Willis, W. D., and Westlund, K. N. (1997). Blockade of joint inflammation and secondary hyperalgesia by L-NAME, a nitric oxide synthase inhibitor. *Neuroreport* **8,** 895–899.

Lefer, D. J., Scalia, R., Campbell, B., Nossuli, T., Hayward, R., Salamon, M., Grayson, J., and Lefer, A. M. (1997). Peroxynitrite inhibits leukocyte–endothelial cell interactions and protects against ischemia–reperfusion injury in rats. *J. Clin. Invest.* **99,** 684–691.

Leist, M., Single, B., Kunstle, G., Volbracht, C., Hentze, H., and Nicotera, P. (1997). Apoptosis in the absence of poly-(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.* **233,** 518–522.

Levine, J. J., Pettei, M. J., Valderrama, E., Gold, D. M., Kessler, B. H., and Trachtman, H. (1998). Nitric oxide and inflammatory bowel disease: Evidence for local intestinal production in children with active colonic disease. *J. Pediatr. Gastroenterol. Nutr.* **26,** 34–38.

Li, J., Billiar, T. R., Talanian, R. V., and Kim, Y. M. (1997). Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem. Biophys. Res. Commun.* **240,** 419–424.

Li, X., De Sarno, P., Song, L., Beckman, J. S., and Jope, R. S. (1998). Peroxynitrite modulates tyrosine phosphorylation and phosphoinositide signalling in human neuroblastoma SH-SY5Y cells: Attenuated effects in human 1321N1 astrocytoma cells. *Biochem. J.* **331,** 599–606.

Lin, K. T., Xue, J. Y., Nomen, M., Spur, B., and Wong, P. Y. (1995). Peroxynitrite-induced apoptosis in HL-60 cells. *J. Biol. Chem.* **270,** 16487–16490.

Lin, K. T., Xue, J. Y., Lin, M. C., Spokas, E. G., Sun, F. F., and Wong, P. Y. (1998). Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. *Am. J. Physiol.* **274,** C855–C860.

Ling, H., Gengaro, P. E., Edelstein, C. L., Martin, P. Y., Wangsiripaisan, A., Nemenoff, R., and Schrier, R. W. (1998). Effect of hypoxia on proximal tubules isolated from nitric oxide synthase knockout mice. *Kidney Int.* **53,** 1642–1646.

Linn, S. C., Morelli, P. A., Edry, I., Cotbugim, S. E., Szabo, C., and Salzman, A. L. (1998). Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am. J. Physiol.* **272,** G1499–G1508.

Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.

Lipton, S. A., Rayudu, P. V., Choi, Y. B., Sucher, N. J., and Chen, H. S. (1998). Redox modulation of the NMDA receptor by NO-related species. *Prog. Brain Res.* **118,** 73–82.

Liu, P., Ioannides, C., Symons, A. M., and Parke, D. V. (1993). Role of tissue glutathione in prevention of surgical trauma. *Xenobiotica* **23,** 899–911.

Lizasoain, I., Moro, M. A., Knowles, R. G., Darley-Usmar, V., and Moncada, S. (1996). Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *Biochem. J.* **314,** 877–880.

Lohinai, Z., Benedek, P., Fehér, E., Györfy, A., Rosivall, L., Fazekas, Á., Zelles, T., Salzman, A. L., and Szabó, C. (1998). Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase in ligature-induced periodontitis in the rat. *Br. J. Pharmacol.* **123,** 353–360.

McCartney-Francis, N., Allen, J. B., Mizel, D. E., Albina, J. E., Xie, Q. W., Nathan, C. F., and Wahl, S. M. (1994). Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* **178,** 749–754.

McDaniel, M. L., Kwon, G., Hill, J. R., Marshall, C. A., and Corbett, J. A. (1996). Cytokines and nitric oxide in islet inflammation and diabetes. *Proc. Soc. Exp. Biol. Med.* **211,** 24–32.

McInnes, I. B., Leung, B. P., Field, M., Wei, X. Q., Huang, F. P., Sturrock, R. D., Kinninmonth, A., Weidner, J., Mumford, R., and Liew, F. Y. (1996). Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. *J. Exp. Med.* **184,** 1519–1524.

McInnes, I. B., Leung, B., Wei, X. Q., Gemmell, C. C., and Liew, F. Y. (1998). Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase. *J. Immunol.* **160,** 308–315.

MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q. W., Sokol, K., and Hutchinson, N. (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81,** 641–650.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997a). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15,** 323–350.

MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K., and Nathan, C. F. (1997b). Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 5243–5248.

MacMillan-Crow, L. A., Crow, J. P., Kerby, J. D., Beckman, J. S., and Thompson, J. A. (1996). Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11853–11858.

MacRitchie, A. N., Jun, S. S., Chen, Z., German, Z., Yuhanna, I. S., Sherman, T. S., and Shaul, P. W. (1997). Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circ. Res.* **81,** 355–362.

Mallozzi, C., Di Stasi, A. M., and Minetti, M. (1997). Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *FASEB J.* **11,** 1281–1290.

Malyshev, I. Yu., Malugin, A. V., Golubeva, L. Yu., Zenina, T. A., Manukhina, E. B., Mikoyan, V. D., and Vanin, A. F. (1996). Nitric oxide donor induces HSP70 accumulation in the heart and in cultured cells. *FEBS Lett.* **391,** 21–23.

Manfield, L., Jang, D., and Murrell, G. A. (1996). Nitric oxide enhances cyclooxygenase activity in articular cartilage. *Inflamm. Res.* **45,** 254–258.

Marczin, N., Tekeres, M., Salzman, A. L., and Szabó, C. (1996). Methylene blue infusion in septic shock. *Crit. Care Med.* **23,** 1936–1938.

Martin, P. Y., Xu, D. L., Niederberger, M., Weigert, A., Tsai, P., St. John, J., Gines, P., and Schrier, R. W. (1996). Upregulation of endothelial constitutive NOS: A major role in the increased NO production in cirrhotic rats. *Am. J. Physiol.* **270,** F494–F499.

Masutani, M., Suzuki, H., Kamada, N., Watanabe, M., Ueda, O., Nozaki, T., Jishage, Ki., Watanabe, T., Sugimoto, T., Nakagama, H., Ochiya, T., and Sugimura, T. (1999). Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 2301–2304.

Mayer, B., Schrammel, A., Klatt, P., Koesling, D., and Schmidt, K. (1995). Peroxynitrite-induced accumulation of cyclic GMP in endothelial cells and stimulation of purified soluble guanylyl cyclase. Dependence on glutathione and possible role of S-nitrosation. *J. Biol. Chem.* **270,** 17355–17360.

Medeiros, M. V., Binhara, I. M., Moreno Junior, H., Zatz, R., De Nucci, G., and Antunes, E. (1995). Effect of chronic nitric oxide synthesis inhibition on the inflammatory responses induced by carrageenin in rats. *Eur. J. Pharmacol.* **285,** 109–114.

Melino, G., and Piacentini, M. (1998). "Tissue' transglutaminase in cell death: A downstream or a multifunctional upstream effector? *FEBS Lett.* **430,** 59–63.

Melino, G., Bernassola, F., Knight, R. A., Corasaniti, M. T., Nistico, G., and Finazzi-Agro, A. (1997). S-nitrosylation regulates apoptosis. *Nature* **388,** 432–433.

Miesel, R., Dietrich, A., Brandl, B., Ulbrich, N., Kurpisz, M., and Kroger, H. (1994). Suppression of arthritis by an active center analogue of Cu2Zn2-superoxide dismutase. *Rheumat. Int.* **14,** 119–126.

Miesel, R., Kurpisz, M., and Kröger, H. (1996). Suppression of inflammatory arthritis by simultaneous inhibition of nitric oxide synthase and NADPH oxidase. *Free Radicals Biol. Med.* **20,** 75–81.

Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hansert, B., Wink, D. A., and Grisham, M. B. (1996). Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J. Biol. Chem.* **271,** 40–47.

Miller, M. J. S., Thompson, J. H., Zhang, X. J., Sadowska-Krowicka, H., Kakkis, J. L., Munshi, U. K., Sandoval, M., Rossi, J., Elobi-Childress, S., Beckman, J., Ye, Y. Z., Rodi, C. P., Manning, P., Currie, M., and Clark, D. A. (1995). Role of inducible nitric oxide synthase expression and peroxynitrite formation in guinea pig ileitis. *Gastroenterology* **109,** 1475–1483.

Minghetti, L., Nicolini, A., Polazzi, E., Creminon, C., Maclouf, J., and Levi, G. (1997). Inducible nitric oxide synthase expression in activated rat microglial cultures is downregulated by exogenous prostaglandin E_2 and by cyclooxygenase inhibitors. *Glia* **19,** 152–160.

Mizumura, K. (1997). Peripheral mechanism of hyperalgesia—Sensitization of nociceptors. *Nagoya J. Med. Sci.* **60,** 69–87.

Mogil, J. S., and Grisel, J. E. (1998). Transgenic studies of pain. *Pain* **77,** 107–128.

Mohr, S., Stamler, J. S., and Brune, B. (1994). Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite and related nitrosating agents. *FEBS Lett.* **348,** 223–227.

Molina-Holgado, F., Lledo, A., and Guaza, C. (1995). Evidence for cyclooxygenase activation by nitric oxide in astrocytes. *Glia* **15,** 167–172.

Mollace, V., Colasanti, M., Muscoli, C., Lauro, G. M., Iannone, M., Rotiroti, D., and Nistico, G. (1998). The effect of nitric oxide on cytokine-induced release of PGE_2 by human cultured astroglial cells. *Br. J. Pharmacol.* **124,** 742–746.

Moro, M. A., Darley-Usmar, V. M., Goodwin, D. A., Read, N. G., Zamora-Pino, R., Feelisch, M., Radomski, M. W., and Moncada, S. (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 6702–6706.

Moro, M. A., Darley-Usmar, V. M., Lizasoain, I., Su, Y., Knowles, R. G., Radomski, M. W., and Moncada, S. (1995). The formation of nitric oxide donors from peroxynitrite. *Br. J. Pharmacol.* **116,** 1999–2004.

Moro, M. A., Russel, R. J., Cellek, S., Lizasoain, I., Su, Y., Darley-Usmar, V. M., Radomski, M. W., and Moncada, S. (1996). cGMP mediates the vascular and platelet actions of nitric oxide: Confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1480–1485.

Murphy, M. E., and Brayden, J. E. (1995). Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J. Physiol.* **486,** 47–58.

Murphy, M. E., and Sies, H. (1997). Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10860–10864.

Mustafa, S. B., and Olson, M. S. (1998). Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. *J. Biol. Chem.* **273,** 5073–5080.

Mustafa, S. B., Howard, K. M., and Olson, M. S. (1996). Platelet-activating factor augments lipopolysaccharide-induced nitric oxide formation by rat Kupffer cells. *Hepatology* **23,** 1622–1630.

Nagase, S., Takemura, K., Ueda, A., Hirayama, A., Aoyagi, K., Kondoh, M., and Koyama, A. (1997). A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine. *Biochem. Biophys. Res. Commun.* **233,** 150–153.

Nathan, C. (1997). Inducible nitric oxide synthase: What difference does it make? *J. Clin. Invest.* **100,** 2417–2423.

Nemath, I., and Boda, D. (1989). The ratio of oxidized/reduced glutathione as an index of oxidative stress in various experimental models of shock syndrome. *Biomed. Biochim. Acta* **48,** S53–S57.

Németh, Z. H., Haskó, G., and Vizi, E. S. (1998). Pyrrolidine dithiocarbamate augments IL-10, inhibits TNF-α, MIP-1α, IL-12, and nitric oxide production and protects from the lethal effect of endotoxin. *Shock* **10,** 49–53.

Ni, Z., Morcos, S., and Vaziri, N. D. (1997). Up-regulation of renal and vascular nitric oxide synthase in iron-deficiency anemia. *Kidney Int.* **52,** 195–201.

Nicholson, S., Bonecini-Almeida, M. da G., Lapa e Silva, J. R., Nathan, C., Xie, Q. W., Mumford, R., Weidner, J. R., Calaycay, J., Geng, J., and Boechat, N. (1996). Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J. Exp. Med.* **183,** 2293–2302.

Nogawa, S., Forster, C., Zhang, F., Nagayama, M., Ross, M. E., and Iadecola, C. (1998). Interaction between inducible nitric oxide synthase and

cyclooxygenase-2 after cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10966–10971.

Nozaki-Taguchi, N., and Yamamoto, T. (1998). Involvement of nitric oxide in peripheral antinociception mediated by κ- and δ-opioid receptors. *Anesth. Analg.* **87,** 388–393.

Nunoshiba, T., DeRojas-Walker, T., Wishnok, J. S., Tannenbaum, S. R., and Demple, B. (1993). Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9993–9997.

Nunoshiba, T., DeRojas-Walker, T., Tannenbaum, S. R., and Demple, B. (1995). Roles of nitric oxide in inducible resistance of *Escherichia coli* to activated murine macrophages. *Infect. Immun.* **63,** 794–798.

O'Connor, M., Salzman, A. L., and Szabó, C. (1997). Role of peroxynitrite in the protein oxidation and apoptotic DNA fragmentation in immunostimulated vascular smooth muscle cells. *Shock* **8,** 439–443.

Oh-Ishi, S., Hayashi, I., Hayashi, M., Yamaki, K., and Utsunomiya, I. (1989). Pharmacological demonstration of inflammatory mediators using experimental inflammatory models: Rat pleurisy induced by carrageenin and phorbol myristate acetate. *Dermatologica* **179**(Suppl. 1), 68–71.

Ohshima, H., Yoshie, Y., Auriol, S., and Gilibert, I. (1998). Antioxidant and pro-oxidant actions of flavonoids: Effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. *Free Radicals Biol. Med.* **25,** 1057–1065.

Ohuchi, Y., Ichinose, M., Miura, M., Kageyama, N., Tomaki, M., Endoh, N., Mashito, Y., Sugiura, H., and Shirato, K. (1998). Induction of nitric oxide synthase by lipopolysaccharide inhalation enhances substance P-induced microvascular leakage in guinea pigs. *Eur. Respir. J.* **12,** 831–836.

Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M., Ichimori, K., and Maeda, H. (1997). Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: A novel mechanism for procollagenase activation involving nitric oxide. *Arch. Biochem. Biophys.* **342,** 261–274.

Okuda, Y., Sakoda, S., Fujimura, H., and Yanagihara, T. (1997). Nitric oxide via an inducible isoform of nitric oxide synthase is a possible factor to eliminate inflammatory cells from the central nervous system of mice with experimental allergic encephalomyelitis. *J. Neuroimmunol.* **73,** 107–116.

Orman, K. L., Shenep, J. L., and English, B. (1998). Pneumococci stimulate the production of the inducible nitric oxide synthase and nitric oxide by murine macrophages. *J. Infect. Dis.* **178,** 1649–1657.

Orucevic, A., Hearn, S., and Lala, P. K. (1997). The role of active inducible nitric oxide synthase expression in the pathogenesis of capillary leak syndrome resulting from interleukin-2 therapy in mice. *Lab. Invest.* **76,** 53–65.

Ou, J., Carlos, T. M., Watkins, S. C., Saavedra, J. E., Keefer, L. K., Kim, Y. M., Harbrecht, B. G., and Billiar, T. R. (1997). Differential effects of nonselective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. *Nitric Oxide Biol. Chem.* **1,** 404–416.

Oudkerk Pool, M., Bouma, G., Visser, J. J., Kolkman, J. J., Tran, D. D., Meuwissen, S. G., and Pena, A. S. (1995). Serum nitrate levels in ulcerative colitis and Crohn's disease. *Scand. J. Gastroenterol.* **30,** 784–788.

Packer, M. A., Porteous, C. M., and Murphy, M. P. (1996). Superoxide production by mitochondria in the presence of nitric oxide forms peroxynitrite. *Biochem. Mol. Biol. Int.* **40,** 527–534.

Padgett, C. M., and Whorton, A. R. (1997). Glutathione redox cycle regulates nitric oxide-mediated glyceraldehyde-3-phosphate dehydrogenase inhibition. *Am. J. Physiol.* **272,** C99–C108.

Pahan, K., Namboodiri, A. M., Sheikh, F. G., Smith, B. T., and Singh, I. (1997). Increasing cAMP attenuates induction of inducible nitric-oxide synthase in rat primary astrocytes. *J. Biol. Chem.* **272,** 7786–7791.

Palmer, R. M. J., Bridge, L., Foxwell, N. A., and Moncada, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.* **105,** 11–12.

Palmer, R. M., Hickery, M. S., Charles, I. G., Moncada, S., and Bayliss, M. T. (1993). Induction of nitric oxide synthase in human chondrocytes. *Biochem. Biophys. Res. Commun.* **193,** 398–405.

Pang, L., and Hoult, J. R. (1996). Induction of cyclooxygenase and nitric oxide synthase in endotoxin-activated J774 macrophages is differentially regulated by indomethacin: Enhanced cyclooxygenase-2 protein expression but reduction of inducible nitric oxide synthase. *Eur. J. Pharmacol.* **317,** 151–155.

Pang, L., and Hoult, J. R. (1997). Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E_2 and other cyclic AMP stimulants in J774 macrophages. *Biochem. Pharmacol.* **53,** 493–500.

Parkinson, J. F., Mitrovic, B., and Merrill, J. E. (1997). The role of nitric oxide in multiple sclerosis. *J. Mol. Med.* **75,** 174–186.

Parks, D. J., Cheung, M. K., Chan, C. C., and Roberge, F. G. (1994). The role of nitric oxide in uveitis. *Arch. Ophthalmol.* **112,** 544–546.

Pasquet, J. P., Zou, M. H., and Ullrich, V. (1996). Peroxynitrite inhibition of nitric oxide synthases. *Biochimie* **78,** 785–791.

Pataricza, J., Toth, G. K., Penke, B., Hohn, J., and Papp, J. G. (1995). Effect of selective inhibition of potassium channels on vasorelaxing response to cromakalim, nitroglycerin and nitric oxide of canine coronary arteries. *J. Pharm. Pharmacol.* **47,** 921–925.

Paya, D., Gray, G. A., and Stoclet, J. C. (1993). Effects of methylene blue on blood pressure and reactivity to norepinephrine in endotoxemic rats. *J. Cardiovascular. Pharmacol.* **21,** 926–930.

Paya, M., Garcia-Pastor, P., Coloma, J., and Alcaraz, M. J. (1997). Nitric oxide synthase and cyclo-oxygenase pathways in the inflammatory response induced by zymosan in the rat air pouch. *Br. J. Pharmacol.* **120,** 1445–1452.

Pelletier, J. P., Jovanovic, D., Fernandes, J. C., Manning, P., Connor, J. R., Currie, M. G., Di Battista, J. A., and Martel-Pelletier, J. (1998). Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. *Arthritis Rheum.* **41,** 1275–1286.

Pender, B. S., Chen, H., Ashton, S., Wise, W. C., Zingarelli, B., Cusumano, V., and Cook, J. A. (1996). Transforming growth factor β1 alters rat peritoneal macrophage mediator production and improves survival during endotoxic shock. *Eur. Cytokine Netw.* **7,** 137–142.

Perkins, D. J., St Clair, E. W., Misukonis, M. A., and Weinberg, J. B. (1998). Reduction of NOS2 overexpression in rheumatoid arthritis patients treated with anti-tumor necrosis factor α monoclonal antibody (cA2). *Arthritis Rheum.* **41,** 2205–2210.

Perrella, M. A., Hsieh, C. M., Lee, W. S., Shieh, S., Tsai, J. C., Patterson, C., Lowenstein, C. J., Long, N. C., Haber, E., Shore, S., and Lee, M. E. (1996). Arrest of endotoxin-induced hypotension by transforming growth factor β1. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2054–2059.

Peskar, B. M., Trautmann, M., Nowak, P., and Peskar, P. A. (1991). Release of 15-hydroxy-5,8,11,13-eicosatetraenoic acid and cysteinyl-leukotrienes in carrageenin-induced inflammation: Effect of non-steroidal anti-inflammatory drugs. *Agents Actions* **33,** 240–246.

Phelps, D. T., Ferro, T. J., Hiiggins, P. J., Shankar, R., Parker, D. M., and Johnson, A. (1995). TNF-α induces peroxynitrite-mediated depletion of lung endothelial glutathione via protein kinase C. *Am. J. Physiol.* **269,** L551–L559.

Pou, S., Nguyen, S. Y., Gladwell, T., and Rosen, G. M. (1995). Does peroxynitrite generate hydroxyl radical? *Biochim. Biophys. Acta* **1244,** 62–68.

Pryor, W., and Squadrito, G. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.* **268,** L699–L722.

Radi, R. (1996). Kinetic analysis of reactivity of peroxynitrite with biomolecules. *Methods Enzymol.* **269,** 354–366.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288,** 481–487.

Radi, R., Rodriguez, M., Castro, L., and Telleri, R. (1994). Inhibition of mitochondrial electron transport by peroxynitrite. *Arch. Biochem. Biophys.* **308,** 89–95.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2,** 1057–1058.

Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 10043–10047.

Raetz, C. R., Ulevich, R. J., Wright, S. D., Sibley, C. H., Ding, A., and Nathan, C. F. (1991). Gram-negative endotoxin: An extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* **5,** 2652–2660.

Ratnam, S., and Mookerjea, S. (1998). The regulation of superoxide generation and nitric oxide synthesis by C-reactive protein. *Immunology* **94,** 560–568.

Ridger, V. C., Greenacre, S. A., Handy, R. L., Halliwell, B., Moore, P. K., Whiteman, M., and Brain, S. D. (1997). Effect of peroxynitrite on plasma extravasation, microvascular blood flow and nociception in the rat. *Br. J. Pharmacol.* **122,** 1083–1088.

Robbins, R. A., Barnes, P. J., Springall, D. R., Warren, J. B., Kwon, O. J., Buttery, L. D., Wilson, A. J., Geller, D. A., and Polak, J. M. (1994). Expression of inducible nitric oxide in human lung epithelial cells. *Biochem. Biophys. Res. Commun.* **203,** 209–218.

Robbins, R. A., Jinkins, P. A., Bryan, T. W., Prado, S. C., and Milligan, S. A. (1998). Methotrexate inhibition of inducible nitric oxide synthase in murine lung epithelial cells in vitro. *Am. J. Respir. Cell. Mol. Biol.* **18,** 853–859.

Roche, A. K., Cook, M., Wilcox, G. L., and Kajander, K. C. (1996). A nitric oxide synthesis inhibitor (L-NAME) reduces licking behavior and Fos-labeling in the spinal cord of rats during formalin-induced inflammation. *Pain* **66,** 331–341.

Rohn, T. T., Nelson, L. K., Sipes, K. M., Swain, S. D., Jutila, K. L., and Quinn, M. T. (1999). Priming of human neutrophils by peroxynitrite: potential role in enhancement of the local inflammatory response. *J. Leukocyte Biol.* **65,** 59–70.

Rothe, H., and Kolb, H. (1999). Strategies of protection from nitric oxide toxicity in islet inflammation. *J. Mol. Med.* **77,** 40–44.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **269,** 26066–26075.

Ruetten, H., and Thiemermann, C. (1997a). Combination immunotherapy which neutralises the effects of TNF α and IL-1 β attenuates the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. *J. Physiol. Pharmacol.* **48,** 605–621.

Ruetten, H., and Thiemermann, C. (1997b). Effect of calpain inhibitor I, and inhibitor of the proteolysis of IκB, on the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. *Br. J. Pharmacol.* **121,** 695–704.

Ruetten, H., and Thiemermann, C. (1997c). Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: A possible role for protein tyrosine kinase. *Br. J. Pharmacol.* **122,** 59–70.

Ruiz, F., Corrales, F. J., Miqueo, C., and Mato, J. M. (1998). Nitric oxide inactivates rat hepatic methionine adenosyltransferase in vivo by S-nitrosylation. *Hepatology* **28,** 1051–1057.

Sadowska-Krowicka, H., Mannick, E. E., Oliver, P. D., Sandoval, M., Zhang, X. J., Eloby-Childess, S., Clark, D. A., and Miller, M. J. (1998). Genistein and gut inflammation: Role of nitric oxide. *Proc. Soc. Exp. Biol. Med.* **217,** 351–357.

Sahrbacher, U. C., Lechner, F., Eugster, H. P., Frei, K., Lassmann, H., and Fontana, A. (1998). Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **28,** 1332–1338.

Saleh, D., Barnes, P. J., and Giaid, A. (1997). Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **155,** 1763–1769.

Salgo, M. G., Bermudez, E., Squadrito, G., and Pryor, W. (1995a). DNA damage and oxidation of thiols peroxynitrite causes in rat thymocytes. *Arch. Biochem. Biophys.* **322,** 500–505.

Salgo, M. G., Squadrito, G. L., and Pryor, W. A. (1995b). Peroxynitrite causes apoptosis in rat thymocytes. *Biochem. Biophys. Res. Commun.* **215,** 1111–1118.

Salkowski, C. A., Detore, G., McNally, R., van Rooijen, N., and Vogel, S. N. (1997). Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide in vivo: The roles of macrophages, endogenous IFN-γ, and TNF receptor-1-mediated signaling. *J. Immunol.* **158,** 905–912.

Salman-Tabcheh, S., Guerin, M. C., and Torreilles, J. (1995). Nitration of tyrosyl-residues from extra- and intracellular proteins in human whole blood. *Free Radicals Biol. Med.* **19,** 695–698.

Salter, M., Knowles, R. G., and Moncada, S. (1991). Widespread tissue distribution, species distribution and changes in activity of calcium-dependent and calcium-independent nitric oxide synthesis. *FEBS Lett.* **291,** 145–149.

Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7240–7244.

Salvemini, D., Seibert, K., Masferrer, J. L., Misko, T. P., Currie, M. G., and Needleman, P. (1994). Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. *J. Clin. Invest.* **93,** 1940–1947.

Salvemini, D., Settle, S. L., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1995). Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *Br. J. Pharmacol.* **114,** 1171–1178.

Salvemini, D., and Masferrer, J. L. (1996). Interactions of nitric oxide with cyclooxygenase: In vitro, ex vivo, and in vivo studies. *Methods Enzymol.* **269,** 12–25.

Salvemini, D., Currie, M. G., and Mollace, V. (1996a). Nitric oxide-mediated cyclooxygenase activation. A key event in the antiplatelet effects of nitrovasodilators. *J. Clin. Invest.* **97,** 2562–2568.

Salvemini, D., Wang, Z. Q., Bourdon, D. M., Stern, M. K., Currie, M. G., and Manning, P. T. (1996b). Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. *Eur. Pharmacol.* **303,** 217–220.

Salvemini, D., Wang, Z. Q., Wyatt, D. M., Bourdon, M. H., Marino, P. T., and Currie, M. G. (1996c). Nitric oxide: A key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.* **118,** 829–838.

Salvemini, D., Wang, Z. Q., Stern, M. K., Currie, M. G., and Misko, T. P. (1998). Peroxynitrite decomposition catalysts: Therapeutics for peroxynitrite-mediated pathology. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2659–2663.

Salzman, A. L. (1995). Nitric oxide in the gut. *New Horizons* **3,** 352–364.

Salzman, A. L., Denenberg, A. G., Ueta, I., O'Connor, M., Linn, S., and Szabó, C. (1996). Characterization of the induction and activity of the human nitric oxide synthase in a transformed intestinal epithelial cell line. *Am. J. Physiol.* **270,** G565–G573.

Salzman, A. L., Eaves-Pyles, T., Linn, S. C., Denenberg, A. G., and Szabo, C. (1998). Bacterial induction of inducible nitric oxide synthase in cultured human intestinal epithelial cells. *Gastroenterology* **114,** 93–102.

Sandoval, M., Zhang, X. J., Liu, X., Mannick, E. E., Clark, D. A., and Miller, M. J. (1996). Peroxynitrite-induced apoptosis in T84 and RAW 264.7 cells: Attenuation by L-ascorbic acid. *Free Radicals Biol. Med.* **22,** 489–495.

Sasaki, K., Hattori, T., Fujisawa, T., Takahashi, K., Inoue, H., and Takigawa, M. (1998). Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. *J. Biochem.* (*Tokyo*) **123,** 431–439.

Sato, T., Kamata, Y., Irifune, M., and Nishikawa, T. (1997). Inhibitory effect of several nitric oxide-generating compounds on purified Na^+,K^+-ATPase activity from porcine cerebral cortex. *J. Neurochem.* **68,** 1312–1318.

Sautebin, L., Ialenti, A., Ianaro, A., and Di Rosa, M. (1995). Endogenous nitric oxide increases prostaglandin biosynthesis in carrageenin rat paw oedema. *Eur. J. Pharmacol.* **286,** 219–222.

Scarim, A. L., Heitmeier, M. R., and Corbett, J. A. (1998). Heat shock inhibits cytokine-induced nitric oxide synthase expression by rat and human islets. *Endocrinology* **139,** 5050–5057.

Schmidt, H. H. H. W., Lohmann, S. M., and Walter, U. (1993). The nitric oxide and cGMP signal transduction system: Regulation and mechanism of action. *Biochim. Biophys. Acta* **1178,** 153–175.

Schwarz, M. A., Lazo, J. S., Yalowich, J. C., Allen, W. P., Whitmore, M., Bergonia, H. A., Tzeng, E., Billiar, T. R., Robbins, P. D., and Lancaster, J. R., Jr. (1995). Metallothionein protects against the cytotoxic and DNA-damaging effects of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4452–4456.

Schwartz, D., Mendonca, M., Schwartz, I., Xia, Y., Satriano, J., Wilson, C. B., and Blatz, R. C. (1997). Inhibition of constitutive NOS by NO generated by iNOS after CPS administration provokes renal dysfunction in rods. *J. Clin. Invest.* **100,** 439–448.

Scott, G. S., Hake, P., Salzman, A. L., and Szabó, C. (1998). Suppression of experimental allergic encephalomyelitis by administration of 5-iodo-6-amino-1,2-benzopyrone, a novel inhibitor of poly(ADP-ribose) synthetase. *Crit. Care Med.* **26,**(Suppl.), A36.

Selden, L. A., Gersham, L. C., Estes, J. E., Ferro, T. J., and Johnson, A. (1995). Peroxynitrite-induced tyrosine nitration mediates decreased polymerization of actin. *Mol. Biol. Cell* **6,** 20.

Semos, M. L., and Headley, P. M. (1994). The role of nitric oxide in spinal nociceptive reflexes in rats with neurogenic and non-neurogenic peripheral inflammation. *Neuropharmacology* **33,** 1487–1497.

Shi, X., Rojanasakul, Y., Gannett, P., Liu, K., Mao, Y., Daniel, L. N., Ahmed, N., and Saffiotti, U. (1994). Generation of thiyl and ascorbyl radicals in the reaction of peroxynitrite with thiols and ascorbate at physiological pH. *J. Inorg. Biochem.* **56,** 77–86.

Shindo, T., Ikeda, U., Ohkawa, F., Kawahara, Y., Yokoyama, M., and Shimada, K. (1995). Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. *Cardiovasc. Res.* **29,** 813–819.

Shultz, P. J., and Raij, L. (1992). Endogenously synthesized nitric oxide prevents endotoxin-induced glomerular thrombosis. *J. Clin. Invest.* **90,** 1718–1725.

Simon, D. I., Mullins, M. E., Jia, L., Gaston, B., Singel, D. J., and Stamler, J. S. (1996). Polynitrosylated proteins: Characterization, bioactivity, and functional consequences. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 4736–4741.

Singer, I. I., Kawka, D. W., Scott, S., Weidner, J. R., Mumford, R. A., Riehl, T. E., and Stenson, W. F. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology* **111,** 871–885.

Smith, F. S., Ceppi, E. D., and Titheradge, M. A. (1997). Inhibition of cytokine-induced inducible nitric oxide synthase expression by glucagon and cAMP in cultured hepatocytes. *Biochem. J.* **326,** 187–192.

Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S., and Perry, G. (1997). Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17,** 2653–2657.

Smith, F. R., Hart, P. H., Coster, D. J., and Williams, K. A. (1998). Mice deficient in TNF receptors p55 and p75, IL-4 or iNOS are susceptible to endolotin-induced ureitis. *Invest. Opthalmol. Vis. Sci.* **39,** 658–661.

So, H. S., Park, R. K., Kim, M. S., Lee, S. R., Jung, B. H., Chung, S. Y., Jun, C. D., and Chung, H. T. (1998). Nitric oxide inhibits c-Jun N-terminal kinase 2 (JNK2) via S-nitrosylation. *Biochem. Biophys. Res. Commun.* **247,** 809–813.

Southan, G. J., and Szabó, C. (1996). Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem. Pharmacol.* **51,** 383–394.

Spencer, J. P., Wong, J., Jenner, A., Aruoma, O. I., Cross, C. E., and Halliwell, B. (1996). Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem. Res. Toxicol.* **9,** 1152–1158.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 444–448.

Stamler, J. S., Jia, L., Eu, J. P., McMahon, T. J., Demchenko, I. T., Bonaventura, J., Gernert, K., and Piantadosi, C. A. (1997). Blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient. *Science* **276,** 2034–2037.

Stefanovic-Racic, M., Stadler, J., and Evans, C. H. (1993). Nitric oxide and arthritis. *Arthritis Rheum.* **36,** 1036–1044.

Stefanovic-Racic, M., Stadler, J., Georgescu, H. I., and Evans, C. H. (1994). Nitric oxide and energy production in articular chondrocytes. *J. Cell. Physiol.* **159,** 274–280.

Stefanovic-Racic, M., Meyers, K., Meschter, C., Coffey, J. W., Hoffman, R. A., and Evans, C. H. (1995). Comparison of the nitric oxide synthase inhibitors methylarginine and aminoguanidine as prophylactic and therapeutic agents in rat adjuvant arthritis. *J. Rheumatol.* **22,** 1922–1928.

Stein, H. J., Hinder, R. A., and Oosthuizen, M. M. (1990). Gastric mucosal injury caused by hemorrhagic shock and reperfusion: Protective role of the antioxidant glutathione. *Surgery* **108,** 467–473.

Stichtenoth, D. O., and Frolich, J. C. (1998). Nitric oxide and inflammatory joint diseases. *Br. J. Rheumatol.* **37,** 246–257.

Stichtenoth, D. O., Fauler, J., Zeidler, H., and Frolich, J. C. (1995). Urinary nitrate excretion is increased in patients with rheumatoid arthritis and reduced by prednisolone. *Ann. Rheum. Dis.* **54,** 820–824.

Stratman, N. C., Carter, D. B., and Sethy, V. H. (1997). Ibuprofen: Effect on inducible nitric oxide synthase. *Brain Res. Mol. Brain Res.* **50,** 107–112.

Swartz *et al.* (1997).

Szabó, C. (1995). Alterations in nitric oxide production in various forms of circulatory shock. *New Horizons* **3,** 2–32.

Szabó, C. (1996). The role of peroxynitrite in the pathophysiology of shock, inflammation and ischemia–reperfusion injury. *Shock* **6,** 79–88.

Szabó, C. (1998). Role of poly (ADP-ribose) synthetase in inflammation. *Eur. J. Pharmacol.* **350,** 1–19.

Szabó, C., and Dawson, V. L. (1998). Role of poly (ADP-ribose) synthetase activation in inflammation and reperfusion injury. *Trends Pharmacol. Sci.* **19,** 287–298.

Szabó, C., and Ohshima, H. (1997). DNA injury induced by peroxynitrite. *Nitric Oxide Biol. Chem.* **1,** 373–385.

Szabó, C., and Salzman, A. L. (1995). Endogenous peroxynitrite is involved in the inhibition of cellular respiration in immuno-stimulated J774.2 macrophages. *Biochem. Biophys. Res. Commun.* **209,** 739–743.

Szabó, C., and Thiemermann, C. (1995). Mechanism of the induction of nitric oxide synthase. *Adv. Pharmacol.* **34,** 113–153.

Szabó, C., Faragó, M., Horváth, I., Lohinai, Z., and Kovách, A. G. B. (1992a). Hemorrhagic hypotension impairs endothelium-dependent relaxations in the renal artery of the cat. *Circ. Shock* **36,** 238–241.

Szabó, C., Csáki, C., Benyó, Z., Reivich, M., and Kovách, A. G. B. (1992b). Role of the L-arginine–nitric oxide pathway in the changes in cerebrovascular reactivity following hemorrhagic hypotension and retransfusion. *Circ. Shock* **37,** 307–316.

Szabó, C., Wu, C. C., Mitchell, J. A., Gross, S. S., Thiemermann, C., and Vane, J. R. (1993a). Platelet-activating factor contributes to the induction of nitric oxide synthase by bacterial lipopolysaccharide. *Circ. Res.* **73,** 991–999.

Szabó, C., Wu, C. C., Gross, S. S., Thiemermann, C., Perretti, M., and Vane, J. R. (1993b). Interleukin-1 contributes to the induction of nitric oxide synthase by endotoxin in vivo. *Eur. J. Pharmacol.* **250,** 157–160.

Szabó, C., Thiemermann, C., and Vane, J. R. (1993c). Inhibition of the production of nitric oxide and vasodilator prostaglandins attenuates the cardiovascular response to bacterial endotoxin in adrenalectomized rats. *Proc. R. Soc. B London* **253,** 233–238.

Szabó, C., Mitchell, J. A., Thiemermann, C., and Vane, J. R. (1993d). Nitric oxide mediated hyporeactivity to noradrenaline precedes nitric oxide synthase induction in endotoxin shock. *Br. J. Pharmacol.* **108,** 786–792.

Szabó, C., Thiemermann, C., Wu, C. C., Perretti, M., and Vane, J. R. (1994a). Inhibition of nitric oxide synthase induction by endogenous

glucocorticoids accounts for endotoxin tolerance in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 271–275.

Szabó, C., Southan, G. J., Thiemermann, C., and Vane, J. R. (1994b). Spermine inhibits the production of nitric oxide in immuno-stimulated J774.2 macrophages: Requirement of a serum factor. *Br. J. Pharmacol.* **112,** 355–356.

Szabó, C., Southan, G. J., and Thiemermann, C. (1994c). Beneficial effects and improved survival in rodent models of septic shock with *S*-methylisothiourea sulfate, a novel, potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 2472–2476.

Szabó, C., Salzman, A. L., and Ischiropoulos, H. (1995a). Endotoxin triggers the expression of an inducible isoform of NO synthase and the formation of peroxynitrite in the rat aorta in vivo. *FEBS Lett.* **363,** 235–238.

Szabó, C., Salzman, A. L., and Ischiropoulos, H. (1995b). Peroxynitrite-mediated oxidation of dihydrorhodamine 123 occurs in early stages of endotoxic and hemorrhagic shock and ischemia–reperfusion injury. *FEBS Lett.* **372,** 229–232.

Szabó, C., Zingarelli, B., and Salzman, A. L. (1996a). Role of poly-ADP ribosyltransferase activation in the nitric oxide- and peroxynitrite-induced vascular failure. *Circ. Res.* **78,** 1051–1063.

Szabó, C., Zingarelli, B., O'Connor, M., and Salzman, A. L. (1996b). DNA strand breakage, activation of poly-ADP ribosyl synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1753–1758.

Szabó, C., Bryk, R., Zingarelli, B., Southan, G. J., Gahman, T. C., Bhat, V., Salzman, A. L., and Wolff, D. J. (1996c). Pharmacological characterization of guanidinoethyldisulphide (GED), a novel inhibitor of nitric oxide synthase with selectivity towards the inducible isoform. *Br. J. Pharmacol.* **118,** 1659–1668.

Szabó, C., Wong, H. R., and Salzman, A. L. (1996d). Heat shock phenotype inhibits peroxynitrite-induced activation of poly(ADP) ribosyl synthetase and protects against peroxynitrite cytotoxicity in J774 macrophages. *Eur. J. Pharmacol.* **315,** 221–226.

Szabó, C., Day, B. J., and Salzman, A. L. (1996e). Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages, using a novel mesoporphyrin superoxide dismutase analog and peroxynitrite scavenger. *FEBS Lett.* **381,** 82–86.

Szabo, C., Hasko, G., Zingarelli, B., Nemeth, Z. H., Salzman, A. L., Kvetan, V., Pastores, S. M., and Vizi, E. S. (1997a). Isoproterenol regulates tumour necrosis factor, interleukin-10, interleukin-6 and nitric oxide production and protects against the development of vascular hyporeactivity in endotoxaemia. *Immunology* **90,** 95–100.

Szabó, C., Cuzzocrea, S., Zingarelli, B., O'Connor, M., and Salzman, A. L. (1997b). Endothelial dysfunction in endotoxic shock: Importance of the activation of poly(ADP-ribose) synthetase (PARS) by peroxynitrite. *J. Clin. Invest.* **100,** 723–735.

Szabó, C., Saunders, C., O'Connor, M., and Salzman, A. L. (1997c). Peroxynitrite causes energy depletion and increases permeability via activation of poly-ADP ribosyl synthetase in pulmonary epithelial cells. *Am. J. Resp. Mol. Cell. Biol.* **16,** 105–109.

Szabó, C., Lim, L. H., Cuzzocrea, S., Getting, S. J., Zingarelli, B., Flower, R. J., Salzman, A. L., and Perretti, M. (1997d). Inhibition of poly(ADP-ribose) synthetase exerts anti-inflammatory effects and inhibits neutrophil recruitment. *J. Exp. Med.* **186,** 1041–1049.

Szabó, C., O'Connor, M., and Salzman, A. L. (1997e). Endogenously produced peroxynitrite induces the oxidation of mitochondrial and nuclear proteins in immunostimulated macrophages. *FEBS Lett.* **409,** 147–150.

Szabó, C., Wong, H. R., Bauer, P. I., Kirsten, E., O'Connor, M., Zingarelli, B., Mendeleyev, J., Hasko, G., Vizi, E. S., Salzman, A. L., and Kun, E. (1997f). Regulation of components of the inflammatory response by 5-iodo-6-amino-1,2-benzopyrone, and inhibitor of poly(ADP-ribose) synthetase and pleiotropic modifier of cellular signal pathways. *Int. J. Oncol.* **10,** 1093–101.

Szabó, C., Virág, L., Cuzzocrea, S., Scott, G. J., Hake, P., O'Connor, M. P., Zingarelli, B., Salzman, A. L., and Kun, E. (1998). Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly(ADP-ribose) synthetase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 3867–3872.

Tabuchi, A., Sano, K., Oh, E., Tsuchiya, T., and Tsuda, M. (1994). Modulation of AP-1 activity by nitric oxide (NO) in vitro: NO-mediated modulation of AP-1. *FEBS Lett.* **351,** 123–127.

Tarpey, M. M., Beckman, J. S., Ischiropoulos, H., Gore, J. Z., and Brock, T. A. (1995). Peroxynitrite stimulates vascular smooth muscle cell cyclic GMP synthesis. *FEBS Lett.* **364,** 314–318.

Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M., Jr., Billiar, T. R., and Geller, D. A. (1998). Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* **273,** 15148–15156.

Ter Steege, J. C., van de Ven, M. W., Forget, P. P., Brouckaert, P., and Buurman, W. A. (1998). The role of endogenous IFN-γ, TNF-α and IL-10 in LPS-induced nitric oxide release in a mouse model. *Cytokine* **10,** 115–123.

Thiemermann, C., Wu, C. C., Szabó, C., Perretti, M., and Vane, J. R. (1993a). Tumor necrosis factor is an endogenous mediator of the induction of nitric oxide synthase in endotoxin shock in the rat. *Br. J. Pharmacol.* **110,** 177–182.

Thiemermann, C., Szabó, C., Mitchell, J. A., and Vane, J. R. (1993b). Vascular hyporeactivity to vasoconstrictor agents and haemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 267–271.

Trachtman, H., Futterweit, S., Greenwald, R., Moak, S., Singhal, P., Franki, N., and Amin, A. R. (1996). Chemically modified tetracyclines inhibit inducible nitric oxide synthase expression and nitric oxide production in cultured rat mesangial cells. *Biochem. Biophys. Res. Commun.* **229,** 243–248.

Trajkovic, V., Badovinac, V., Jankovica, V., and Stojkovic, M. M. (1999). Cyclosporin A inhibits activation of inducible nitric oxide synthase in C6 glioma cell line. *Brain Res.* **816,** 92–98.

Tsukahara, Y., Morisaki, T., Horita, Y., Torisu, M., and Tanaka, M. (1998). Expression of inducible nitric oxide synthase in circulating neutrophils of the systemic inflammatory response syndrome and septic patients. *World J. Surg.* **22,** 771–777.

Twart, C. H. C., and Van Breemen, C. (1998). Cyclic guanosine monophosphate enhanced sequestration of Ca^{2+} by sarcoplasmic reticulum in vascular smooth muscle. *Circ. Res.* **62,** 962–964.

Uesugi, M., Hayashi, T., and Jasin, H. E. (1998). Covalent cross-linking of immune complexes by oxygen radicals and nitrite. *J. Immunol.* **161,** 1422–1427.

Uppu, R. M., Cueto, R., Squadrito, G. L., Salgo, M. G., and Pryor, W. A. (1996). Competitive reactions of peroxynitrite with 2'-deoxyguanosine and 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG): Relevance to the formation of 8-oxodG in DNA exposed to peroxynitrite. *Free Radicals Biol. Med.* **21,** 407–411.

Vallette, G., Jarry, A., Lemarre, P., Branka, J. E., and Laboisse, C. L. (1997). NO-dependent and NO-independent IL-1 production by a human colonic epithelial cell line under inflammatory stress. *Br. J. Pharmacol.* **121,** 187–192.

Van de Loo, F. A., Arntz, O. J., van Enckevort, F. H., van Lent, P. L., and van den Berg, W. B. (1998). Reduced cartilage proteoglycan loss during zymosan-induced gonarthritis in NOS2-deficient mice and in anti-interleukin-1-treated wild-type mice with unabated joint inflammation. *Arthritis Rheum.* **41,** 634–646.

Van Der Veen, R., Hinto, D. R., Incardona, F., and Hofman, F. M. (1997). Extensive peroxynitrite activity during progressive stages of central nervous system inflammation. *J. Neuroimmunol.* **77,** 1–7.

Van der Vliet, A., Smith, D., O'Neill, C. A., Kaur, H., Darley-Usmar, V., Cross, C. E., and Halliwell, B. (1994). Interactions of peroxynitrite with human plasma and its constituents: Oxidative damage and antioxidant depletion. *Biochem. J.* **303,** 295–301.

Van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997). Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272,** 7617–7625.

Van der Vliet, A., Hristova, M., Cross, C. E., Eiserich, J. P., and Goldkorn, T. (1998a). Peroxynitrite induces covalent dimerization of epidermal growth factor receptors in A431 epidermoid carcinoma cells. *J. Biol. Chem.* **273,** 31860–31866.

Van der Vliet, A., 't Hoen, P. A., Wong, P. S., Bast, A., and Cross, C. E. (1998b). Formation of *S*-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J. Biol. Chem.* **273,** 30255–30262.

Vatassery, G. T. (1996). Oxidation of vitamin E, vitamin C, and thiols in rat brain synaptosomes by peroxynitrite. *Biochem. Pharmacol.* **52,** 579–586.

Villa, L. M., Salas, E., Darley-Usmar, M., Radomski, M. W., and Moncada, S. (1994). Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12383–12387.

Viner, R. I., Huhmer, A. F., Bigelow, D. J., and Schoneich, C. (1996). The oxidative inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase by peroxynitrite. *Free Radical Res.* **24,** 243–259.

Virág, L., Haskó, G., Salzman, A. L., and Szabó, C. (1998a). NADPH-diaphorase histochemistry detects inducible nitric oxide synthetase (iNOS) activity in the thymus of naive and staphylococcal enterotoxin B (SEB)-stimulated mice. *J. Histochem. Cytochem.* **46,** 787–792.

Virág, L., Marmer, D. J., and Szabó, C. (1998b). The crucial role of apopain/caspase-3 in the peroxynitrite-induced apoptotic DNA fragmentation. *Free Radicals Biol. Med.* **25,** 1075–1082.

Virág, L., Scott, G. S., Salzman, A. L., and Szabó, C. (1998c). Peroxynitrite-induced thymocyte apoptosis: The role of caspases and poly-(ADP-ribose) synthetase (PARS) activation. *Immunology* **94,** 345–355.

Vodovotz, Y., Geiser, A. G., Chesler, L., Letterio, J. J., Campbell, A., Lucia, M. S., Sporn, M. B., and Roberts, A. B. (1996). Spontaneously increased production of nitric oxide and aberrant expression of the inducible nitric oxide synthase in vivo in the transforming growth factor β 1 null mouse. *J. Exp. Med.* **183,** 2337–2342.

Vodovotz, Y., Kopp, J. B., Takeguchi, H., Shrivastav, S., Coffin, D., Lucia, M. S., Mitchell, J. B., Webber, R., Letterio, J., Wink, D., and Roberts, A. B. (1998). Increased mortality, blunted production of nitric oxide, and increased production of TNF-α in endotoxemic TGF-β1 transgenic mice. *J. Leukocyte Biol.* **63,** 31–39.

Wang, P., and Zweier, J. L. (1996). Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. *J. Biol. Chem.* **271,** 29223–29230.

Wang, P., Ba, Z. F., and Chaudry, I. H. (1995). ATP-$MgCl_2$ restores depressed endothelial cell function after hemorrhagic shock and resuscitation. *Am. J. Physiol.* **268,** H1390–H1396.

Watkins, S. C., Macaulay, W., Turner, D., Kang, R., Rubash, H. E., and Evans, C. H. (1997). Identification of inducible nitric oxide synthase in human macrophages surrounding loosened hip prostheses. *Am. J. Pathol.* **150,** 1199–1206.

Watts, B. P., Barnard, M., and Turrens, J. F. (1995). Peroxynitrite-dependent chemiluminescence of amino acids, proteins, and intact cells. *Arch. Biochem. Biophys.* **317,** 324–330.

Webb, D. J., Wen, J., Lysiak, J. J., Umans, L., Van Leuven, F., and Gonias, S. L. (1996). Murine α-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor-β and as inducers of nitric oxide synthesis. A possible mechanism for the endotoxin insensitivity of the α_2-macroglobulin gene knock-out mouse. *J. Biol. Chem.* **271,** 24982–24988.

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* **375,** 408–411.

Weinberg, J. B., Granger, D. L., Pisetsky, D. S., Seldin, M. F., Misukonis, M. A., Mason, S. N., Pippen, A. M., Ruiz, P., Wood, E. R., and Gilkeson, G. S. (1994). The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: Increased nitric oxide production and nitric oxide synthase expression in MRL-*lpr/lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J. Exp. Med.* **179,** 651–660.

Wheeler, M. A., Smith, S. D., Garcia-Cardena, G., Nathan, C. F., Weiss, R. M., and Sessa, W. C. (1997). Bacterial infection induces nitric oxide synthase in human neutrophils. *J. Clin. Invest.* **99,** 110–116.

Whittle, B. J. (1997). Nitric oxide—a mediator of inflammation or mucosal defence. *Eur. J. Gastroenterol. Hepatol.* **9,** 1026–1032.

Wildhirt, S. M., Dudek, R. R., Suzuki, H., and Bing, R. J. (1995). Involvement of inducible nitric oxide synthase in the inflammatory process of myocardial infarction. *Int. J. Cardiol.* **50,** 253–261.

Wildhirt, S. M., Suzuki, H., Wolf, W. P., Dudek, R., Horstman, D., Weismueller, S., and Reichart, B. (1996). *S*-Methylisothiourea inhibits inducible nitric oxide synthase and improves left ventricular performance after acute myocardial infarction. *Biochem. Biophys. Res. Commun.* **227,** 328–333.

Wildhirt, S. M., Suzuki, H., Horstman, D., Weismuller, S., Dudek, R. R., Akiyama, K., and Reichart, B. (1997). Selective modulation of inducible nitric oxide synthase isoenzyme in myocardial infarction. *Circulation* **96,** 1616–1623.

Wink, D. A., Feelisch, M., Fukuto, J., Chistodoulou, D., Jourd'heuil, D., Grisham, M. B., Vodovotz, Y., Cook, J. A., Krishna, M., DeGraff, W. G., Kim, S., Gamson, J., and Mitchell, J. B. (1998). The cytotoxicity of nitroxyl: Possible implications for the pathophysiological role of NO. *Arch. Biochem. Biophys.* **351,** 66–74.

Witthoft, T., Eckmann, L., Kim, J. M., and Kagnoff, M. F. (1998). Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. *Am. J. Physiol.* **275,** G564–G571.

Wizemann, T., Gardner, C., Laskin, J., Quinones, S., Durham, S., Goller, N., Ohnishi, T., and Laskin, D. (1994). Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J. Leukocyte Biol.* **56,** 759–768.

Wolin, M. S., Xie, Y. W., and Hintze, T. H. (1999). Nitric oxide as a regulator of tissue oxygen consumption. *Curr. Opin. Nephrol. Hypertens.* **8,** 97–103.

Wong, H. R., Finder, J. D., Wasserloos, K., and Pitt, B. R. (1995). Expression of iNOS in cultured rat pulmonary artery smooth muscle cells is inhibited by the heat shock response. *Am. J. Physiol.* **269,** L843–L848.

Wong, H. R., Ryan, M., and Wispe, J. R. (1997a). The heat shock response inhibits inducible nitric oxide synthase gene expression by blocking IκB degradation and NF-κB nuclear translocation. *Biochem. Biophys. Res. Commun.* **231,** 257–263.

Wong, H. R., Ryan, M., Menendez, I. Y., Denenberg, A., and Wispe, J. R. (1997b). Heat shock protein induction protects human respiratory epithelium against nitric oxide-mediated cytotoxicity. *Shock* **8,** 213–218.

Wu, C. C., Szabó, C., Chen, S. J., Thiemermann, C., and Vane, J. R. (1994). Activation of soluble guanylyl cyclase by a factor other than nitric oxide or carbon monoxide contributes to the vascular hyporeactivity to vasoconstrictor agents in the aorta of rats treated with endotoxin. *Biochem. Biophys. Res. Commun.* **201,** 436–442.

Wu, M. S., Yang, C. W., Bens, M., Yu, H. M., Huang, J. Y., Wu, C. H., Huang, C. C., and Vandewalle, A. (1998). Cyclosporin inhibits nitric oxide production in medullary ascending limb cultured cells. *Nephrol. Dial. Transplant.* **13,** 2814–2820.

Xia, Y., and Zweier, J. L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 6954–6958.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6770–6774.

Xie, Q., and Nathan, C. (1994). The high-output nitric oxide pathway: Role and regulation. *J. Leukocyte Biol.* **56,** 576–582.

Xie, Y. W., and Wolin, M. S. (1996). Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration. Involvement in response to hypoxia/reoxygenation. *Circulation* **94,** 2580–2586.

Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998). Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* **279,** 234–237.

Xu, Q., Hu, Y., Kleindienst, R., and Wick, G. (1997). Nitric oxide induces heat-shock protein 70 expression in vascular smooth muscle cells via activation of heat shock factor 1. *J. Clin. Invest.* **100,** 1089–1097.

Yan, Z. Q., Yokota, T., Zhang, W., and Hansson, G. K. (1996). Expression of inducible nitric oxide synthase inhibits platelet adhesion and restores blood flow in the injured artery. *Circ. Res.* **79,** 38–44.

Yermilow, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995). Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis* **16,** 2045–2050.

Yermilow, V., Yoshie, Y., Rubio, J., and Ohshima, H. (1996). Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. *FEBS Lett.* **399,** 67–70.

Yin, M. J., Yamamoto, Y., and Gaynor, R. B. (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature* **396,** 77–80.

Zielasek, J., Jung, S., Gold, R., Liew, F. Y., Toyka, K. V., and Hartung, H. P. (1995). Administration of nitric oxide synthase inhibitors in experimental autoimmune neuritis and experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **58,** 81–88.

Zingarelli, B., O'Connor, M., Wong, H., Salzman, A. L., and Szabó, C. (1996). Peroxynitrite-mediated DNA strand breakage activates poly-ADP ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipopolysaccharide. *J. Immunol.* **156,** 350–358.

Zingarelli, B., Cuzzocrea, S., Zsengeller, Z., Salzman, A. L., and Szabó, C. (1997a). Beneficial effect of inhibition of poly-ADP ribose synthetase activity in myocardial ischemia–reperfusion injury. *Cardiovasc. Res.* **36,** 205–215.

Zingarelli, B., Day, B. J., Crapo, J., Salzman, A. L., and Szabó, C. (1997b). The potential involvement of peroxynitrite in the pathogenesis of endotoxic shock. *Br. J. Pharmacol.* **120,** 259–267.

Zingarelli, B., Ischiropoulos, H., Salzman, A. L., and Szabó, C. (1998a). Improved vascular contractility and cellular energetics in the delayed phase of hemorrhagic shock by mercaptoethylguanidine, a novel inhibitor of the inducible isoform of nitric oxide synthase. *Eur. J. Pharmacol.* **338,** 5–65.

Zingarelli, B., Salzman, A. L., and Szabó, C. (1998b). Genetic disruption of poly (ADP ribose) synthetase inhibits the expression of P-selectin and intercellular adhesion molecule-1 in myocardial ischemia–reperfusion injury. *Circ. Res.* **83,** 85–94.

Zingarelli, B., Virág, L., Szabó, A., Cuzzocrea, S., Salzman, A. L., and Szabó, C. (1998c). Oxidation, nitration and cytostasis induction in the absence of inducible nitric oxide synthase. *Int. J. Mol. Med.* **1,** 787–795.

Zingarelli, B., Szabo, C., and Salzman, A. L. (1999). Blockade of poly(ADP-ribose) synthetase inhibits neutrophil recruitment, oxidant generation, and mucosal injury in murine colitis. *Gastroenterology* **116,** 335–345.

Zweier, J. L., Wang, P., Samouilov, A., and Kuppusamy, P. (1995). Enzyme-independent formation of nitric oxide in biological tissues. *Nat. Med.* **1,** 804–809.

CHAPTER 53

Nitric Oxide, Chronic Joint Inflammation, and Pain

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RHEUMATOID ARTHRITIS (RA) IS A CHRONIC SYSTEMIC DISEASE OF UNKNOWN ETIOLOGY CHARACTERIZED BY SYMMETRIC POLYARTICULAR INFLAMMATION OF SYNOVIA-LINED JOINTS, REMARKABLE JOINT SWELLING, DEFORMATION, AND PAIN. INDEED, CHRONIC PAIN IS ONE OF THE MOST SERIOUS SYMPTOMS ASSOCIATED WITH RA AND AFFECTS THE QUALITY OF LIFE OF RA PATIENTS. PROMINENT HISTOPATHOLOGICAL FEATURES OF THE CHRONICALLY INFLAMED SYNOVIA INCLUDE NEOVASCULARIZATION, HYPEREMIA, INFILTRATION OF LARGE NUMBERS OF LEUKOCYTES, AND BONE AND CARTILAGE DEGRADATION. COINCIDENT WITH THIS EXTENSIVE INFLAMMATORY INFILTRATE IS THE ENHANCED EXPRESSION OF THE INDUCIBLE ISOFORM OF NITRIC OXIDE SYNTHASE (iNOS) AND THE SUSTAINED OVERPRODUCTION OF THE FREE RADICAL NITRIC OXIDE (NO). BECAUSE NO IS KNOWN TO DIRECTLY OR INDIRECTLY MODULATE THE INFLAMMATORY RESPONSE AS WELL AS TO PLAY AN IMPORTANT ROLE IN PAIN PERCEPTION (HYPERALGESIA), THERE IS INCREASING INTEREST IN DEFINING THE ROLE THAT NO MAY PLAY IN THE PATHOGENESIS OF RA AND CHRONIC PAIN. THE OBJECTIVE OF THIS CHAPTER IS TO REVIEW SOME BASIC CONCEPTS OF NO BIOLOGY THAT MAY HELP IN UNDERSTANDING THE ROLE THAT NO MAY PLAY IN THE PATHOPHYSIOLOGY OF CHRONIC JOINT INFLAMMATION AND PAIN PERCEPTION.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic disease of unknown etiology characterized by symmetric polyarticular inflammation of synovia-lined joints and progressive loss of articular cartilage and subchondral bone (Firestein, 1998). Corresponding to these pathophysiological events is remarkable joint swelling, deformation, and pain. The healthy joint is normally encapsulated by a thin, four- to five-cell-thick synovial membrane (Fig. 1). Chronic inflammation of this membrane initiates a cascade of molecular and cellular events that culminate in hyperplasia of the synovial membrane, resulting in the formation of an invasive, tumor-like extension of the synovial membrane called pannus (Fig. 1). It is thought that pannus-derived hydrolytic enzymes (e.g., metalloproteinases) are responsible for the cartilage and bone degradation observed in RA (Firestein, 1998). Other studies have demonstrated that angiogenesis may be crucial for the development of pannus in RA (Koch, 1998).

Other prominent pathological features of the chronically inflamed synovia include neovascularization, hyperemia, and the infiltration of large numbers of leukocytes. Indeed, one of the hallmark features of RA is the infiltration of large numbers of mononuclear leukocytes (monocytes, plasma cells, and lymphocytes) into the inflamed synovia. Interestingly, extensive polymorphonuclear leukocyte (PMN) extravasation is observed in synovial fluid but not in inflamed synovial tissue. Because this inflammatory infiltrate is

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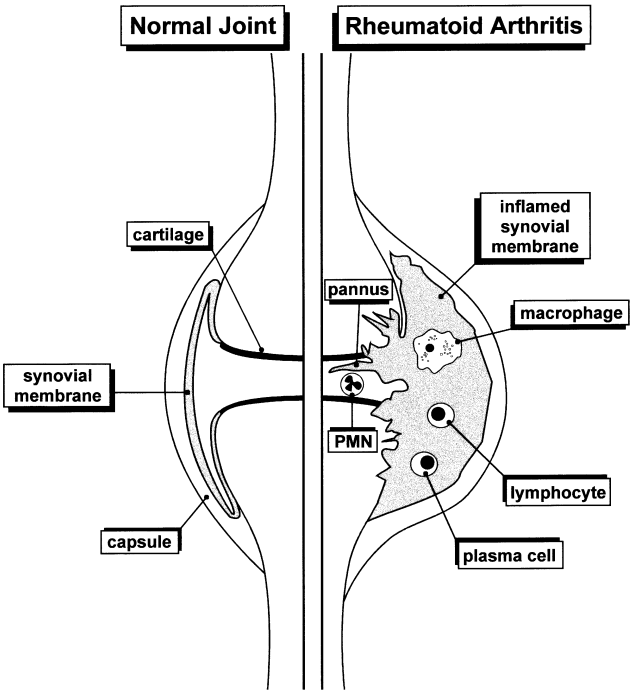


Figure 1 Schematic representation of the normal and chronically inflamed synovia.

accompanied by extensive articular damage including cartilage and bone erosion, edema, and enhanced vascular permeability, it has been suggested that these leukocytes play an important role in initiating and perpetuating the synovitis as well as cartilage and bone degradation observed in RA (Firestein, 1998; Koch, 1998).

Studies have demonstrated that the enhanced leukocyte infiltrate and some of the pathophysiology observed in different models of synovitis and in human RA may be mediated by the interaction between leukocytes and specific endothelial cell adhesion molecules (ECAMs) (Issekutz and Issekutz, 1995; Issekutz *et al.,* 1996, 1996; Kavanaugh *et al.,* 1994, 1996, 1997; Schimmer *et al.,* 1997). In addition to promoting adhesion and extravasation of potentially damaging leukocytes into the synovia, certain ECAMs such as P- and L-selectin have been shown to play an important role in mediating the recruitment of β-endorphin-containing, memory-type T lymphocytes into inflamed sites (Machelska *et al.,* 1998). Thus, these types of leukocyte–endothelial cell interactions may be important in controlling inflammatory pain. Coincident with this extensive inflammatory infiltrate is the enhanced expression of the inducible isoform of nitric oxide synthase (iNOS) and the sustained overproduction of the free radical nitric oxide (NO) (Clancy *et al.,* 1998; Cochran *et al.,* 1996; Evans *et al.,* 1995; Jang and Murrell, 1998; Stichtenoth and Frolich, 1998). Because NO is known to directly or indirectly modulate the inflammatory response as well as play an important role in pain perception (hyperalgesia), there is increasing interest in defining the role that NO may play in the pathogenesis of RA and chronic pain. This chapter will review some basic concepts of NO biology that may help in understanding the role that NO may play in the pathophysiology of chronic joint inflammation and pain perception.

Role of Cytokines in the Pathogenesis of Rheumatoid Arthritis

Although the etiology of RA remains undefined, it is thought that the initiation and pathogenesis of this disease is multifactorial, involving interactions among genetic, immune, and possibly infectious agents (Firestein, 1998; Weyand and Goronzy, 1997). Indeed, there is a growing body of experimental and clinical evidence to suggest an immune-mediated pathogenesis of RA.

The first step in the immune response to antigen is the uptake, processing, and presentation of antigen by macrophages and/or other antigen presenting cells (Fig. 2). Antigen recognition by T lymphocytes activates these cells to synthesize and release interleukin 2 (IL-2) and γ-interferon (IFN-γ). IL-2 promotes the clonal expansion of cytotoxic T cells and enhances the function of helper T cells and B cells, whereas IFN-γ interacts with and activates antigen presenting cells and macrophages to produce IL-12 (Trinchieri, 1998). IL-12 feeds back onto the T cells to enhance further production of IFN-γ. IFN-γ can then activate endothelial cells and enhance ECAM expression on endothelial cells. Interferon-activated macrophages produce large amounts of T-helper cell-1 (Th1)-type cytokines such as tumor necrosis factor α (TNF-α), IL-1, IL-6, IL-8, and IL-12 as well as reactive oxygen metabolites, all of which are thought to be important in initiating and/or promoting the inflammatory response via promotion of angiogenesis and pannus growth as well as recruitment and activation of PMNs and macrophages. Indeed, RA is thought to be associated with an imbalance in cytokine formation resulting in the overproduction of Th1-type or proinflammatory cytokines (Odeh, 1997; Schulze-Koops *et al.,* 1995) (Fig. 3).

The importance of cytokines as mediators of chronic joint inflammation can be seen in clinical studies where investigators have demonstrated that administration of IL-1 receptor antagonist or antibodies specific for Th1-derived cytokines such as TNF-α attenuate the chronic joint inflammation observed in different animal models of synovitis as well as human RA (Elliott *et al.,* 1994; Lebsack *et al.,* 1991). In addition, Th1-type cytokines are also known to upregulate the expression of iNOS, resulting in enhanced production of NO (Clancy *et al.,* 1998; Cochran *et al.,* 1996; Evans *et al.,* 1995; Jang and Murrell, 1998; Stichtenoth and Frolich, 1998). It is interesting to note that some of the dramatic anti-inflammatory activity demonstrated in clinical studies using the anti-TNF monoclonal antibody (cA2) is associated with a reduction in expression of iNOS in patients with RA (Perkins *et al.,* 1998). The role that iNOS-derived NO plays in modulating joint inflammation is currently under active investigation, the results of which are not entirely clear. An

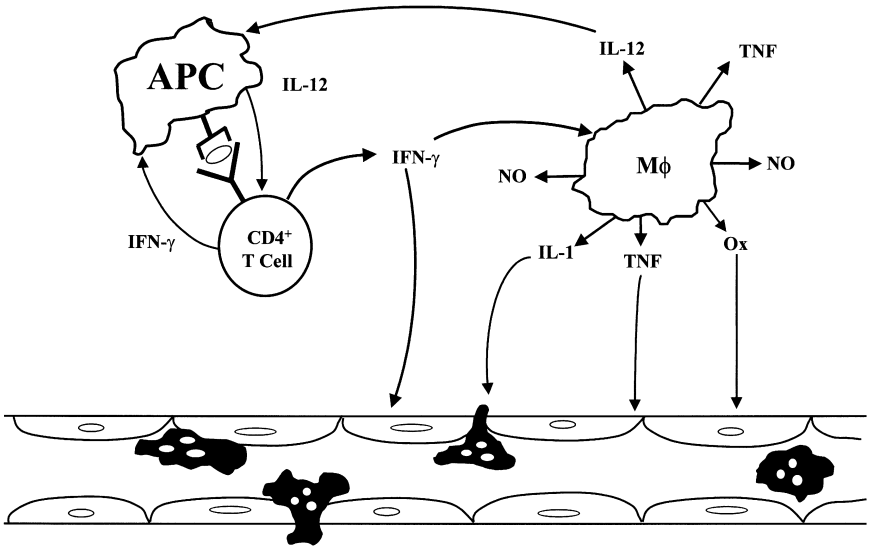


Figure 2 Interaction between antigen presenting cell (APC) and $CD4^+$ T lymphocytes to produce cytokines. Ox represents reactive oxygen species such as superoxide and hydrogen peroxide, and Mϕ stands for macrophage.

understanding of some of the basic concepts involved in regulation of the inflammatory response may prove useful in ultimately defining the role of NO in the pathophysiology of RA.

Regulation of Chronic Joint Inflammation

It has been appreciated for some time that chronic joint inflammation is associated with increased production of reactive oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and possibly hydroxyl radical (HO·) (Biemond *et al.,* 1984; Blake *et al.,* 1990; Chapman *et al.,* 1989; Clancy *et al.,* 1998; Cochran *et al.,* 1996; Dabbagh *et al.,* 1991; Edwards *et al.,* 1988; Evans *et al.,* 1995; Halliwell, 1995; Jang and Murrell, 1998; Shingu *et al.,* 1994; Skaleric *et al.,* 1991; Stichtenoth and Frolich, 1998). Historically, it has been thought that these oxidants and free radicals promote synovitis as well as bone and cartilage damage directly via their ability to degrade important cellular constituents and biopolymers such as hyaluronic acid proteoglycans and collagen (Halliwell, 1995).

More recent data suggests that reactive oxygen species may also initiate and/or perpetuate chronic joint inflammation by activating the transcription of a variety of different

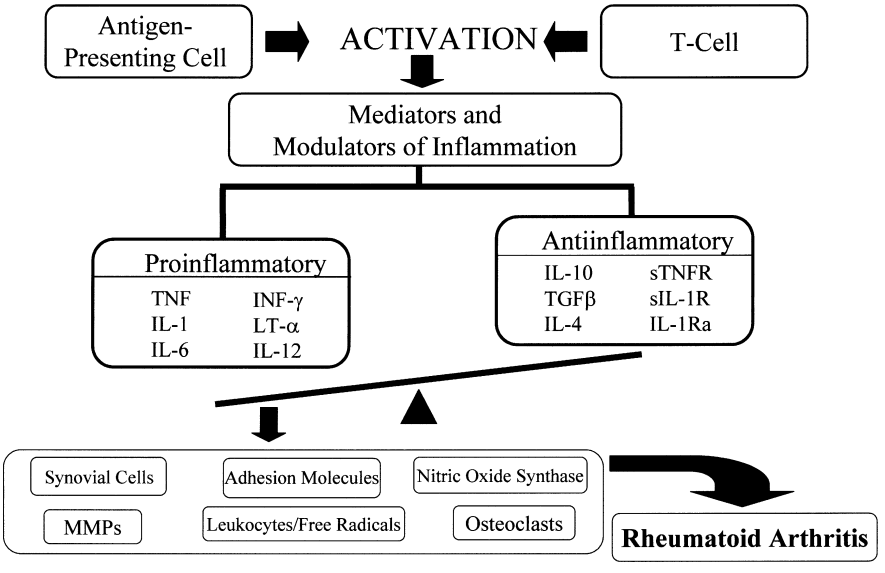


Figure 3 Proposed cytokine imbalance produced by immune cell activation in rheumatoid arthritis. MMPs represent matrix metalloproteinases, whereas sTNFR, sIL-1R, and IL-1Ra represent soluble TNF receptor, soluble IL-1 receptor, and IL-1 receptor antagonist, respectively.

genes known to be important in the inflammatory response. For example, certain reactive oxygen species are known to activate specific transcription factors such as nuclear transcription factor κ B (NF-κB). It is well appreciated that Th1-derived cytokines such as TNF-α, TGF-β (lymphotoxin-α), and IFN-γ either alone or in combination promote leukocyte adhesion *in vitro* and *in vivo* as well as upregulate the expression of iNOS (Baeuerle and Henkle, 1994; Xie *et al.,* 1994). The mechanisms by which this diverse group of proinflammatory agents promote leukocyte adhesion and enhance NO production *in vivo* are not clear; however, recent *in vitro* data suggest that cytokine–receptor interaction may activate NF-κB. This heterodimeric protein is a ubiquitous transcription factor and pleiotropic regulator of numerous inflammatory and immune responses. Once activated, NF-κB translocates to the nucleus of the cell where it binds to its consensus sequence on the promoter–enhancer region of different genes, thereby activating the transcription of genes known to be important in the immune and inflammatory responses (Baeuerle and Henkle, 1994). For example, NF-κB appears to regulate the transcription of a variety of different cytokines (e.g., IL-1, IL-2, TNF, IL-6, IL-8), certain ECAMs such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), as well as iNOS (Baeuerle and Henkle, 1994; Collins *et al.,* 1995; Palombella *et al.,* 1998; Read *et al.,* 1995; Xie *et al.,* 1994). Thus, NF-κB may regulate inflammation directly or indirectly via its ability to activate the transcription of inflammatory cytokines.

NF-κB belongs to the Rel family of transcription factors (Baeuerle and Henkle, 1994), whose members share a region of about 300 amino acids known as the Rel homology domain. The heterodimeric NF-κB is composed of p50 and p65 subunits and is normally sequestered in the cytoplasm in association with its inhibitor IκB (Baeuerle and Baltimore, 1995). A large number of different bacterial and viral products, cytokines, and lipid mediators activate NF-κB (Baeuerle and Henkle, 1994). It is unlikely that each of these stimuli activates the cytoplasmic NF-κB–IκB complex via completely different pathways. Indeed, there is a growing body of experimental data to suggest that many, if not all, of these stimuli activate multiple signaling pathways that converge to enhance reactive oxygen metabolism within the cell (Fig. 4) (Schmidt *et al.,* 1995; Schreck *et al.,* 1991, 1992a,b; Sen and Packer, 1996). This has been shown for the NF-κB activators TNF, IL-1, lipopolysaccharide, phorbol esters, UV light, γ radiation, anti-immunoglobulin M (IgM), okadaic

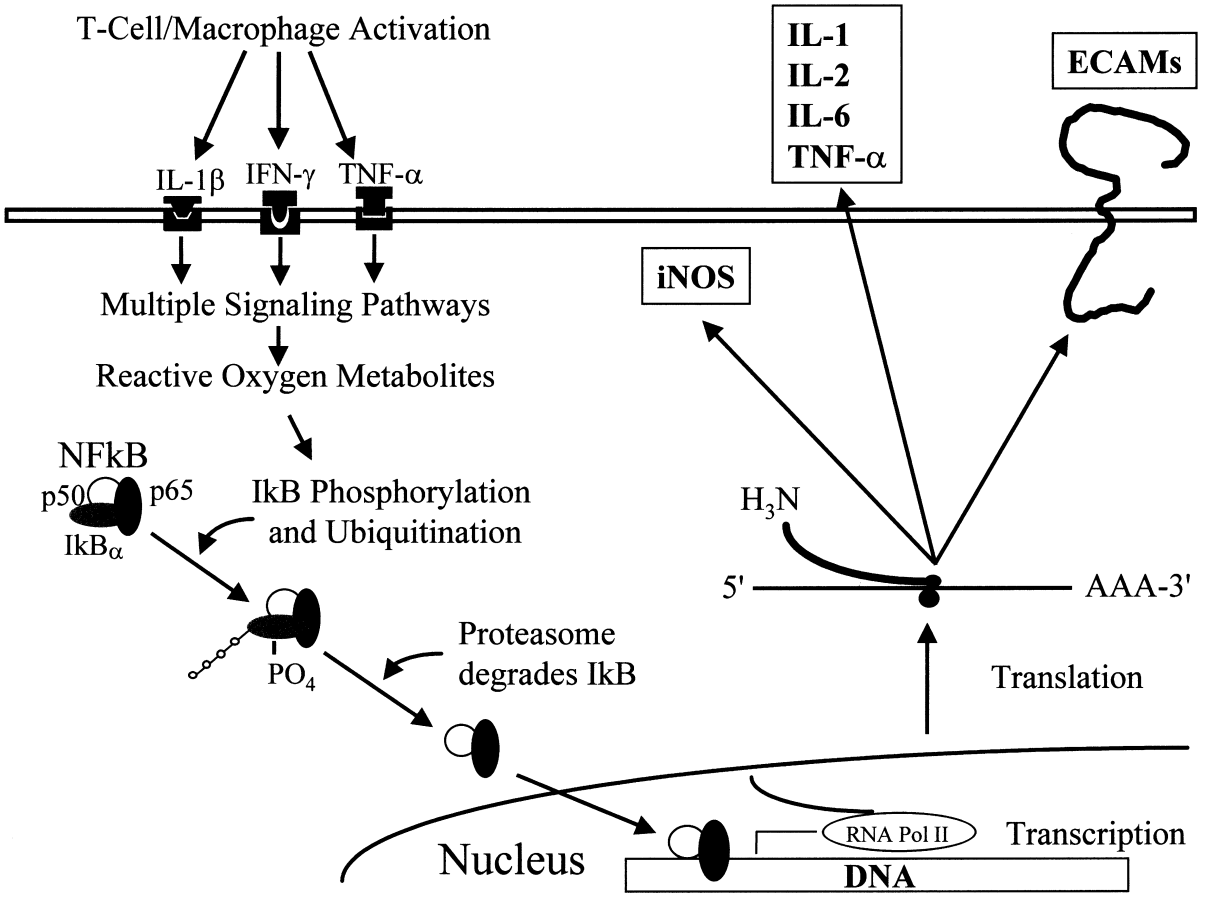


Figure 4 Role of cytokines, reactive oxygen species, and the 26S proteasome in the activation and translocation of nuclear factor κB (NF-κB).

acid, and anti-CD28. Further support for this concept is the recognition that certain lipophilic, membrane-permeable oxidants, such as H_2O_2 and oxidant-producing xenobiotics (e.g., menendione), active NF-κB as well (Sen and Packer, 1996).

The specific intracellular source(s) for this enhanced oxidative metabolism has not been identified, but prostaglandin synthase, xanthine oxidase, mitochondria, NADPH oxidase, and cytochrome P-450 are likely candidates (Munroe *et al.,* 1995; Schulze-Osthoff *et al.,* 1993; Suzuki *et al.,* 1992; Weber *et al.,* 1994). Sources of exogenous oxidants *in vivo* that could activate NF-κB include activated phagocytic leukocytes (e.g., PMNs, monocytes, macrophages, eosinophils). Furthermore, NF-κB activation has been shown to be inhibited *in vitro* by a wide variety of structurally diverse enzymatic or nonenzymatic antioxidants or free radical scavengers such as superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase, *N*-acetylcysteine, vitamin E derivatives, α-lipoic acid, and certain dithiocarbamates (reviewed in Schmidt *et al.,* 1995; Schreck *et al.,* 1991, 1992a,b; Sen and Packer, 1996). Furthermore, two studies have demonstrated that certain antioxidants inhibit NF-κB *in vivo* and protect the lung from inflammatory tissue injury (Blackwell *et al.,* 1996; Ye and Malik, 1997). Indeed, it is intriguing to speculate that the observed protective effects of antioxidants in various models of RA (Shingu *et al.,* 1994; Skaleric *et al.,* 1991) may be due more to inhibition of NF-κB activation than inhibition of oxidant-induced toxicity. One study demonstrated activation and nuclear translocation of NF-κB in synovia obtained from patients with RA (Handel *et al.,* 1995).

The mechanisms by which oxidants activate NF-κB have not been defined. This intracellular oxidative stress is thought to then activate, via several intermediate reactions, one or more redox-sensitive kinases that specifically phosphorylate IκB (Mercurio *et al.,* 1997; Woronicz *et al.,* 1997) (Fig. 4). Once phosphorylated, IκB is selectively ubiquinated and then degraded via the nonlysosomal, ATP-dependent 26S proteolytic complex (Goldberg, 1995; Palombella *et al.,* 1994). Thus, the 26S proteasome represents an important step in the activation of NF-κB (Fig. 4).

Because the proteolytic degradation of the posttranslationally modified IκB is known to be mediated by the 26S proteasome complex, the therapeutic anti-inflammatory activity of a selective proteasome inhibitor in a model of chronic polyarthritis was assessed (Palombella *et al.,* 1998). Chronic polyarthritis with granulomatous liver inflammation was induced in female Lewis rats via the intraperitoneal injection of peptidoglycan polysaccharide (PG/PS). Twenty-one rats were randomized into three groups consisting of a saline-injected control group, a PG/PS arthritic group given vehicle (methylcellulose) orally (p.o.) daily beginning 7 days following the induction of arthritis, and a PG/PS arthritic group given 0.3 mg kg^{-1} day^{-1} PS-341 (proteasome inhibitor; $K_i = 0.6$ n*M*) p.o. daily beginning 7 days following the induction of arthritis and continuing daily for an additional 3 weeks. Arthritic symptoms were monitored throughout the course of the study and were quantified using the total arthritis index score and measurement of paw volume. PS-341 was found to attenuate polyarthritis induced by the intraperitoneal injection of PG/PS as assessed by significant reductions in the total arthritis and average hind paw volume (Palombella *et al.,* 1998). Histologically, drug treatment attenuated the cellular infiltration, synovial thickening, and pannus formation as well as the bone and cartilage erosion typical of PG/PS-induced arthritis. This inhibition of polyarthritis correlated with significant reductions in plasma levels of nitrate and nitrite and IL-6 compared to vehicle-treated controls. The gross liver inflammation score was also attenuated by drug treatment. Furthermore, PG/PS-induced upregulation of liver inducible nitric oxide synthase and VCAM-1 expression were significantly attenuated by therapeutic treatment with PS-341 (Palombella *et al.,* 1998). It was concluded that the 26S proteasome and thus NF-κB play important roles in regulating the acute and chronic inflammation responses *in vivo.* These data also suggest that the antiadhesive and anti-inflammatory properties of antioxidants, free radical scavengers, and proteasome inhibitors may be due to their abilities to inhibit the activation of NF-κB and the subsequent upregulation of ECAM expression on the endothelium.

Although it is tempting to conclude that inhibition of NF-κB activation attenuates joint inflammation via the pathways described, it should be remembered that chronic NF-κB inhibition also results in apoptosis (see Grisham, 1999, and references therein). Some of the anti-inflammatory activity observed using PS-341 may in fact be due to selective apoptosis of microvascular endothelial cells and/or pannus.

Role of Nitric Oxide as an Endogenous Modulator of Joint Inflammation

One of the most consistent findings in experimental or human RA is the large and significant overproduction of NO in the inflamed joint (Clancy *et al.,* 1998; Cochran *et al.,* 1996; Evans *et al.,* 1995; Issekutz *et al.,* 1996; Issekutz and Issekutz, 1995; Jang and Murrell, 1998; Kavanaugh *et al.,* 1994, 1996, 1997; Koch, 1998; Schimmer *et al.,* 1997; Stichtenoth and Frolich, 1998). From a physiological perspective, NO is a particularly interesting bimolecular species in that it possesses both anti-inflammatory as well as proinflammatory properties (Table I). For example, exogenous NO donor agents have been shown to attenuate the adhesion and recruitment of leukocytes in postcapillary venules exposed to a variety of different acute inflammatory stimuli (Gaboury *et al.,* 1993; Kurose *et al.,* 1994; Liao and Granger, 1995).

One explanation that has been offered to explain the antiadhesion properties of NO relates to the ability of this molecule to rapidly interact with and decompose O_2^- (Gaboury *et al.,* 1993). Since O_2^- reacts with NO three times faster than with superoxide dismutase (Huie and Padmaja, 1993), it has been proposed that NO may act as a physiological scavenger of O_2^-. However, depending on the relative fluxes

Table I Modulatory Role of Nitric Oxide in Chronic Joint Inflammation[a]

Anti-inflammatory properties	Proinflammatory properties
Inhibits leukocyte–endothelial cell adhesion and extravasation	Acts as vasodilator (increases blood flow)
Inhibits metal-catalyzed oxidative reactions	Enhances vascular permeability
Inhibits ECAM expression	Induces TNF-α synthesis
Inhibits lymphocyte proliferation	Stimulates metalloproteinases
Inhibits platelet aggregation	Inhibits proteoglycan and collagen synthesis
	Inhibits actin polymerization and integrin signaling
	Induces chrondrocyte apoptosis
	Downregulates IL-1 receptor antagonist receptor expression
	Increases susceptibility of cells to oxidant injury
	Inhibits mitochondrial respiration
	Inhibits DNA synthesis
	Increases bone resorption

[a] Derived from Clancy *et al.* (1998), Cochran *et al.* (1996), Evans *et al.* (1995), and Jang and Murrell (1998), Koch (1998), and Stichtenoth and Frolich (1998).

of each radical, NO may inhibit or enhance oxidant production via the degradation or synthesis of peroxynitrite ($ONOO^-$) (Grisham *et al.,* 1999). This possibility is supported by studies demonstrating that NO donor compounds are only effective in inhibiting leukocyte–endothelial cell adhesion in models of inflammation in which SOD is also antiadhesive (Gaboury *et al.,* 1993; Kurose *et al.,* 1994).

A second mechanism by which NO may attenuate leukocyte adhesion during the acute inflammatory response involves modulation of ECAM expression. It has been shown that NO donor compounds prevent the mobilization of preformed P-selectin that is observed within 30 min after reperfusion of the ischemic intestine (Gauthier *et al.,* 1994). Furthermore, it has been shown that exogenous NO donor compounds induce the expression of and/or stabilize IκB, thereby maintaining NF-κB in its inactive form bound to IκB (DeCaterina *et al.,* 1995; Khan *et al.,* 1996; Peng *et al.,* 1995). Although this mechanism has been proposed to account for the ability of NO to attenuate surface expression of certain ECAMs on human endothelial cells *in vitro,* we have been unable to demonstrate this anti-inflammatory effect of NO on ECAM expression in models of chronic inflammation *in vivo* where both ECAMs and iNOS are coexpressed (Kawachi *et al.,* 1999).

Although several studies have demonstrated potent antiadhesive activity of NO *in vitro* and in acute inflammation *in vivo,* the role of NO in chronic joint inflammation is far from clear-cut. In fact, numerous studies have indicated that chronic joint inflammation may be attenuated by administration of a variety of NOS inhibitors, indicating that NO may directly or indirectly promote rather than inhibit chronic inflammation (Conner *et al.,* 1995; Fletcher *et al.,* 1998; Gilkeson *et al.,* 1997; Ialenti *et al.,* 1993; Lawand *et al.,* 1997; McCartney-Francis *et al.,* 1993; McInnes *et al.,* 1998; Oyanagui *et al.,* 1994; Pozza *et al.,* 1998; Sakiniene *et al.,* 1997; Salvemini *et al.,* 1996; Santos *et al.,* 1997; Stefanovic-Racic *et al.,* 1994, 1995; Van de Loo *et al.,* 1998; Verissimo de Mello *et al.,* 1997; Weinberg *et al.,* 1994) (Table II). The mechanisms by which the sustained overproduction of iNOS-derived NO promotes chronic joint inflammation, or more precisely the mechanisms by which NOS inhibitors attenuate experimental RA, have not been clearly delineated; however, there are several possibilities (Table II). For example, chondrocyte, macrophage, and synovial fibroblasts are thought to produce large amounts of NO within the inflamed synovia (Clancy *et al.,* 1998; Cochran *et al.,* 1996; Evans *et al.,* 1995; Issekutz *et al.,* 1996; Issekutz and Issekutz, 1995; Jang and Murrell, 1998; Kavanaugh *et al.,* 1994, 1996, 1997; Koch, 1998; Schimmer *et al.,* 1997; Stichtenoth and Frolich, 1998).

The sustained overproduction of NO could contribute to the pathophysiology of RA by several mechanisms. First, NO has been implicated in the enhanced microvascular permeability induced by IL-2 (Clancy *et al.,* 1998; Cochran *et al.,* 1996; Evans *et al.,* 1995; Jang and Murrell, 1998; Koch, 1998; Stichtenoth and Frolich, 1998). Increases in vascular permeability could account for the edema and swelling observed during acute flares of RA. Second, a substantial number of studies have demonstrated some rather dramatic effects of NO on chondrocyte function. For example, enhanced production of NO has been shown to (a) stimulate metalloproteinase activity, (b) inhibit proteoglycan and collagen synthesis, (c) inhibit actin polymerization and β1 integrin signaling, (d) enhance susceptibility to reactive oxygen-induced cytotoxicity, (e) decrease expression of IL-1 receptor antagonist, and (f) induce apoptosis (Clancy *et al.,* 1998; Cochran *et al.,* 1996; Evans *et al.,* 1995; Jang and Murrell, 1998; Koch, 1998; Stichtenoth and Frolich, 1998). In addition, NO may enhance bone resorption and inhibit osteoblast function (Clancy *et al.,* 1998; Stichtenoth and Frolich, 1998). Another potentially important mechanism by which iNOS-derived NO may promote RA is via its ability

Table II Effects of Nitric Oxide Synthase Inhibitors on Different Animal Models of Arthritis

Model species[a]	Inhibitor[b]	Route of administration	Effect[c]	Reference
SCW (rat)	L-NMMA	i.v. (prophylactic)	↓	McCartney-Francis *et al.* (1993)
Adjuvant (rat)	L-NAME	i.p. (prophylactic)	↓	Oyanagui *et al.* (1994)
	L-NMMA	i.p. (prophylactic)	↓	Oyanagui *et al.* (1994)
	L-NMMA	p.o. (prophylactic)	↓	Stefanovic-Racic *et al.* (1994)
	L-NIL	p.o. (prophylactic)	↓	Conner *et al.* (1995)
	L-NMMA	p.o. (prophylactic)	↓	Stefanovic-Racic *et al.* (1995)
	L-NMMA	p.o. (therapeutic)	−↓	Stefanovic-Racic *et al.* (1995)
	AG	p.o. (therapeutic)	−	Stefanovic-Racic *et al.* (1995)
	AG	p.o. (therapeutic)	−	Stefanovic-Racic *et al.* (1995)
	L-NIO	i.p. (therapeutic)	↓	Santos *et al.* (1997)
	AG	p.o. (therapeutic)	−	Pozza *et al.* (1998)
	7-NI	i.p. (therapeutic)	↓	Pozza *et al.* (1998)
	L-NIL	p.o. (prophylactic)	↓	Connor *et al.* (1995)
	L-NAME	p.o. (prophylactic)	−	Tanaka *et al.* (1998)
	AG	subQ (prophylactic)	↓	Tanaka *et al.* (1998)
Carrageenan (rat)	L-NAME	i.v. (prophylactic)	↓	Salvemini *et al.* (1996)
	L-NMMA	i.v. (prophylactic)	↓	Salvemini *et al.* (1996)
	L-NIL	i.v. (prophylactic)	↓	Salvemini *et al.* (1996)
	AG	i.v. (prophylactic)	↓	Salvemini *et al.* (1996)
	L-NIL	i.v. (therapeutic)	↓	Salvemini *et al.* (1996)
	AG	i.v. (therapeutic)	↓	Salvemini *et al.* (1996)
	L-NAME	i.a. (prophylactic)	↓	Lawand *et al.* (1997)
	L-NI	i.a. (prophylactic)	−	Lawand *et al.* (1997)
AIA (rabbit)	L-NAME	p.o. (prophylactic)	↓	Verissimo de Mello *et al.* (1997)
MRL-*lpr/lpr* (mouse)	L-NMMA	p.o. (prophylactic?)	↓	Weinberg *et al.* (1994)
	iNOS$^{-/-}$	—	−	Gilkeson *et al.* (1997)
Bacterial (mouse)	L-NAME	i.v. (prophylactic)	↑	Sakiniene *et al.* (1997)
	L-NMMA	i.v. (prophylactic)	↑	Sakiniene *et al.* (1997)
	iNOS$^{-/-}$	—	↑	Van de Loo *et al.* (1998)
Zymosan (mouse)	iNOS$^{-/-}$	—	↑↓	McInnes *et al.* (1998)

[a] Streptococcal cell wall-induced (SCW), autoimmune disease (MRL-*lpr/lpr*), antigen-induced arthritis (AIA), and *Staphylococcus aureus*-induced arthritis (bacterial).

[b] N^G-monomethyl-L-NMMA), N^ω-ntro-L-arginine methyl ester (L-NAME), *N*-iminoethyl-L-lysine (L-NIL), aminoguandine (AG), *N*-iminoethyl-L-omithine (L-NIO), 7-nitroimidazole (7-NI), and inducible nitric oxide synthase-deficient mice (iNOS$^{-/-}$).

[c] Decreased inflammation (↓), no effect (−), or increased inflammation (↑).

to modulate cyclooxygenase 2 (COX-2)-dependent production of prostaglandins (Clancy *et al.,* 1998; Jang and Murrell, 1998; Stichtenoth and Frolich, 1998). Other mechanisms include NO-dependent promotion of chemotaxis of certain leukocytes *in vitro* (Beauvais *et al.,* 1995; Kaplan *et al.,* 1989).

Nitric oxide or NO-derived metabolites may also promote leukocyte infiltration in an indirect manner by enhancing the production of proinflammatory mediators such as IL-8 or TNF (Lander *et al.,* 1996; Villarete and Remick, 1995). Lander and co-workers have demonstrated that NO or one of its auto-oxidation products activates lymphocytes to produce TNF (Lander *et al.,* 1996). Furthermore, NO may rapidly interact with certain reactive oxygen species such as O_2^- to yield the potent oxidant $ONOO^-$ (Grisham *et al.,* 1999). Because of the large influx of phagocytic leukocytes such as PMNs, monocytes, and macrophages, it is not unreasonable to suggest that iNOS-derived NO could promote chronic gut inflammation by reacting with leukocyte-derived O_2^- to form $ONOO^-$, which in turn upregulates cytokine and/or ECAM expression and injures the tissue.

One observation that has proved particularly troublesome when trying to delineate the ''anti-inflammatory'' mechanisms observed with certain NOS inhibitors is the inability

(or relative lack of efficacy) of certain NOS inhibitors when administered therapeutically, that is, when administered to animals with established disease (Table II). Two important concepts may help in clarifying this apparent paradoxical observation. First, NO is a potent vasodilator that enhances blood flow to most tissues (Ignarro, 1989; Radomski and Moncada, 1993). By and large most NOS inhibitors, especially those that inhibit the endothelial NOS (eNOS), will promote vasoconstriction and thus may remarkably reduce blood flow to a variety of different tissues (Conner *et al.,* 1999). Although selective iNOS inhibitors would not be expected to promote vasoconstriction via inhibition of eNOS, they could still theoretically reduce the hyperemia mediated by elevated levels of iNOS-derived NO. Because the delivery of leukocytes and inflammatory mediators to the inflamed synovia is blood flow dependent, a reduction in blood flow to the pannus via inhibition of NO production would be expected to inhibit the development of joint inflammation when the NOS inhibitor is administered prophylactically but not therapeutically (Fig. 5).

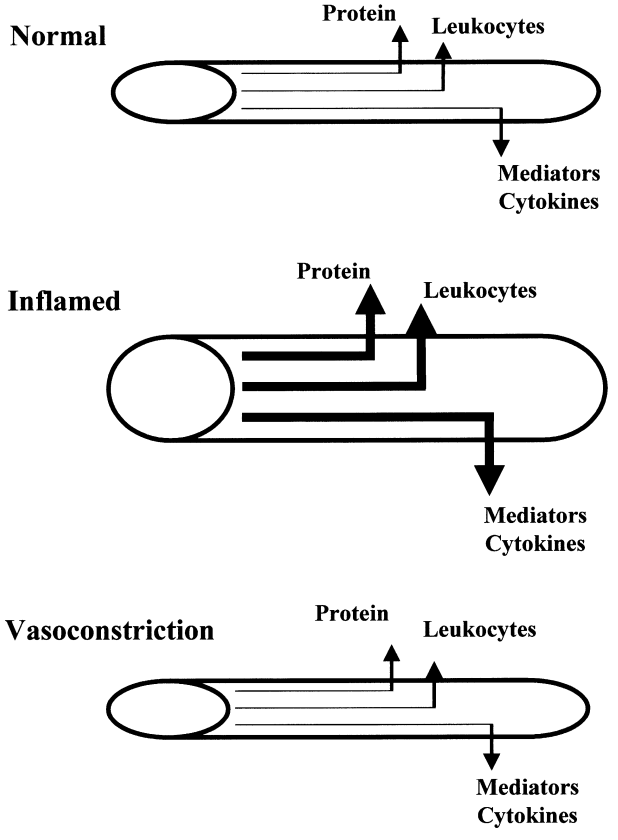


Figure 5 Role of blood flow in inflammation. Inflammation produces vasoactive mediators such as prostaglandins and NO, both of which will enhance blood flow and the delivery of leukocytes, mediators, and cytokines to the affected tissue. NOS inhibitors may attenuate this NO-dependent hyperemia, thereby limiting the delivery of leukocytes, cytokines, and mediators to the inflamed tissue and attenuating inflammation.

In addition to its vasoactive properties, NO is also thought to be an important mediator of angiogenesis, that is, it has been shown to promote blood vessel growth (Montrucchio *et al.,* 1997; Papapetropoulos *et al.,* 1997). If this is true, then NOS inhibitors may inhibit the development of pannus and thus attenuate joint inflammation when administered prophylactically but not therapeutically. Indeed, Cheresh and co-workers have demonstrated, using selective inhibitors of angiogenesis, significant anti-inflammatory activity in an experimental model of arthritis in rabbits (Storgard *et al.,* 1999). Furthermore, our data suggest that the selective proteasome inhibitor PS-341 may attenuate polyarthritis via a similar mechanism (Palombella *et al.,* 1998). Taken together, the literature demonstrates that NO possesses both anti-inflammatory as well as proinflammatory properties. The role that NO plays in the pathophysiology of chronic joint inflammation remains the subject of active debate.

Role of Nitric Oxide in Pain and Pain Perception

Chronic pain is one of the most serious symptoms associated with RA, and it can affect the quality of life of RA patients. Because chronic joint pain is difficult to treat, an understanding of the pathophysiological mechanisms that underlie pain is essential in order to design new therapeutic strategies to treat this debilitating disease.

Pain and Pain Pathways

Pain is defined by the International Association for the Study of Pain as ''an unpleasant sensory and emotional experience arising from actual or potential tissue damage or described in terms of such damage.'' This broad definition includes pain that arises from acute injury as well as pain that occurs chronically as a result of ongoing pathophysiological processes. A key component of the definition is the emotional experience, because pain is always a perceived event and because individuals often respond differently to the same aversive stimulus, depending on their emotional state (Stimmel, 1997). The experience of pain is important to the survival of the individual because anticipation of pain produces aversion to a potentially harmful stimulus, and the pain experience prompts the individual to seek pain alleviation to enhance healing.

Tissue injury or pathophysiological processes such as inflammation activate peripheral nerve endings known as nociceptors that are responsive to noxious thermal, mechanical, or chemical stimuli. Thus, response to a stimulus that activates nociceptors is referred to as nociception in studies that utilize animal models, while humans can describe the actual experience of perceived pain. Untreated, persistent nociceptive pain continuously activates nociceptors and can lead to development of hyperalgesia and allodynia. Hyperalgesia is defined as enhanced pain to a noxious stimulus, whereas allodynia is described as pain produced by stimuli that are normally nonnoxious. Two types of hyperalgesia, primary

and secondary, have been described (Balter, 1992). Enhanced pain at the site of injury or inflammation is referred to as primary hyperalgesia. Secondary hyperalgesia occurs when enhanced pain is perceived after stimulation of sites away from the site of injury or inflammation.

Activation of peripheral nociceptors by noxious stimuli activates bipolar sensory afferent nerves with cell bodies in the dorsal root ganglia (DRG) (Fig. 6) and terminals in both the periphery and the central nervous system (CNS) (Bonica, 1990). The axons of these nerves are either nonmyleinated, slow (1 m/s) conducting C fibers or myleinated, fast (15 m/s) conducting Aδ fibers. These nerves terminate in superficial layers of the dorsal spinal cord. Afferent input is integrated and modulated within the spinal cord through release of various mediators (see later). Nociceptive signals are then conducted to the brain through the spinothalamic tract to the thalamus and from the thalamus to areas of the cortex. The perception of pain likely occurs within the thalamus and surrounding areas (Fig. 6). Other spinal ascending systems, such as the spinoreticulothalamic tract, also contribute to overall pain transmission. Sensing of pain by the brain activates descending pain inhibitory control systems through corticospinal and reticulospinal tracts (Fig. 6). Signal integration occurs at several levels in the midbrain and medulla, in areas such as the periaqueductal gray (PAG) and the reticular formation. The hypothalamus and brain stem nuclei such as the locus coeruleus send input to the PAG, which provides descending input to the dorsal horn. These pathways terminate in the spinal cord to modulate the incoming nociceptive signals.

A number of substances are released in the periphery and in the spinal cord in response to nociceptive stimulation. Tissue injury and inflammation stimulate release of mediators such as histamine, prostaglandins (PGs), substance P (SP), bradykinin (BK), and leukotrienes that activate nociceptors. Stimulated nociceptors activate Aδ and C fibers to stimulate the release in the spinal dorsal horn of peptides such as SP, neurokinin A, somatostatin, galanin, and calcitonin gene-related peptide (CGRP) as well as nonpeptide excitatory amino acids (EAAs, such as glutamate and aspartate), serotonin (5-HT), and PGs, among others (Coderre *et al.,* 1993; Wilcox, 1991). These mediators activate receptors in the dorsal horn to stimulate the ascending pain pathways to the brain. Perception of pain by the brain activates the descending pain inhibition pathways to release mediators in regions such as the PAG and in the spinal dorsal horn. These include opioid peptides, 5-HT, norepinephrine (NE), and adenosine as well as others. Release of these mediators alleviates pain, possibly by attenuating the spinal release of nociceptive mediators. Since the late 1980s, it has become evident that NO also has an important role in pain pathways.

The pain that follows actual nerve damage is referred to as neuropathic pain. This type of pain is characterized by causalgia (burning sensation), feelings of pin pricks, numbness, and tingling sensations (paresthesias) as well as hyperalgesia and allodynia. Neuropathic pain is reported after stroke (central pain), after limb amputation (phantom limb pain), after deafferentiation, during disease states such as diabetes and AIDS, and with other types of nerve injury. The mediators responsible for neuropathic pain are not well characterized. However, NE and 5-HT may be involved, since antidepressant drugs that inhibit reuptake of these neurotransmitters can alleviate some types of neuropathic pain. This type of pain is particularly difficult to treat because it is resistant to conventional agents such as nonsteroidal antiinflammatory drugs (NSAIDS) and opioids that are useful in nociceptive pain.

Development of hyperalgesia and allodynia following chronic pain is postulated to depend on sensitization of peripheral receptors as well as central sensitization (Coderre *et al.,* 1993). A key site of central sensitization is the spinal cord, considering that sensitization of spinal dorsal horn neurons is coincident with spreading areas of hyperalgesia (secondary hyperalgesia). Other CNS regions such as the thalamus and somatosensory cortex also show sensitized neuronal responses. The mechanism for central sensitization is not completely understood, but it is postulated to involve both neuropeptides and EAAs acting thorough second messenger systems and increases in intracellular calcium. Because increased intracellular calcium stimulates NOS to increase formation of NO, a role for NO may also be found in central sensitization. Increased expression of proto-oncogenes, particularly c-Fos, and transcriptional regulation of peptides such as dynorphin may also contribute to the hyperalgesic response.

Methods Used in the Study of Pain

Because pain is perceived, the most appropriate model for understanding mechanisms of pain transmission is the human. However, there are important caveats in human studies. One consideration is the individual variability of responses to the same noxious stimulus, as mentioned earlier. Owing to the underlying emotional component of pain, there is not a simple relationship between perceived pain intensity and noxious stimulus intensity. Also, many methods utilized for evaluating pain are not particularly objective. In spite of these factors, numerous devices such as visual analog scales, questionnaires, pictures of facial expressions, or other types of subjective pain grading methods have been used with reasonable success in clinical situations (Chapman and Syrjala, 1990; McGrath, 1989). Nonsubjective tests include electrodiagnostic evaluations of nerves and muscles (Stolov, 1990) and thermography (LeRoy and Filask, 1990). Investigations of experimentally induced pain in humans are also problematic. Studies measuring pain threshold (ability to discriminate when a stimulus becomes painful) and pain tolerance (unwillingness to receive more intense stimuli) in normal volunteers showed that although threshold and tolerance are related, they are different indices of pain perception (Harris and Rollman, 1983). The evaluation of experimentally induced pain should include measurements of both threshold and tolerance. Also, patients suffering pathophysiological pain have higher tolerance levels for experimentally induced

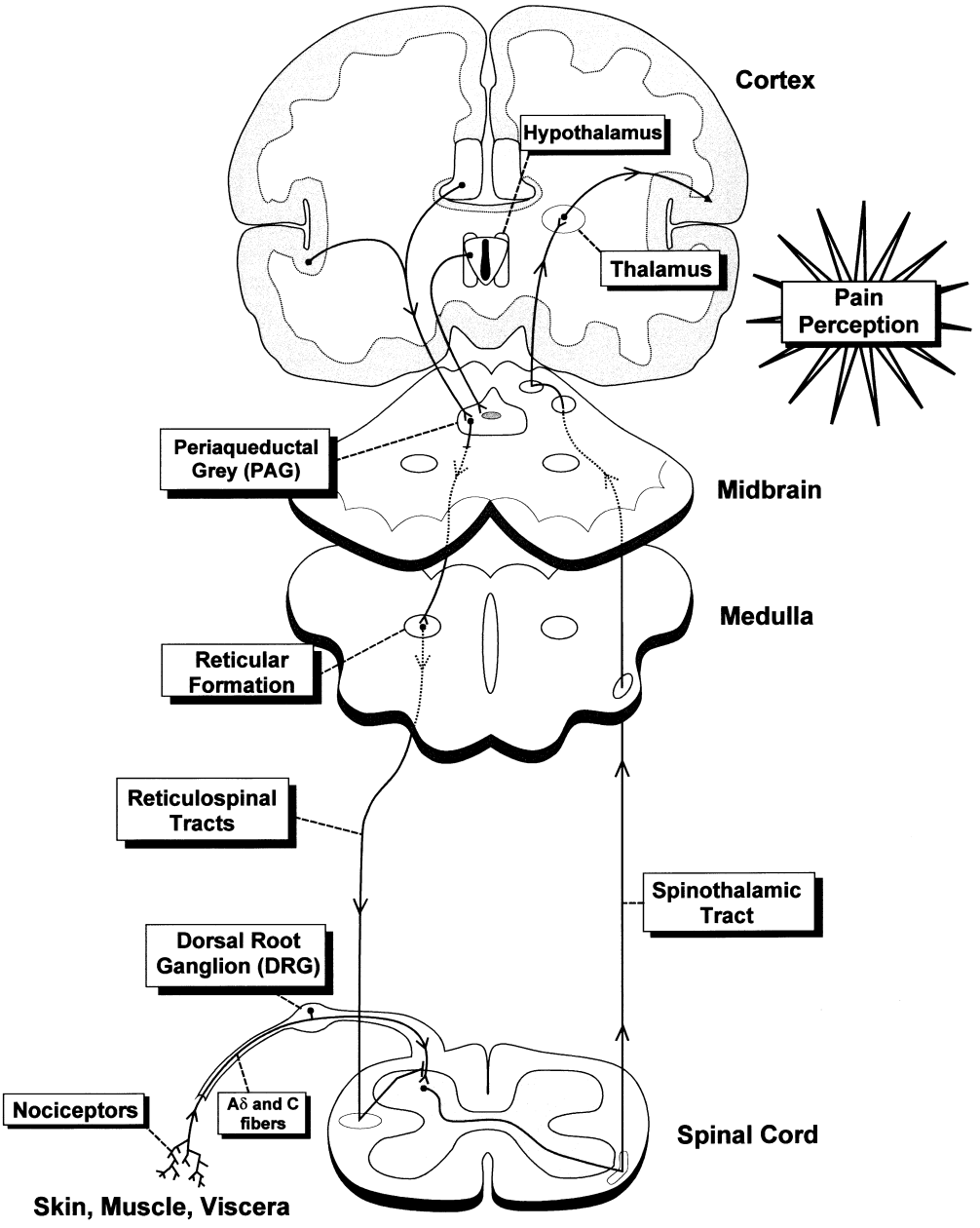


Figure 6 Major neuronal pathways that participate in pain transmission/modulation. In the ascending pain transmission pathway, painful stimuli activate peripheral nociceptors in skin, muscle, and viscera to activate Aδ and C fibers that terminate in the dorsal horn of the spinal cord. Neurotransmitters are released here that activate the ascending spinothalamic tract, which terminates in the thalamus. Projections from the thalamus extend to specific areas in the cortex. Pain perception occurs in these brain areas. In the descending pain modulation pathway, pain perceived in brain structures activates neuronal pathways that synapse in various brain stem areas such as the periaqueductal gray (PAG) and reticular formation. Projections from the hypothalamus and other rostral structures also converge in the PAG to modulate signal transmission. Fibers descend to the spinal cord, where other neurotransmitters are released that modulate incoming painful stimuli. This is a very simplified diagram; there are many more pathways and brain stem nuclei involved in the integration of pain (Bonica, 1990).

pain than do patients without ongoing pain (Lipman *et al.,* 1987). Clearly, objective measurement of pain in humans is inherently difficult.

To facilitate the study of pain pathways, a number of animal models have been developed. Models for evaluation of nociceptive pain after noxious mechanical, thermal, or chemical stimulation have been developed, and some of these are described in Table III. The time required (seconds) for the animal to respond is an indication of the nociceptive threshold of that animal. These tests are commonly used to evaluate antinociceptive effects of administered drugs that will increase the response latency times (hypoalgesia or

Table III Animal Models of Acute and Chronic Pain

Stimulus type	Species	Stimulus[a]	Response	Reference
Acute				
Thermal				
Heat	Mouse/rat	Radiant heat (tail)	Tail flick	D'Amour and Smith (1941)
	Rat	Radiant heat (paw)	Paw withdrawal	Hargreaves *et al.* (1988)
		Hot water (55°C)	Tail flick	Sewell and Spencer (1976)
	Mouse/rat	Hot plate	Jump or lick hind paw	Ankier (1974)
Cold	Rat	Cold water (−10°C)	Tail flick	Pizziketti *et al.* (1985)
Mechanical	Rat	von Frey filaments	Paw withdrawal	Chaplan *et al.* (1994)
	Mouse/rat	Mechanical force	Paw withdrawal	Randall and Selitto (1957)
Chemical	Mouse/rat	Acetic acid, NaCl, BK, phenylquinone (i.p.)	Writhing	Vyklicky (1979)
		SP, EAAs (i.t.)	Licking, scratching	Wilcox (1988)
Chronic				
Inflammatory				
Acute	Mouse/rat	Formalin (i.pl.)	Licking, shaking	Murray *et al.* (1988), Wheeler-Aceto and Cowan (1991)
		Carrageenan (i.pl.)	Thermal hyperalgesia	Garry *et al.* (1994a)
		CFA (i.pl)	Hyperalgesia	Millan *et al.* (1998)
		Yeast suspension (i.pl.)	Decreased mobility	Langford *et al.* (1972)
Chronic	Rat	CFA (tail base)	Vocalization, hyperalgesia	Colpaert (1987); Calvino *et al.* (1987)
		PG/PS (i.p.)	Hyperalgesia	Fuseler *et al.* (1998)
Neuropathic				
	Rat	CCI	Hyperalgesia/allodynia	Bennett and Xie (1988)
		Partial sciatic nerve ligation	Hyperalgesia/allodynia	Seltzer *et al.* (1990)
		L5/L6 nerve ligation	Hyperalgesia/allodynia	Kim and Chung (1992)
		Spinal cord irradiation	Mechanical allodynia	Xu *et al.* (1992)

[a] i.pl., intraplantar; i.p., intraperitoneal; i.t., intrathecal; CFA, Freund's complete adjuvant; BK, bradykinin; SP, substance P; EAA, excitatory amino acids; PG/PS, peptidoglycan polysaccaride; CCI, chronic constriction injury, sciatic nerve.

analgesia). A decrease in response latency time (hyperalgesia and allodynia) is observed in animal models of inflammation.

A number of models of acute inflammatory pain in which behaviors are quantified have also been described (Table III). One of the most widely used is the formalin test. In this test, formalin injected into a rat or mouse paw (intraplantar, i.pl.) elicits a two-phase behavioral response. In the first 5 min after formalin injection, there is flinching/shaking of the paw, and the animal licks the paw. These responses decline by 10 min and are followed by a secondary phase of the same responses beginning at about 15 min, peaking at around 20 to 30 min, and ending at about 60 min (Wheeler-Aceto and Cowan, 1991; Murray *et al.,* 1988). The early phase is thought to be due to direct activation of nociceptors, whereas the secondary phase likely represents an inflammatory response (Hunskaar and Hole, 1987). In other models, inflammatory responses consist of a single acute behavioral phase that subsides within hours or days.

Evaluation of chronic inflammatory pain has been performed in animal models of arthritis (Table III). Administration of a heat-killed *Mycobacterium butyricum* (Freund's adjuvant) suspension intradermally into a rat tail base produces paw inflammation in about 7 days that peaks at around day 18–20 and gradually subsides (Colpaert, 1987). Increased vocalization, decreased mobility (Colpaert, 1987), as well as mechanical hyperalgesia (Calvino *et al.,* 1987) occur with a similar time course, suggesting development of chronic pain associated with the inflammation. Preliminary studies measuring pain thresholds have been performed in the PG/PS model of polyarthritis described earlier (Wilder *et al.,* 1983). Results obtained thus far suggest that both mechanical allodynia and thermal hyperalgesia develop along with the increases in paw volume and arthritis index (Fuseler *et al.,* 1998).

Several animal models have been developed as mimics of neuropathic pain. Those listed in Table III include injury both to peripheral nerves and to the CNS. Mechanical and thermal allodynia and hyperalgesia as well as affected paw guarding and licking behaviors are observed in these models. These preparations are believed to represent models of human causalgia, dysesthesia (spontaneous pain), and paresthesias.

Nitric Oxide as a Pain Mediator/Modulator

Among the chemical mediators of pain, a role for NO in both nociceptive and neuropathic pain is gradually being

revealed. Studies in human volunteers show that when solutions containing NO are injected or infused intravenously (i.v.) into the hand, pain is produced, with the intensity dependent on the NO concentration (Holthausen and Arndt, 1995). The vasodilating activity of NO is at least partially responsible for development of pain. In many painful disorders characterized by inflammation, such as rheumatoid arthritis, enhanced NO production occurs, and there is a strong correlation between pain and skin hyperthermia (due to enhanced local vasodilation) (Anbar and Gratt, 1997). Release of NO is also hypothesized to be a trigger for spontaneous migraine pain (Thomsen and Olesen, 1998). The NO donor compound glycerol trinitrate (GTN, nitroglycerin) reliably produces headache in normal volunteers as well as patients who suffer from migraine (Iversen *et al.,* 1989). Although the mechanisms underlying NO involvement in migraine are unknown, dilation of cerebral arteries and direct activation of sensory nerve fibers have been suggested (Thomsen and Olesen, 1998). Nitric oxide thus appears to be a chemical link between peripheral and vascular nociception (Anba and Gratt, 1998).

A CNS role for NO in pain is shown in animal studies. Injection of NO donor compounds such as sodium nitroprusside (SNP), hydroxylamine, and NOC-18 into the spinal cord produce a dose-dependent hyperalgesic response in the tail flick test that is blocked by hemoglobin (Hb), an NO scavenger (Inoue *et al.,* 1997; Kitto *et al.,* 1992). Similar effects are found for intracerebroventricular (i.c.v.) NOC-18 administration, but at doses that are almost 15-fold greater (Shibuta *et al.,* 1995), indicating a predominant role for spinal NO in tail flick hyperalgesia. When administered spinally, low (picomolar) doses of L-arginine, the endogenous NO precursor, facilitate tail flick nociception in accord with the proposed role of NO in mediating pain (Meller *et al.,* 1992). More direct evidence is derived from studies showing an increase in NO metabolites in spinal microdialysates after i.pl. injection of capsaicin, the component of hot peppers that produces secondary hyperalgesia and allodynia (Wu *et al.,* 1998a). This increase is blocked by pretreatment with a NOS inhibitor, suggesting that formation of spinal NO is increased in peripheral pain.

Chronic neuropathic pain induces changes in NOS expression and activity in the CNS. Increased expression of NOS protein (measured by immunoreactivity) is found in L5 and L6 DRG 2 weeks after L5/L6 spinal nerve ligation (Steel *et al.,* 1994). Activity of NOS also increases at this time point, but after 4 weeks it returns to prelesion values even though mechanical allodynia is still present (Choi *et al.,* 1996). A bilateral decrease in NOS activity is found in lumbar spinal cord tissue in the same animals at both the 2- and 4-week time points (Choi *et al.,* 1996). Others have found increased expression of NOS in neurons in the lumbar DRG and in ipsilateral dorsal roots, fibers, and terminals in the dorsal horn after sciatic nerve lesion (Zhang *et al.,* 1993). In these same animals, the numbers of NOS-immunopositive neurons in dorsal horn lamina were decreased. Thus, NOS in sensory neurons is upregulated, whereas NOS in dorsal horn appears to decline. Studies using antisera selective for NOS isoforms have also shown changes in immunoreactivity in neuropathic pain models (see later). These alterations in NOS activity suggest that NO has an important role in neuropathic pain processing, but the exact relationships between pain and NO synthesis in discrete CNS regions are not clear.

Effects of Nitric Oxide Synthase Inhibitors on Pain

Inhibitors of NOS activity provide experimental evidence for the role of NO in pain. The pain evoked by injection of hyperosmolar solutions into human hand veins is completely blocked by pretreatment with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) (Kindgen-Milles and Arndt, 1996). Injection of BK into hand veins induces pain that is blocked by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), suggesting that BK-induced effects are mediated by release of NO (Holthausen, 1997). Clinical studies show that L-NMMA (546C88) is effective in acute treatment of migraine and chronic tension-type headache, although systemic side effects such as increased blood pressure may limit its therapeutic potential (Ashina *et al.,* 1999; Lassen *et al.,* 1997). Development of selective nNOS inhibitors may provide effective therapy for headache pain.

In animal models of neuropathic pain, inhibition of NOS activity alleviates pain behaviors. When administered systemically and continuously for 12 days in the chronic constriction injury (CCI) model, L-NAME is ineffective in reversing thermal (heat) hyperalgesia. However, when applied directly and continuously to the site of CCI, L-NAME prevents the development and maintenance of hyperalgesia (Thomas *et al.,* 1996). Systemically administered L-NAME reverses mechanical allodynia in other neuropathic pain models, an effect that is reversed by L-arginine (Hao *et al.,* 1994; Yoon *et al.,* 1998). Systemic L-NAME also reverses cold allodynia and cold-stress-exacerbated ongoing pain. As mentioned earlier, the EAAs released in the spinal cord after nerve injury activate *N*-methyl-D-aspartate (NMDA) receptors to increase intracellular calcium and activate NOS. Indeed, spinal administration of L-NAME as well as both NMDA and non-NMDA antagonists reduces nerve lesion-induced behaviors thought to be indicative of pain (Wong *et al.,* 1998). Also, in an animal model of painful radiculopathy from lumbar disk herniation, spinally administered L-NMMA reduces mechanical hyperalgesia (Kawakami *et al.,* 1998). Electrophyisological recordings from dorsal root ganglia show that L-NAME suppresses neuronal activity following sciatic nerve ligation, but not in nonligated conditions (Wiesenfeld-Hallin *et al.,* 1993). These and other studies indicate that NO plays an important role in pain produced by nerve injury.

Studies with NOS inhibitors have also suggested a role for NO in inflammatory pain. Both local and systemic administration of NOS inhibitors are effective in alleviating nociception. The thermal hyperalgesia found in the rat arthritis model of intraarticular (i.a.) kaolin/carrageenan is reversed by i.a. L-NAME (Lawand *et al.,* 1997). Mechanical

hyperalgesia induced by i.pl. PGE_2 is reversed by i.pl. L-NMMA, suggesting that the effects of peripheral PGE_2 are mediated through NO (Aley *et al.,* 1998). Similarly, i.pl. coinjection of L-NAME with formalin attenuates the second phase of the behavioral response in the formalin test (Kawabata *et al.,* 1994). Systemic L-NAME attenuates both mechanical and thermal hyperalgesia induced by i.pl. carrageenan (Handy and Moore, 1998) and inhibits the second phase of the formalin test as well (Morgan *et al.,* 1992). NOS activity in the CNS may be involved in some of the actions of systemic L-NAME, as spinally administered L-NAME attenuates the maintenance of thermal but not mechanical hyperalgesia in this model (Meller *et al.,* 1994). These effects of NOS inhibitors may be partially due to effects other than NOS inhibition, because L-NAME also inhibits production of PGs and interleukin (IL-1β) (deMello *et al.,* 1997).

Electrophysiological studies in the spinal cord show that systemic L-NAME reduces the second but not the first peak of the neuronal firing response observed following i.pl. formalin (Haley *et al.,* 1992). Noxious mechanical stimulation of inflamed rat paws (from i.pl. carrageenan or mustard oil) produces an increase in spinal single motor unit reflex activity that is blocked by i.v. L-NAME (Semos and Headley, 1994). In noninflamed paws in these studies, L-NAME has no effect on the activity produced by mechanical stimulation, whereas it blocks noxious thermal nociceptive reflexes equally in inflamed and noninflamed paws. In addition, L-NAME has no effect on mechanical nociception in spinalized rats with inflamed paws. These results suggest a role for central NO in peripheral inflammation-induced mechanical, but not thermal, nociception. However, because the behavioral results implicate spinal NO in thermal but not mechanical hyperalgesia, the exact relationship between electrophysiological results and behavioral observations remains to be clarified.

Although most studies using NOS inhibitors to examine pain mechanisms have utilized models of neuropathic or inflammatory pain, these agents have also been tested in acute nociceptive pain models. When administered systemically, i.c.v., or spinally, L-NAME produces antinociception in the tail flick, hot plate, and acetic acid writhing tests (Moore *et al.,* 1991; Shibuta *et al.,* 1995; Yamaguchi *et al.,* 1996). Although the effect produced by i.c.v. L-NAME in the tail flick test is slight (15% analgesia), it is reversed by NOC-18 (Shibuta *et al.,* 1995). When compared to morphine, spinal L-NAME is 20- to 60-fold less potent, and i.v. L-NAME is about 30-fold less potent in this test (Yamaguchi and Naito, 1996). However, others have reported that spinally administered L-NAME does not produce tail flick antinociception in a dose that was effective in previous studies (Machelska *et al.,* 1997a,b). These differences in results point to other possible interpretations for NO actions in pain pathways.

Inhibitors of NOS activity also show that NO may not be a mediator for all types of pain. For example, in humans, i.v. L-NAME does not affect pain evoked by heat, cold, or stretch (Holthausen and Ding, 1997). The site of NO release is important; peripherally administered NOS inhibitors generally inhibit pain from peripheral stimuli. However, the role of centrally released NO may vary, depending on the type of pain stimulus. For instance, the tactile allodynia observed in a rat diabetic model is not reversed by spinally administered L-NAME (Calcutt and Chaplan, 1997). Also, spinally administered L-NAME is ineffective in reversing pain behaviors in a rat model of postoperative pain, a plantar incision (Zahn and Brennan, 1998). Further studies are needed to clarify the role of CNS NO in pain.

There is evidence that NO is also antinociceptive. When administered i.c.v., the precursor of NO, L-arginine (a constituent of kyotorphin, L-tyrosyl-L-arginine), produces antinociception that is proposed to be mediated via kyotorphin-induced release of endogenous opioid peptides (Kawabata *et al.,* 1993) as well as via activation of the NO–cGMP pathway (Ji and Zhu, 1993). Antinociception produced by i.c.v. or systemic L-arginine is blocked by systemic opioid antagonists (Kawabata *et al.,* 1992, 1993), suggesting involvement of CNS opioids. In support of the latter hypothesis, iontophoresis of a stable analog of cGMP to the rostroventral medulla, the brain stem region involved in pain suppression, produces cellular excitation that is inhibited by NOS inhibition (Hantall, 1995). In behavioral studies, i.c.v. administration of cGMP analogs produces antinociception in the tail flick test (Vocci *et al.,* 1978). At peripheral sites, methylene blue (MB, a guanylyl cyclase inhibitor), but not nalxone (an opioid antagonist), blocks antinociception produced by L-arginine in the carrageenan model (Kawabata *et al.,* 1992). Thus, NO may be important in antinociception as well as nociception. Indeed, i.c.v. L-arginine has been postulated to play a dual role in nociceptive processing, being nociceptive under conditions where NOS activity is inhibited and where either kyotorphin or opioid receptors are blocked (Kawabata *et al.,* 1993).

Evidence for NO as an antinociceptive mediator is found in studies using muscarinic agonists and NOS inhibitors. Antinociception in the hot plate and tail flick tests produced by muscarinic agonists administered both spinally and supraspinally is attenuated by L-N^G-nitroarginine (L-NNA) a nonselective NOS inhibitor (Iwamoto *et al.,* 1994a,b). Muscarinic agonist antinociception is also blocked by MB, suggesting involvement of the cGMP system. Endogenous agonists at muscarinic receptors could potentially activate the L-arginine/NO/cGMP cascade at multiple sites in the CNS during nociceptive stimulation to suppress pain.

Studies show that L-NAME inhibits the increase in c-Fos expression induced by GTN in brain areas associated with pain pathways, such as the nucleus tractus solitaris, the spinal trigeminal nucleus caudalis (NTC), and the periaqueductal gray, among others (Tassorelli *et al.,* 1997). Interestingly, L-NAME, which inhibits both neuronal and vascular endothelial NOS activity, is effective in most brain areas, whereas 7-nitroindazole (7-NI), a more specific inhibitor for neuronal NOS (nNOS), is effective in only in specific areas, such as the NTC. L-NAME also completely prevents the increase in c-Fos immunoreactivity found in spinal dorsal horn after i.pl. formalin injection (Roche *et al.,* 1996) and

after noxious mechanical stimulation (Lee *et al.,* 1992). The decrease in c-Fos production is associated with decreased behaviors in the formalin test, a measure of antinociception (Roche *et al.,* 1996). The relationship between c-Fos induction, NO, and pain is not well understood, but NO induction of c-Fos may be involved in activation of pain pathways.

Which NOS Is Involved?

Isoform-selective inhibitors of NOS suggest roles for both nNOS and iNOS in pain arising from inflammation. It is well established that iNOS production is associated with inflammatory situations that are accompanied by pain (Anbar and Gratt, 1997). A role for nNOS is also postulated. The nNOS-selective inhibitors 7-NI and 1-(2-trifluromethyphenyl)imidazole (TRIM) inhibit carrageenan-induced thermal and mechanical hyperalgesia (Handy and Moore, 1998). Late phase formalin-induced behaviors are also reversed by 7-NI (Moore *et al.,* 1993). Involvement of eNOS in this action was discounted because 7-NI has no effects on vascular responses. However, these decreased pain behaviors may also result from the activity of 7-NI as an anti-inflammatory agent, because it also inhibits synthesis of PGs and cellular infiltration as well as nNOS activity (Salvemini *et al.,* 1995). In accordance with the decreased pain behaviors, 7-NI but not aminoguanidine, an iNOS inhibitor, reduces joint inflammation when administered following development of polyarthritis after injection of Freund's complete adjuvant (Pozza *et al.,* 1998). Although pretreatment with aminoguanidine or *N*-iminoethyl-L-lysine (L-NIL, a selective iNOS inhibitor) prevents development of adjuvant-induced arthritis (Connor *et al.,* 1995), when given after the polyarthritis has developed, L-NIL has no effect on joint inflammation (Fletcher *et al.,* 1998). These findings suggest that nNOS but not iNOS is involved in maintenance of established inflammation and pain.

Studies using transgenic mice deficient in nNOS further refine the role for NO in pain. In nNOS-deficient mice, the nociceptive responses to formalin are not different from those in wild-type mice (Crosby *et al.,* 1995). Interestingly, although L-NAME completely prevents the second phase of the formalin test in wild-type mice, the NOS inhibitor does not have this effect in the nNOS-deficient mice. These results suggest that NO is sufficient but not essential for expression of nociception.

The role of NOS isoforms in neuropathic pain has also been studied using selective inhibitors. The mechanical allodynia in spinal cord lesioned rats is reversed by systemically administered 7-NI (Hao and Xu, 1996). The effects of 7-NI are reversed by L-arginine, and the NOS inhibitor has no effects on blood pressure at the doses used, suggesting that the antiallodynic effects are not mediated through eNOS. In the CCI model, both L-NAME and aminoguanidine reverse the increase in local blood flow that occurs at the nerve constriction (Levy and Zochodne, 1998). Others, using 7-NI in electrophysiological studies after spinal nerve L5 lesions, have suggested that nNOS is not involved in the generation of spontaneous activity of lesioned afferents, but other NOS isoforms are probably activated (Habler *et al.,* 1998). The investigators postulated that nNOS activity may be more important as a faciliator of nociceptive transmission than in the generation of spontaneous activity.

Expression of NOS immunoreactivity in neuronal tissues has been measured in attempts to determine roles of various isoforms in pain. In rat neuropathic pain models (CCI or tight ligature), nNOS immunoreactivity decreases in the spinal dorsal horn ipsilateral to the injury and increases in the DRG (Goff *et al.,* 1998). Activity of peripheral NOS may also increase, as both eNOS and nNOS immunoreactivity increases in the endoneurium of the ligated nerve in this model. Similar results were reported in a model of polyarthritis (Pozza *et al.,* 1998). The appearance of iNOS immunoreactivity at the nerve constriction site in the same model parallels the appearance of macrophages following nerve injury (Levy and Zochodne, 1998). The role of spinal nNOS in peripheral inflammation is not clear, as some investigators report no change in nNOS after inflammation induced by i.pl. Freund's adjuvant (Goff *et al.,* 1998) or carrageenan (Traub *et al.,* 1994), but others show an increase in dorsal horn nNOS-immunopositive neurons (Lam *et al.,* 1996). No iNOS immunoreactivity was detected in the dorsal horn after either neuropathic or inflammatory pain induction (Goff *et al.,* 1998). Other studies using an arthritis model of i.a. Freund's adjuvant show an increase in nNOS immunoreactivity in the lumbar (but not cervical) spinal dorsal horn and an increase in iNOS staining in ependymal cells around the central canal of the lumbar spinal cord (Wu *et al.,* 1998b). A recent review summarizes these and other findings and concludes that weak nociceptive input induces mainly an increase in nNOS that may be followed by a decrease after several days, whereas a strong nociceptive input causes mainly a persistent decrease in nNOS (Callsen-Cencic *et al.,* 1999).

Mechanisms of NO in Producing or Alleviating Pain

The exact mechanisms by which NO produces pain are not well understood. Because NO activates soluble guanylyl cyclase and increases cGMP concentrations, involvement of the NO–cGMP pathway in nociception has been suggested. Indeed, MB, a guanylyl cyclase inhibitor, blocks i.v. BK-induced pain in humans (Holthausen and Ding, 1997). Animal studies support this hypothesis. For example, the tail flick hyperalgesia produced by i.c.v. NOC-18 (Shibuta *et al.,* 1995) and the i.c.v. NOC-18-induced enhancement of nociception in the second phase of formalin-induced inflammation are completely abolished by MB (Shibuta *et al.,* 1996). Intradermal injections of the NO donor compound 3-(4-morphinolinyl)sydnonimine (SIN-1) into rat paws produces a mechanical hyperalgesia that is blocked by the guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) (Aley *et al.,* 1998). Immunohistochemical studies show high levels of cGMP-dependent kinase I (cGKI) expression in a subpopulation of rat DRG

neurons that partially overlap with SP- and CGRP-containing neurons (Qian *et al.,* 1996). Neuronal NOS is coexpressed with cGKI in these sensory neurons during embryonic development. The NO donor agent SNP causes an increase in release of immunoreactive SP, CGRP, and cGMP from spinal dorsal horn slices. The release of both cGMP and CGRP is inhibited by MB, suggesting an association between CGRP release and activation of guanylyl cyclase (Garry *et al.,* 1994b).

There is also evidence that additional mechanisms not involving cGMP may be involved in NO-induced pain, possibly depending on the site of NO release. For instance, MB does not affect the hyperalgesia induced by spinally administered NOC-18, indicating that cGMP is not involved in spinal NO-mediated nociception (Inoue *et al.,* 1997). Similarly, in the CCI model of neuropathic pain, spinally administered NOC-18 shortens the time to paw thermal hyperalgesia, an effect blocked by Hb, but not by MB (Inoue *et al.,* 1998). Also, the mechanical hyperalgesia produced by i.pl. PGE_2 is not reversed by coinjection with ODQ, but it is blocked by NOS inhibition (Aley *et al.,* 1998).

The pain produced by NO may be due to effects on ion channel activity (reviewed in Fagni and Bockaert, 1996). These effects may be through direct actions on glutamate-gated channels or through indirect actions mediated by cGMP. Nitric oxide-induced nociception may depend on formation of free radical species such as peroxynitrite, because a free radical scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), blocks thermal hyperalgesia in the CCI model (Tal, 1996). However, although inflammation occurs following injection of peroxynitrite directly into a rat paw, mechanical hyperalgesia is not observed (Ridger *et al.,* 1997).

Nitric oxide may also produce nociception indirectly through release of other nociceptive mediators. The SNP-induced release of immunoreactive SP and CGRP from rat spinal dorsal horn is attenuated by Hb, indicating dependence on NO. However, release of these peptides is also stimulated by sodium ferricyanide, which does not generate NO, suggesting that both NO-dependent and NO-independent mechanisms are involved in release (Garry *et al.,* 1994b). Superfusion of SNP onto parietal cortex of cats results in arterial vasodilation that is blocked by a CGRP antagonist and by LY83583, an inhibitor of guanylyl cyclase activity (Wei *et al.,* 1992). Interestingly, although LY83583 completely blocks vasodilation induced by applied CGRP, the SNP-induced vasodilation is only partially attenuated, suggesting an effect of SNP that is not mediated by cGMP. In the periphery, NO donor compounds also induce release of CGRP, possibly through formation of PGs (Holzer *et al.,* 1995).

Although nociceptive stimuli induce the release of BK and SP, pain produced by these peptides is likely mediated through NO because BK- or SP-induced pain is blocked after inhibition of NOS (Kindgen-Milles and Arndt, 1996). Inhibitors of NOS also block peripheral SP-induced vasodilation and plasma extravasation, symptoms associated with pain (Ralevic *et al.,* 1995). The expression of NOS in neurons in the lumbar dorsal root ganglia and in the spinal cord dorsal horn is increased after sciatic nerve lesion, suggesting enhanced activity in neuropathic pain. Some of these same DRG neurons also express CGRP and SP (Zhang *et al.,* 1993).

It must be remembered that some types of pain are alleviated by increasing the levels of NO. The vasodilator activity of oral nitrates, by increasing NO levels, is well known to alleviate anginal pain (Thadani, 1997). In addition, transdermal GTN has been shown to be effective in treatment of pain associated with primary dysmenorrhea (Anonymous, 1997). However, headache (which may be the result of cerebral vasodilation; see earlier) was reported as a side effect in about 20% of patients studied. In patients with interstitial cystitis, pain is relieved and urinary NOS products are increased after treatment with oral L-arginine, a NO precursor (Smith *et al.,* 1997; Wheeler *et al.,* 1997). Lower pain scores in patients with acute vaso-occlusive sickle-cell crisis are associated with elevated blood levels of NO metabolites, suggesting that lowering NO may enhance the pain in this disease (Lopez *et al.,* 1996). Animal studies utilizing the formalin test show that L-arginine, the precursor for NO, is hyperalgesic or analgesic, depending on the dose, when injected into a paw. Low doses are hyperalgesic, an effect reversed by L-NAME, whereas a 10-fold higher dose is analgesic (Kawabata *et al.,* 1994).

Role of NO in Activity of Analgesic Drugs

OPIOIDS

In the postulated role of NO as a mediator of antinociception, it has been suggested that NO mediates the analgesia produced by morphine and other opioids. Interplantar morphine in rats reverses the hyperalgesia produced by interplantar PGE_2, an action that is blocked by pretreatment with MB and NOS inhibition (Ferreira *et al.,* 1991). These results suggest a role for the NO–cGMP pathway in peripheral morphine antinociception. In acute thermal pain tests, i.c.v. and spinally administered NOS inhibitors attenuate and L-arginine enhances morphine-induced antinociception, suggesting a central as well as a peripheral role for NO in morphine effects (Pataki and Telegdy, 1998; Song *et al.,* 1998; Xu and Tseng, 1995). In support of these behavioral studies, systemic morphine was shown to increase release of nitrite (a stable NO metabolite) in spinal cord microdialysates (Xu *et al.,* 1997). In contrast, others have shown that inhibition of spinal NOS enhances the antinociceptive effects of morphine and opioid receptor-selective agonists in both the acute thermal and pressure nociceptive tests as well as in inflammatory models (Machelska *et al.,* 1997a,b; Yamaguchi and Naito, 1996). These conflicting results suggest a role for NO in opioid effects, but the relationships are not clear.

Studies utilizing antisense oligodeoxynucleotides (ODN) to selectively downregulate isoforms of NOS have provided additional support for a role of NO in morphine effects and suggest possible explanations for previous conflicting results

using NOS inhibitors. Downregulation of a truncated form of nNOS at both the spinal and supraspinal levels blocks tail flick antinociception induced by systemic morphine (Kolesnikov *et al.,* 1997). Treatment with antisense ODN for the full-length nNOS does not affect acute morphine antinociception, but it blocks development of tolerance to the chronically administered opioid. Use of antisense ODN to iNOS has no effect on morphine antinociception. The antinociceptive effects of morphine are thus regulated in complex ways by the expression of various forms of NOS.

The role of NOS activity in development of tolerance to the antinociceptive effects of opioids is also complex. Several reports show that NOS inhibitors attenuate morphine-induced tolerance (Kolesnikov *et al.,* 1993; Pataki and Telegdy, 1998) and physical dependence (Adams *et al.,* 1993; Dambisya and Lee, 1996; Vaupel *et al.,* 1997). In these studies, morphine tolerance was induced by chronic treatment with subcutaneous morphine. However, continuous spinal infusion of L-NAME with morphine showed development of tolerance and physical dependence to morphine that was not substantially different from that which developed after infusion of morphine alone (Dunbar and Yaksh, 1996). The differences between these results could be due to the different routes of morphine administration, namely, peripheral versus central. When total cellular NOS mRNA or tissue homogenate NOS activity was measured, no change in brain or spinal cord mRNA or NOS activity in brain stem and cerebellum is found after chronic systemic morphine treatment (Babey *et al.,* 1994). However, repeated treatment with peripheral morphine increases NOS mRNA and NOS immunoreactivity in spinal cord slices measured by *in situ* hybridization and immunohistochemical methods (Machelska *et al.,* 1997a,b). Thus, adaptive changes of NOS probably occur in selective neurons throughout the CNS during development of morphine tolerance.

Pretreatment with a nonanalgesic dose of L-arginine has been shown to enhance (Pataki and Telegdy, 1998) and to have no effect on (Xu and Tseng, 1993) morphine-induced antinociception. Repeated treatment with i.c.v. or peripheral L-arginine is reported to enhance development of tolerance to systemic morphine (Pataki and Telegdy, 1998; Babey *et al.,* 1994). The mechanism for this effect may be attributed to findings that chronic treatment with systemic L-arginine decreases levels of morphine in specific brain regions and spinal cord (Bhargava *et al.,* 1997). The decreased morphine levels in the CNS result in a decreased antinociceptive potency of morphine that may appear to be an enhancement of tolerance.

Studies show that the NO-releasing drug FK409, injected locally, enhances the antinociceptive actions of systemically administered opioids in the formalin test (Nozaki-Taguchi *et al.,* 1998a,b). These results are difficult to reconcile with studies showing that systemic L-NAME plus morphine produce synergistic antinociception in the tail flick test (Yamaguchi and Naito, 1996). Thus, both increased NO and decreased NOS activity enhance morphine antinociception. This apparent discrepancy could be due to the different types of pain measured, namely, inflammatory versus acute thermal nociceptive. Resolution of these differences awaits further studies.

NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

It has been proposed that one mechanism by which some nonsteroidal anti-inflammatory drugs (NSAIDs) alleviate pain and inflammation is by inhibition of expression of iNOS and decreased production of NO as shown in lipopolysaccharide (LPS)-stimulated macrophages (Aeberhard *et al.,* 1995; Amin *et al.,* 1995). This effect may or may not be due to direct inhibition of iNOS catalytic activity, but NSAID-induced decreased levels of iNOS mRNA and decreased protein expression are a common finding. Interestingly, antinociceptive synergism between peripheral L-NAME and NSAIDs in the formalin test and in the acetic acid writhing test has been reported (Morgan *et al.,* 1992). These effects may be mediated through decreased production of NO, decreased release of PGs, or both.

Effects of NSAIDs in the CNS may also be mediated through NOS inhibition. Ibuprofen inhibits iNOS mRNA and protein expression in glial cell cultures, where it has no effect on cyclooxygenase mRNA expression (Stratman *et al.,* 1997). The greater potency for ibuprofen to inhibit protein expression and iNOS activity than to inhibit mRNA production suggests that NSAIDs may inhibit posttranslational processing. However, the NSAID dipyrone has no significant effect on brain nNOS activity (Bierith *et al.,* 1998), and a role for inhibition of nNOS activity in NSAID actions has not been reported.

In fact, the NO–cGMP pathway has been proposed to mediate the analgesic effects of NSAIDs. Local administration of NOS inhibitor or MB abolishes diclofenac-induced analgesia in the animal models of acute inflammatory pain (Tonussi and Ferreira, 1994). Others showed that dipyrone administered i.c.v. has no effect on intraplantar PG-induced hyperalgesia, but systemic, intraplantar, and spinally administered dipyrone reverse the hyperalgesia. Both L-NMMA and MB abolish the antinociceptive effects of dipyrone administered by all these routes, suggesting involvement of the NO–cGMP pathway in dipyrone effects. Thus, spinal and peripheral, but not brain, NO may mediate the analgesia produced by this NSAID (Lorenzetti and Ferreira, 1996).

Following these and other studies, a class of NO-releasing NSAIDs are being developed that produce less gastrointestinal injury than the NSAIDs. For example, NO-naproxan shows greater analgesic activity than naproxan in the acetic acid writhing test (Davies *et al.,* 1997). Although these drugs may represent improved therapy for pain, further studies are needed to clarify the role of NO in NSAID analgesia.

OTHER DRUGS

As mentioned earlier, neuropathic pain is often resistant to treatment by standard analgesic agents, namely, NSAIDs and opioids, but it can be alleviated by administration of antidepressants. Amitriptyline, imipramine, or other tricyclic antidepressants are effective in neuropathic pains. More

recently, imipramine, desipramine, and nortriptyline were shown to attenuate the nociceptive jaw opening reflex, and L-NAME blocked this activity (Ahn *et al.,* 1998). The role of NO in the analgesic action of antidepressants remains to be defined.

Inhibitors of NOS decrease the minimum alveolar concentration required for isoflurane anesthesia, suggesting augmentation of analgesic/anesthetic effects (Pajewski *et al.,* 1996). Of the two inhibitors tested, L-NAME produced a significant increase in blood pressure, whereas the nNOS-selective inhibitor 7-NI did not. Both drugs have similar effects on the isoflurane anesthesia. These studies suggest that nNOS, but not eNOS, is involved in the observed effect. Additional experiments will determine whether clinical advantage in anesthesia can be obtained with selective NOS inhibitors.

Capsaicin, the active component of hot peppers, is effective in relieving both neuropathic and inflammatory pain when applied to the site of pain origin. This analgesic effect is believed to be due to depletion of neuronal SP because capsaicin also produces pain by releasing SP. Animal studies show that antinociception produced by spinally administered capsaicin is prevented by inhibition of NOS activity and guanylyl cyclase activity (Kreeger *et al.,* 1994). Thus, the NO–cGMP pathway may also be involved in the analgesic activity of capsaicin.

Although the actions of many analgesics appear to involve NO in some way, not all drugs that produce analgesia have been shown to act by this mediator. For example, the antinociceptive effects of Δ^9-tetrahydrocannibinol are not affected by NOS inhibitors (Thorat and Bhargava, 1994).

Conclusion

There is a great deal of evidence supporting a role for NO in pain and nociception as well as in analgesia and antinociception. However, the exact mechanisms by which NO affects pain pathways remain to be clarified. The use of NOS inhibitors or NO releasers as analgesic agents to treat specific pains without the undesirable side effects of other currently used drugs remains an intriguing possibility.

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References

Adams, M. L., Kalicki, J. M., Meyer, E. R., and Cicero, T. J. (1993). Inhibition of the morphine withdrawal syndrome by a nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester. *Life Sci.* **52,** 245–249.

Aeberhard, E. E., Henderson, S. A., Arabolos, N. S., Griscavage, J. M., Castro, F. E., Barrett, C. T., and Ignarro, L. J. (1995). Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.* **208,**1053–1059.

Ahn, D. K., Kim, Y. S., and Park, J. S. (1998). Central NO is involved in the antinociceptive action of intracisternal antidepressants in freely moving rats. *Neurosci. Lett.* **243,** 105–108.

Aley, K. O., McCarter, G., and Levine, J. D. (1998). Nitric oxide signaling in pain and nociceptor sensitization in the rat. *J. Neurosci.* **18,** 7008–7014.

Amin, A. R., Vyas, P., Attur, M., Leszczynska-Piziak, J., Patel, I. R., Weissmann, G., and Abramson, S. B. (1995). The mode of action of aspirin-like drugs: Effect on inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7926–7930.

Anbar, M., and Gratt, B. M. (1997). Role of nitric oxide in the physiopathology of pain. *J. Pain Symptom Manage.* **14,** 225–254.

Anbar, M., and Gratt, B. M. (1998). The possible role of nitric oxide in the physiopathology of pain associated with temporomandibular joint disorders. *J. Oral Maxillofac. Surg.* **56,** 872–882.

Ankier, S. I. (1974). New hot plate tests to quantify antinociceptive and narcotic antagonists activities. *Eur. J. Pharmacol.* **27,** 1–4.

Anonymous (1997). Transdermal nitroglycerine in the management of pain associated with primary dysmenorrhoea: A multinational pilot study. The Transdermal Nitroglycerine/Dysmenorrhoea Study Group. *J. Int. Med. Res.* **25,** 41–44.

Ashina, M., Lassen, L. H., Bendtsen, L., Jensen, R., and Olesen, J. (1999). Effect of inhibition of nitric oxide synthase on chronic tension-type headache: A randomised crossover trial. *Lancet* **353,** 287–289.

Babey, A. M., Kolesnikov, Y., Cheng, J., Inturrisi, C. E., Trifilletti, R. R., and Pasternak, G. W. (1994). Nitric oxide and opioid tolerance. *Neuropharmacology* **33,** 1463–1479.

Baeuerle, P. A., and Baltimore, D. (1995). IkB: A specific inhibitor of NF-κB transcription factor. *Science* **268,** 522–523.

Baeuerle, P. A., and Henkle, T. (1994). Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* **58,** 1–27.

Balter, K. (1992). Tutorial 8: A review of pain anatomy and physiology. *Pain Digest* **2,** 306–330.

Beauvais, F., Michel, L., and Dubertret, L. (1995). Exogenous nitric oxide elicits chemotaxis of neutrophils *in vitro. J. Cell. Physiol.* **165,** 610–614.

Bennett, G. J., and Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33,** 87–107.

Bhargava, H. N., Bian, J.-T., and Kumar, S. (1997). Mechanism of attenuation of morphine antinociception by chronic treatment with L-arginine. *J. Pharmacol. Exp. Ther.* **281,** 707–712.

Biemond, P., Swaak, A. J. G., and Koster, J. F. (1984). Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid. *Arthritis Rheum.* **27,** 760–765.

Bierith, A., Santos, A. R., Rodrigues, A. L., Creczynski-Pasa, T. B., and Calixto, J. B. (1998). Spinal and supraspinal antinociceptive action of dipyrone in formalin, capsaicin and glutamate tests: Study of the mechanism of action. *Eur. J. Pharmacol.* **345,** 233–245.

Blackwell, T. S., Blackwell, T. R., Holden, E. P., Christman, B. W., and Christman, J. w. (1996). *In vivo* antioxidant treatment suppresses nuclear factor-κB activation and neutrophilic lung inflammation. *J. Immunol.* **157,** 1630.

Blake, D. R., Merry, P., Stevens, C., Dabbagh, A., Sahinoglu, T., Allen, R., and Morris, C. (1990). Iron free radicals and arthritis. *Proc. Nutr. Soc.* **49,** 239–245.

Bonica, J. J. (1990). Anatomic and physiologic basis of nociception and pain. *In* ''The Management of Pain'' (J. J. Bonica, ed.), Vol. 1, pp. 28–94. Lea & Febiger, Philadelphia, Pennsylvania.

Calcutt, N. A., and Chaplan, S. R. (1997). Spinal pharmacology of tactile allodynia in diabetic rats. *Br. J. Pharmacol.* **122,** 1478–1482.

Callsen-Cencic, P., Hoheisel, U., Kaske, A., Mense, S., and Tenschert, S. (1999). The controversy about spinal neuronal nitric oxide synthase: Under which conditions is it up- or downregulated? *Cell Tissue Res.* **295,** 183–194.

Calvino, B., Crepon-Bernard, M.-O., and LeBars, D. (1987). Parallel clinical and behavioural studies of adjuvant-induced arthritis in the rat: Possible relationship with 'chronic pain.' *Behav. Brain Res.* **24,** 11–29.

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., and Yaksh, T. L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **53,** 55–63.

Chapman, C. R., and Syrjala, K. L. (1990). Measurement of pain. *In* ''The Management of Pain'' (J. J. Bonica, ed.), Vol. 1, pp. 580–594. Lea & Febiger, Philadelphia, Pennsylvania.

Chapman, M. L., Rubin, B. R., and Gracy, R. W. (1989). Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. *J. Rheumatol.* **16,** 15–18.

Choi, Y., Rafa, S. N., Moore, L. C., and Tobin, J. R. (1996). Neuropathic pain in rats is associated with altered nitric oxide snythase activity in neural tissue. *J. Neurol. Sci.* **138,** 14–20.

Clancy, R. M., Amin, A. R., and Abramson, S. B. (1998). The role of nitric oxide in inflammation and immunity. *Arthritis Rheum.* **41,** 1141–1151.

Cochran, F. R., Selph, J., and Sherman, P. (1996). Insights into the role of nitric oxide in inflammatory arthritis. *Med. Res. Rev.* **16,** 547–563.

Coderre, T. J., Katz, J., Vaccarino, A. L., and Melzack, R. (1993). Contribution of central neuroplasticity to pathological pain: Review of clinical and experimental evidence. *Pain* **52,** 259–285.

Collins, T., Read, M. A., Neish, A. S., Whitby, M. Z., Thanos, D., and Maniatis, T. (1995). Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J.* **9,** 899–909.

Colpaert, F. C. (1987). Evidence that adjuvant arthritis in the rat is associated with chronic pain. *Pain* **28,** 201–222.

Conner, E. M., Aiko, S., Fernandez, M., Battarbee, H. D., and Grisham, M. B. (2000). Duration of the hemodynamic effects of L-NAME *in vivo. Nitric Oxide: Biology and Chemistry,* in press.

Connor, J. R., Manning, P. T., Settle, S. L., Moore, W. M., Jerome, G. M., Webber, R. K., Tjoeng, F. S., and Currie, M. G. (1995). Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur. J. Pharmacol.* **273,** 15–24.

Crosby, G., Marota, J. J. A., and Huang, P. L. (1995). Intact nociception-induced neuroplasticity in transgenic mice deficient in neuronal nitric oxide synthase. *Neuroscience* **69,** 1013–1017.

Dabbagh, A. J., Blake, D. R., and Morris, C. J. (1992). Effect of iron complexes on adjuvant arthritis in rats. *Ann. Rheum. Dis.* **51,** 516–521.

Dambisya, Y. M., and Lee, T. L. (1996). Role of nitric oxide in the induction and expression of morphine tolerance and dependence in mice. *Br. J. Pharmacol.* **117,** 914–918.

D'Amour, F. E., and Smith, D. L. (1941). A method for determining pain sensation. *J. Pharmacol. Exp. Ther.* **72,** 74–79.

Davies, N. M., Roseth, A. G., Appleyard, C. B., McKnight, W., DelSoldato, P., Calignano, A., Cirino, G., and Wallace, J. L. (1997). NO-Naproxen vs. naproxen: ulcerogenic, analgesic and anti-inflammatory effects. *Aliment. Pharmacol. Ther.* **11,** 69–79.

DeCaterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M., Shin, W. S., and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial cell activation: Nitric oxide selectively reduces endothelial cell expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* **96,** 60–68.

deMello, S. B. V., Novaes, G. S., Laurindo, I. M. M., Muscara, M. N., Maciel, F. M., and Cossermelli, W. (1997). Nitric oxide synthase inhibitor influences prostaglandin and interleukin-1 production in experimental arthritic joints. *Inflammation Res.* **46,** 72–77.

Dunbar, S., and Yaksh, T. L. (1996). Effect of spinal infusion of L-NAME, a nitric oxide synthase inhibitor, on spinal tolerance and dependence induced by chronic intrathecal morphine in the rat. *Neurosci. Lett.* **207,** 33–36.

Edwards, S. W., Hughes, V., Barlow, J., and Bucknall, R. (1988). Immunological detection of myeloperoxidase in synovial fluid from patients with rheumatoid arthritis. *Biochem. J.* **250,** 81–85.

Elliott, M. J., Maini, R. N., Feldmann, M., Kalden, J. R., Antoni, C., Smolen, J. S., Leeb, B., Breedveld, F. C., Macfarlane, J. D., Bilj, H., and Woody, J. N. (1994). Randomized double-blind comparison of chimeric monoclonal antibody to tumor necrosis factor α (cA2) versus placebo in rheumatoid arthritis. *Lancet* **344,** 1105–1110.

Evans, C. H., Stefanovic-Racic, M., and Lancaster, J. (1995). Nitric oxide and its role in orthopaedic disease. *Clin. Orthop. Relat. Res.* **312,** 275–294.

Fagni, L., and Bockaert, J. (1996). Effects of nitric oxide on glutamate-gated channels and other ionic channels. *J. Chem. Neuroanat.* **10,** 231–240.

Ferreira, S. H., Duarte, I. D. G., and Lorenzetti, B. B. (1991). The molecular mechanism of action of peripheral morphine analgesia: Stimulation of the cGMP system via nitric oxide release. *Eur. J. Pharmacol.* **201,** 121–122.

Firestein, G. S. (1998). Rheumatoid synovitis and pannus. *In* ''Rheumatology'' (J. H. Klippel and P. A. Dieppe, eds.), pp. 513.1–513.24. Mosby, London.

Fletcher, D. S., Widmer, W. R., Luell, S., Christen, A., Orevillo, C., Shah, S., and Visco, D. (1998). Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats. *J. Pharmacol. Exp. Ther.* **284,** 714–721.

Fuseler, J. W., Roerig, S. C., Grisham, M. B., Hall, V., Jourd'heil, S., Laroux, S., and Wolf, R. E. (1998). Inhibition of inducible nitric oxide synthase exacrbates established joint inflammation and increases interleukin-6 levels in synovial tissue and serum. *Arthritis Rheum.* **41,** 599.

Gaboury, J., Woodman, R. C., Granger, D. N., Reinhart, P., and Kubes, P. (1993). Nitric oxide prevents leukocyte adherence: Role of superoxide. *Am. J. Physiol.* **265,** H862–H867.

Garry, M. G., Richardson, J. D., and Hargreaves, K. M. (1994a). Carrageenan-induced inflammation alters the content of I-cGMP and I-cAMP in the dorsal horn of the spinal cord. *Brain Res.* **646,** 135–139.

Garry, M. G., Richardson, J. D., and Hargreaves, K. M. (1994b). Sodium nitroprusside evokes the release of immunoreactive calcitonin gene-related peptide and substance P from dorsal horn slices via nitric oxide-dependent and nitric oxide-independent mechanisms. *J. Neurosci.* **14,** 4329–4337.

Gauthier, T. W., Davenpeck, K. L., and Lefer, A. M. (1994). Nitric oxide attenuates leukocyte–endothelial interaction via P-selectin in splanchnic ischemia–reperfusion. *Am. J. Physiol.* **267,** G562–G568.

Gilkeson, G. S., Mudgett, J. S., Seldin, M. F., Ruiz, P., Alexander, A. A., Misukonis, M. A., Pisetsky, D. S., and Weinberg, J. B. (1997). Clinical and serologic manifestations of autoimmune disease in MRL-*lpr/lpr* mice lacking nitric oxide synthase type 2. *J. Exp. Med.* **186,** 365–373.

Goff, J. R., Burkey, A. R., Goff, D. J., and Jasmin, L. (1998). Reorganization of the spinal dorsal horn in models of chronic pain: Correlation with behavior. *Neuroscience* **82,** 559–574.

Goldberg, A. L. (1995). Functions of the proteasome: The lysis at the end of the tunnel. *Science* **268,** 522–523.

Grisham, M. B. (1999). NF-κB activation in acute pancreatitis: Protective, detrimental or inconsequential? *Gastroenterology* **116,** 420–430.

Grisham, M. B., Jourd'heuil, D., and Wink, D. A. (1999). Physiological chemistry of nitric oxide and its metabolites: Implications in inflammation. *Am. J. Physiol.* **276,** G315–G321.

Habler, H.-J., Liu, Z., Eschenfelder, S., and Janig, W. (1998). Responses of axotomized afferents to blockade of nitric oxide snythesis after spinal nerve lesion in the rat. *Neurosci. Lett.* **254,** 33–36.

Haley, J. E., Dickenson, A. H., and Schachter, M. (1992). Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociceptin in the rat. *Neuropharmacology* **31,** 251–258.

Halliwell, B. (1995). Free radicals and rheumatic disease. *In* ''Mechanisms and Models in Rheumatoid Arthritis'' (B. Henderson, J. C. W. Edwards, and E. R. Pettipher, eds.), pp. 301–306. Academic Press, San Diego.

Handel, M. L., McMorrow, L. B., and Gravallese, E. M. (1995). Nuclear factor-κB in rheumatoid synovium. *Arthritis Rheum.* **38,** 1762–1770.

Handy, R. L. C., and Moore, P. K. (1998). Effects of selective inhibitors of neuronal nitric oxide synthase on carrageenan-induced mechanical and thermal hyperalgesia. *Neuropharmacology* **37,** 37–43.

Hantall, I. D. (1995). Excitation of cells in the rostral medial medulla of the

rat by the nitric oxide–cyclic guanosine monophosphate messenger system. *Neurosci. Lett.* **195,** 155–158.

Hao, J.-X., and Xu, X.-J. (1996). Treatment of a chronic allodynia-like response in spinally injured rats: Effects of systemically administered nitric oxide synthase inhibitors. *Pain* **66,** 313–319.

Hao, J. X., Xu, X. J., and Wiesenfeld-Hallin, Z. (1994). Systemic *N*-nitro-L-arginine ester (L-NAME), inhibitor of nitric oxide synthase, relieves chronic allodynia-like symptoms in rats with spinal cord lesion. *Acta Physiol. Scand.* **150,** 457–458.

Hargreaves, K., Dubner, R., Brown, F., and Flores, C. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32,** 77–88.

Harris, G., and Rollman, G. B. (1983). The validity of experimental pain measures. *Pain* **17,** 369–376.

Holthausen, H. (1997). Involvement of the NO/cyclic GMP pathway in bradykinin-evoked pain from veins in humans. *Pain* **69,** 87–92.

Holthausen, H., and Arndt, J. O. (1995). Nitric oxide evokes pain at nociceptors of the paravascular tissue and veins in humans. *J. Physiol.* **487.1,** 253–258.

Holthausen, H., and Ding, Z. (1997). Nitric oxide is not involved in vascular nociception of noxious physical stimuli in humans. *Neurosci. Lett.* **227,** 111–114.

Holzer, P., Jocic, M., and Peskar, B. A. (1995). Mediation by prostaglandins of the nitric oxide-induced neurogenic vasodilatation in rat skin. *Br. J. Pharmacol.* **116,** 2365–2370.

Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radical Res. Commun.* **18,** 195–199.

Hunskaar, S., and Hole, K. (1987). The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. *Pain* **30,** 103–114.

Ialenti, A., Moncada, S., and Di Rosa, M. (1993). Modulation of adjuvant arthritis by endogenous nitric oxide. *Br. J. Pharmacol.* **110,** 701–706.

Ignarro, L. J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ. Res.* **65,** 1–21.

Inoue, T., Mashimo, T., Shibuta, S., and Yoshiya, I. (1997). Intrathecal administration of a new nitric oxide donor, NOC-18, produces acute thermal hyperalgesia in the rat. *J. Neurol. Sci.* **153,** 1–7.

Inoue, T., Mashimo, T., Shibata, M., Shibuta, S., and Yoshiya, I. (1998). Rapid development of nitric oxide-induced hyperalgesia depends on an alternate to the cGMP-mediated pathway in the rat neuropathic pain model. *Brain Res.* **792,** 263–270.

Issekutz, A. C., and Issekutz, T. B. (1995). Monocyte migration to arthritis in rats utilizes both CD11/CD18 and very late activation antigen 4 integrin mechanisms. *J. Exp. Med.* **181,** 1197–1203.

Issekutz, A. C., Ayer, L., Miyasaka, M., and Issekutz, T. B. (1996). Treatment of established adjuvant arthritis in rats with monoclonal antibody to CD18 and very late activation antigen-4 integrins suppresses neutrophil and T-lymphocyte migration to the joints and improves clinical disease. *Immunology* **88,** 569–576.

Iversen, H. K., Olesen, J., and Tfelt-Hansen, P. (1989). Intravenous nitroglycerin as an experimental model of vascular headache. Basic characteristics. *Pain* **38,** 17–24.

Iwamoto, E. T., and Marion, L. (1994a). Pharmacologic evidence that spinal muscarinic analgesia is mediated by an L-arginine/nitric oxide/cyclic GMP cascade in rats. *J. Pharmacol. Exp. Ther.* **271,** 601–608.

Iwamoto, E. T., and Marion, L. (1994b). Pharmacological evidence that nitric oxide mediates the antinociception produced by muscarinic agonists in the rostral ventral medulla of rats. *J. Pharmacol. Exp. Ther.* **269,** 699–708.

Jang, D., and Murrell, G. A. C. (1998). Nitric oxide in arthritis. *Free Radicals Biol. Med.* **24,** 1511–1519.

Ji, X. Q., and Zhu, X. Z. (1993). Possible involvement of nitric oxide in arginine-induced analgesia. *Acta Pharm. Sin.* (*Yaoxue Xuebao*) **14,** 289–291.

Kaplan, S. S., Billiar, T., Curran, R. D., Zdziarski, U. E., Simmons, R. L., and Basford, R. E. (1989). Inhibition of chemotaxis with N^G-monomethyl-L-arginine. A role for cyclic GMP. *Blood* **74,** 1885–1887.

Kavanaugh, A. F., Davis, L. S., Nichols, L. A., Norris, S. H., Rothlein, R., Scharschmidt, L. A., and Lipsky, P. E. (1994). Treatment of refractory rheumatoid arthritis with a monoclonal antibody to intercellular adhesion molecule 1. *Arthritis Rheum.* **37,** 992–999.

Kavanaugh, A. F., Davis, L. S., Jain, R. I., Nichols, L. A., Norris, S. H., and Lipsky, P. E. (1996). A Phase I/II open label study of the safety and efficacy of an anti-ICAM-1 (intercellular adhesion molecule-1; CD54) monoclonal antibody in early rheumatoid arthritis. *J. Rheumatol.* **23,** 1338–1344.

Kavanaugh, A. F., Schulze-Koops, H., Davis, L. S., and Lipsky, P. E. (1997). Repeat treatment of rheumatoid arthritis patients with a murine anti-intercellular adhesion molecule 1 monoclonal antibody. *Arthritis Rheum.* **40,** 849–853.

Kawabata, A., Fukuzumi, Y., Fukushima, Y., and Takagi, H. (1992). Antinociceptive effect of L-arginine on the carrageenin-induced hyperalgesia of the rat: Possible involvement of central opioidergic systems. *Eur. J. Pharmacol.* **218,** 153–158.

Kawabata, A., Umeka, N., and Takagi, H. (1993). L-Arginine exerts a dual role in nociceptive processing in the brain: Involvement of the kyotorphin–Met–enkephalin pathway and NO–cyclic GMP pathway. *Br. J. Pharmacol.* **109,** 73–79.

Kawabata, A., Manabe, S., Manabe, Y., and Takagi, H. (1994). Effect of topical administration of L-arginine on formalin-induced anociception in the mouse: A dual role of peripherally formed NO in pain modulation. *Br. J. Pharmacol.* **112,** 547–550.

Kawachi, S., Cockrell, A., Laroux, S., Gray, L., Van der Hyde, H., and Grisham, M. B. (1999). Role of inducible nitric oxide synthese (iNOS) in the regulation of VCAM-1 expression in acute and chronic gut inflammation. *Am. J. Physiol.* **277,** G572–G576.

Kawakami, M., Tamaki, T., Hayashi, N., Hashizume, H., and Nishi, H. (1998). Possible mechanism of painful radiculopathy in lumbar disc herniation. *Clin. Orthop. Relat. Res.* **351,** 241–251.

Khan, B. V., Harrison, D. G., Olbrych, M. T., Alexander, R. W., and Medford, R. M. (1996). Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 9114–9119.

Kim, S. H., and Chung, J. M. (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50,** 355–363.

Kindgen-Milles, D., and Arndt, J. O. (1996). Nitric oxide as a chemical link in the generation of pain from veins in humans. *Pain* **64,** 139–142.

Kitto, K. F., Haley, J. E., and Wilcox, G. L. (1992). Involvement of nitric oxide in spinally mediated hyperalgesia in the mouse. *Neurosci. Lett.* **148,** 1–5.

Koch, A. E. (1998). Angiogenesis: Implications for rheumatoid arthritis. *Arthritis Rheum.* **41,** 951–962.

Kolesnikov, Y. A., Pick, C. G., Ciszewska, G., and Pasternak, G. W. (1993). Blockade of tolerance to morphine but not to κ opioids by a nitric oxide synthase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 5162–5166.

Kolesnikov, Y. A., Pan, Y.-X., Babey, A.-M., Jain, S., Wilson, R., and Pasternak, G. W. (1997). Functionally differentiating two neuronal nitric oxide synthase isoforms through antisense mapping: Evidence for opposing NO actions on morphine analgesia and tolerance. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 8220–8225.

Kreeger, J. S., Kitto, K. F., and Larson, A. A. (1994). Substance P N-terminal metabolites and nitric oxide mediate capsaicin-induced antinociception in the adult mouse. *J. Pharmacol. Exp. Ther.* **271,** 1281–1285.

Kurose, I., Wolf, R., Grisham, M. B., and Granger, D. N. (1994). Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. *Circ. Res.* **74,** 376–382.

Lam, H. H. D., Hanley, D. F., Trapp, B. D., Saito, S., Raja, S., Dawson, T. M., and Yamaguchi, H. (1996). Induction of spinal cord neuronal nitric oxide synthase (NOS) after formalin injection in the rat hind paw. *Neurosci. Lett.* **210,** 201–204.

Lander, H. M., Jacovina, A. T., Davis, R. J., and Tauras, J. M. (1996). Differential activation of mitogen-activated protein kinases by nitric oxide-related species. *J. Biol. Chem.* **271,** 19705–19709.

Langford, F. D., Holmes, P. A., and Emele, J. F. (1972). Objective method for evaluation of analgesic/anti-inflammatory activity. *J. Pharm. Sci.* **61,** 75–77.

Lassen, L. H., Ashina, M., Chrisiansen, I., Ulrich, V., and Olesen, J. (1997). Nitric oxide synthase inhibition in migraine. *Lancet* **349,** 401–402.

Lawand, N. B., Willis, W. D., and Westlund, K. N. (1997). Blockade of joint inflammation and secondary hyperalgesia by L-NAME, a nitric oxide synthase inhibitor. *NeuroReport* **8,** 895–899.

Lebsack, M. E., Paul, C. C., and Bloedow, D. C. (1991). Subcutaneous IL-1 receptor antagonist in patients with rheumatoid arthritis (abstract). *Arthritis Rheum.* **34**(Suppl. 9), S45.

Lee, J. H., Wilcox, G. L., and Beitz, A. J. (1992). Nitric oxide mediates Fos expression in the spinal cord induced by mechanical noxious stimulation. *NeuroReport* **3,** 1–4.

LeRoy, P. L., and Filask, R. (1990). Thermography. *In* ''The Management of Pain'' (J. J. Bonica, ed.), Vol. 1, pp. 610–621. Lea & Febiger, Philadelphia, Pennsylvania.

Levy, D., and Zochodne, D. W. (1998). Local nitric oxide synthase activity in a model of neuropathic pain. *Eur. J. Neurosci.* **10,** 1846–1855.

Liao, L., and Granger, D. N. (1995). Modulation of oxidized low-density lipoprotein-induced microvascular dysfunction by nitric oxide. *Am. J. Physiol.* **268,** H1643–H1650.

Lipman, J. J., Blumenkopf, B., and Parris, W. C. V. (1987). Chronic pain assessment using heat beam dolorimetry. *Pain* **30,** 59–67.

Lopez, B. L., Barnett, J., Ballas, S. K., Cristopher, T. A., Davis-Moon, L., and Ma, X. (1996). Nitric oxide metabolite levels in acute vaso-occlusive sickle-cell crisis. *Acad. Emerg. Med.* **3,** 1098–1103.

Lorenzetti, B. B., and Ferreira, S. H. (1996). Activation of the arginine–nitric oxide pathway in primary sensory neurons contributes to dipyrone-induced spinal and peripheral analgesia. *Inflammation Res.* **45,** 308–311.

McCartney-Francis, N., Allen, J. B., Mizel, D. E., Albina, J. E., Qiao-Wen, X., Nathan, C. F., and Wahl, S. M. (1993). Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* **178,** 749–754.

McGrath, P. A. (1989). Evaluating a child's pain. *J. Pain Symptom Manage.* **4,** 198–214.

Machelska, H., Labuz, D., Przewlicki, R., and Przewlocka, B. (1997a). Inhibition of nitric oxide synthase enhances antinociception mediated by μ, δ, and κ opioid receptors in acute and prolonged pain in the rat spinal cord. *J. Pharmacol. Exp. Ther.* **282,** 977–984.

Machelska, H., Ziolkowska, B., Mika, J., Przewlocka, B., and Przewlocki, R. (1997b). Chronic morphine increases biosynthesis of nitric oxide synthase in the rat spinal cord. *Neuroreport* **8,** 2743–2747.

Machelska, H., Cabot, P. J., Mousa, S. A., Zhang, Q., and Stein, C. (1998). Pain control in inflammation governed by selectins. *Nat. Med.* **4,** 1425–1428.

McInnis, I. B., Leung, B., Wei, X.-Q., Gemmell, C., and Liew, F. Y. (1998). Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase. *J. Immunol.* **160,** 308–315.

Meller, S. T., Dykstra, C., and Gebhart, G. F. (1992). Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for *N*-methyl-D-aspartate-produced facilitation of the nociceptive tail-flick reflex. *Eur. J. Pharmacol.* **214,** 93–96.

Meller, S. T., Cummings, C. P., Graub, R. J., and Gebhart, G. F. (1994). The role of nitric oxide in the development and maintenance of the hyperalgesia produced by intraplantar injection of carrageenan in the rat. *Neuroscience* **60,** 367–374.

Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997). IKK-1 and IKK-2: Cytokine-activated IκB kinases essential for NF-κB activation. *Science* **278,** 860–866.

Millan, J. M., Czlonkowski, A., Morris, B., Stein, C., Arendt, R., Huber, A., Hollt, V., and Herz, A. (1988). Inflammation of the hind limb as a model of unilateral localized pain; influence on multiple opioid systems in the spinal cord of the rat. *Pain* **35,** 299–312.

Montrucchio, G., Lupia, E., De Martino, A., Battaglia, E., Arese, M., Tizzani, A., Bussolino, F., and Camussi, G. (1997). Nitric oxide mediates angiogenesis induced in vivo by platelet-activating factor and tumor necrosis factor-α. *Am. J. Pathol.* **151,** 557–563.

Moore, P. K., Oluyomi, A. O., Babbedge, R. C., Wallace, P., and Hart, S. L. (1991). L-N^G-nitro arginine methyl ester exhibits antinociceptive activity in the mouse. *Br. J. Pharmacol.* **102,** 198–202.

Moore, P. K., Wallace, P., Gaffen, Z., Hart, S. L., and Babbedge, R. C. (1993). Characterization of the novel nitric oxide synthase inhibitor 7-nitroindazole and related indazoles: Antinociceptive and cardiovascular effects. *Br. J. Pharmacol.* **110,** 219–224.

Morgan, C. V. J., Babbedge, R. C., Gaffen, Z. G., Wallace, P., Hart, S. L., and Moore, P. K. (1992). Synergistic anti-nociceptive effect of L-N^G-nitroarginine methyl ester (L-NAME) and flurbiprofen in the mouse. *Br. J. Pharmacol.* **105,** 493–497.

Munroe, D. G., Wang, E. Y., MacIntyre, J. P., Tam, S. S., Lee, D. H., Taylor, G. R., Zhou, L., Plante, R. K., Kazmi, S. M., and Bauerle, P. A. (1995). Novel intracellular signaling function of prostaglandin H synthase-1 in NF-κB activation. *J. Inflamm.* **45,** 260–268.

Murray, C. W., Porreca, F., and Cowan, A. (1988). Methodological refinements to the mouse paw formalin test. *J. Pharmacol. Methods* **20,** 175–186.

Nozaki-Taguchi, N., and Yamamoto, T. (1998a). The interaction of FK409, a novel nitric oxide releaser, and peripherally administered morphine during experimental inflammation. *Anesth. Analg.* **86,** 367–373.

Nozaki-Taguchi, N., and Yamamoto, T. (1998b). Involvement of nitric oxide in peripheral antinociception mediated by κ- and δ-opioid receptors. *Anesth. Analg.* **87,** 388–393.

Odeh, M. (1997). Short analytical review: New insights into the pathogenesis and treatment of rheumatoid arthritis. *Clin. Immunol. Immunopathol.* **83,** 103–116.

Oyanagui, Y. (1994). Nitric oxide and superoxide radical are involved in both initiation and development of adjuvant arthritis in rats. *Life Sci.* **54,** 285–289.

Pajewski, T. N., DeFazio, C. A., Moscicki, J. C., and Johns, R. A. (1996). Nitric oxide synthase inhibitors, 7-nitroindazole and nitro^G-L-arginine methyl ester, dose dependently reduce the threshold for isoflurane anesthesia. *Anesthesiology* **85,** 1111–1119.

Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994). The ubiquitin–proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78,** 773–785.

Palombella, V. J., Conner, E. M., Fuseler, J. W., Destree, A., Davis, J. M., Laroux, F. S., Wolf, R. E., Huang, J., Brand, S., Elliott, P. J., Lazarus, D., McCormack, T., Parent, L., Stein, R., Adams, J., and Grisham, M. B. (1998). Role of the proteasome and NF-κB in streptococcal wall-induced polyarthritis. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15671–15676.

Papapetropoulos, A., Garcia-Cardena, G., Madri, J. A., and Sessa, W. C. (1997). Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100,** 3131–3139.

Pataki, I., and Telegdy, G. (1998). Further evidence that nitric oxide modifies acute and chronic morphine actions in mice. *Eur. J. Pharmacol.* **357,** 157–162.

Peng, H. B., Libby, P., and Liao, J. K. (1995). Induction and stabilization of IκBx by nitric oxide mediates inhibition of NF-κB. *J. Biol. Chem.* **270,** 14214–14219.

Perkins, D. J., St. Clair, E. W., Misukonis, M. A., and Weinberg, J. B. (1998). Reduction of NOS2 overexpression in rheumatoid arthritis patients treated with anti-tumor necrosis factor α monoclonal antibody (cA2). *Arthritis Rheum.* **41,** 2205–2210.

Pizziketti, R. J., Pressman, N. S., Geller, E. B., Cowan, A., and Adler, M. W. (1985). The rat cold water tail-flick: A novel analgesic test that distinguishes opioid agonists from mixed agonist–antagonists. *Eur. J. Pharmacol.* **119,** 23–29.

Pozza, M., Bettelli, C., Magnani, F., Mascia, M. T., Manzini, E., and Calza, L. (1998). Is neuronal nitric oxide involved in adjuvant-induced joint inflammation? *Eur. J. Pharmacol.* **359,** 87–93.

Qian, Y., Chao, D. S., Santillano, D. R., Cornwell, T. L., Nairn, A. C., Greengard, P., Lincoln, T. M., and Bredt, D. S. (1996). cGMP-Dependent protein kinase in dorsal root ganglion: Relationship with nitric oxide synthase and nociceptive neurons. *J. Neurosci.* **16,** 3130–3138.

Radomski, M. W., and Moncada, S. (1993). Regulation of vascular homeostasis by nitric oxide. *Thromb. Haemostasis* **70,** 36–41.

Ralevic, V., Khalil, Z., Helme, R. D., and Dusting, G. J. (1995). Role of nitric oxide in the actions of substance P and other mediators of inflammation in rat skin microvasculature. *Eur. J. Pharmacol.* **284,** 231–239.

Randall, L. O., and Selitto, J. J. (1957). A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther.* **4,** 409–419.

Read, M. A., Neish, A. S., Luscinskas, F. W., Palombella, V. J., Maniatis, T., and Collins, T. (1995). The proteasome pathway is required for cytokine-induced endothelial–leukocyte adhesion molecule expression. *Immunity* **2,** 493–506.

Ridger, V. C., Greenacre, S. A. B., Handy, R. L. C., Halliwell, B., Moore, P. K., Whiteman, M., and Brain, S. D. (1997). Effect of peroxynitrite on plasma extravasation, microvascular blood flow and nociception in the rat. *Br. J. Pharmacol.* **122,** 1083–1088.

Roche, A. K., Cook, M., Wilcox, G. L., and Kajander, K. C. (1996). A nitric oxide synthesis inhibitor (L-NAME) reduces licking behavior and Fos-labeling in the spinal cord of rats during formalin-induced inflammation. *Pain* **66,** 331–341.

Sakiniene, E., Bremell, T., and Tarkowski, A. (1997). Inhibition of nitric oxide synthase (NOS) aggravates *Staphylococcus aureus* septicaemia and septic arthritis. *Clin. Exp. Immunol.* **110,** 370–377.

Salvemini, D., Manning, P. T., Zweifel, B. S., Seibert, K., Connor, J., Currie, M. G., Needleman, P., and Masferrer, J. L. (1995). Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. *J. Clin. Invest.* **96,** 301–398.

Salvemini, D., Wang, Z.-Q., Wyatt, P. S., Bourdon, D. M., Marino, M. H., Manning, P. T., and Currie, M. G. (1996). Nitric oxide: A key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.* **118,** 829–838.

Santos, L. L., Morand, E. F., Yang, Y., Hutchinson, P., and Holdsworth, S. R. (1997). Suppression of adjuvant arthritis and synovial macrophage inducible nitric oxide by *N*-iminoethyl-L-ornithine, a nitric oxide synthase inhibitor. *Inflammation* **21,** 299–311.

Schimmer, R. C., Schrier, D. J., Flory, C. M., Dykens, J., Tung, D. K. L., Jacobson, P. B., Friedl, H. P., Conroy, M. C., Schimmer, B. B., and Ward, P. A. (1997). Streptococcal cell wall-induced arthritis. *J. Immunol.* **159,** 4103–4108.

Schmidt, K. N., Amstad, P., Cerutti, P., and Baeuerle, P. A. (1995). The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB. *Chem. Biol.* **2,** 13–22.

Schreck, R., Rieber, P., and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of NF-κB transcription factor and HIV-1. *EMBO J.* **10,** 2247–2258.

Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992a). Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med.* **175,** 11810.

Schreck, R., Albermann, K., and Baeuerle, P. A. (1992b). NF-κB: An oxidative stress-responsive transcription factor of eukaryotic cells. *Free Radical Res. Commun.* **17,** 221–237.

Schulze-Koops, H., Lipsky, P. E., Kavanaugh, A. F., and Davis, L. S. (1995). Elevated Th1- or Th2-like cytokine mRNA in peripheral circulation of patients with rheumatoid arthritis. *J. Immunol.* **155,** 5029–5037.

Schulze-Osthoff, K., Beyaert, R., Vandervoorde, V., Haegeman, G., and Fiers, W. (1993). Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J.* **12,** 3095–3104.

Seltzer, Z., Dubner, R., and Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* **43,** 205–218.

Semos, M. L., and Headley, P. M. (1994). The role of nitric oxide in spinal nociceptive reflexes in rats with neurogenic and non-neurogenic peripheral inflammation. *Neuropharmacology* **33,** 1487–1497.

Sen, C. K., and Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *FASEB J.* **10,** 709–720.

Sewell, R. D. E., and Spencer, P. S. J. (1976). Antinociceptive activity of narcotic agonist and partial agonist analgesics and other agents in the tail-immersion test in mice and rats. *Neuropharmacology* **15,** 683–688.

Shibuta, S., Mashimo, T., Ohara, A., Zhang, P., and Yoshiya, I. (1995). Intracerebroventricular administration of a nitric oxide-releasing compound, NOC-18, produces thermal hyperalgesia in rats. *Neurosci. Lett.* **187,** 103–106.

Shibuta, S., Mashimo, T., Ohara, P. Z. A., and Yoshiya, I. (1996). A new nitric oxide donor, NOC-18, exhibits a nociceptive effect in the rat formalin model. *J. Neurol. Sci.* **141,** 1–5.

Shingu, M., Takahashi, S., Ito, M., Hamamatsu, N., Suenaga, Y., Ichibangase, Y., and Nobunaga, M. (1994). Anti-inflammatory effects of recombinant human manganese superoxide dismutase on adjuvant arthritis in rats. *Rheumatol. Int.* **14,** 77–81.

Skaleric, U., Allen, J. B., Smith, P. D., Mergenhagen, S. E., and Wahl, S. M. (1991). Inhibitors of reactive oxygen intermediates suppress bacterial cell wall-induced arthritis. *J. Immunol.* **147,** 2559–2564.

Smith, S. D., Wheeler, M. A., Harris, E., Foster, J., and Weiss, R. M. (1997). Improvement in interstitial cystitis symptom scores during treatment with oral L-arginine. *J. Urol.* **158,** 703–708.

Song, H.-K., Pan, H.-L., and Eisenach, J. C. (1998). Spinal nitric oxide mediates antinociception from intravenous morphine. *Anesthesiology* **89,** 215–221.

Steel, J. H., Terenghi, G., Chung, J. M., Na, H. S., Carlton, S. M., and Polak, J. M. (1994). Increased nitric oxide synthase immunoreactivity in rat dorsal root ganglia in a neuropathic pain model. *Neurosci. Lett.* **169,** 81–84.

Stefanovic-Racic, M., Meyers, K., Meschter, C., Coffey, J. W., Hoffman, R. A., and Evans, C. H. (1994). *N*-monomethyl arginine, an inhibitor of nitric oxide synthase, suppresses the development of adjuvant arthritis in rats. *Arthritis Rheum.* **37,** 1062–1069.

Stefanovic-Racic, M., Meyers, K., Meschter, C., Coffey, J. W., Hoffman, R. A., and Evans, C. H. (1995). Comparison of the nitric oxide synthase inhibitors methylarginine and aminoguanidine as prophylactic and therapeutic agents in rat adjuvant arthritis. *J. Rheumatol.* **22,** 1922–1928.

Stichtenoth, D. O., and Frolich, J. C. (1998). Nitric oxide and inflammatory joint diseases. *Br. J. Rheumatol.* **37,** 246–257.

Stimmel, B. (1997). The effects of emotion on pain. *In* ''Pain and Its Relief without Addiction'' (B. Stimmel, ed.), pp. 75–89. Hawarth Medical Press, New York.

Stolov, W. C. (1990). Electrodiagnostic evaluation of acute and chronic pain syndromes. *In* ''The Management of Pain'' (J. J. Bonica, ed.), Vol. 1, pp. 622–640. Lea & Fegiber, Philadelphia, Pennsylvania.

Storgard, C. M., Stupack, D. G., Jonczyk, A., Goodman, S. L., Fox, R. I., and Cheresh, D. A. (1999). Decreased angiogenesis and arthritic disease in rabbits treated with an $\alpha_v\beta_3$ antagonist. *J. Clin. Invest.* **103,** 3–4.

Stratman, N. C., Carter, D. B., and Sethy, V. H. (1997). Ibuprofen: Effect on inducible nitric oxide synthase. *Mol. Brain Res.* **50,** 107–112.

Suzuki, Y., Wang, W., Vu, T. H., and Raffin, T. A. (1992). Effect of NADPH oxidase inhibition on endothelial cell ELAM-1 mRNA expression. *Biochem. Biophys. Res. Commun.* **184,** 1339–1343.

Tal, M. (1996). A novel antioxidant alleviates heat hyperalgesia in rats with an experimental painful peripheral neuropathy. *NeuroReport* **7,** 1382–1384.

Tanaka, S., Matsui, T., Murakami, T., Ishizuka, T., Sugiura, M., Kawashima, K., and Sugita, T. (1998). Immunological abnormality associated with the augmented nitric oxide synthesis in adjuvant-induced arthritis. *Int. J. Immunopharmacol.* **20,** 71–84.

Tassorelli, C., Joseph, S. A., and Napp, G. (1997). Neurochemical mechanisms of nitroglycerin-induced neuronal activation in rat brain: A pharmacological investigation. *Neuropharmacology* **36,** 1417–1424.

Thadani, U. (1997). Oral nitrates: More than symptomatic therapy in coronary artery disease? *Cardiovasc. Drugs Ther.* **11,** 213–218.

Thomas, D. A., Ren, K., Bese, D., Ruda, M. A., and Dubner, R. (1996). Application of nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester, on injured nerve attenuates neuropathy-induced thermal hyperalgesia in rats. *Neurosci. Lett.* **210,** 124–126.

Thomsen, L. L., and Olesen, J. (1998). Nitric oxide theory of migraine. *Clin. Neurosci.* **5,** 28–33.

Thorat, S. N., and Bhargava, H. N. (1994). Effects of NMDA receptor blockade and nitric oxide synthase inhibition on the acute and chronic actions of Δ^9-tetrahydrocannabinol in mice. *Brain Res.* **667,** 77–82.

Tonussi, C. R., and Ferreira, S. H. (1994). Mechanism of doclofenac analgesia: Direct blockade of inflammatory sensitization. *Eur. J. Pharmacol.* **251,** 173–179.

Traub, R. J., Solodkin, A., Meller, S. T., and Gebhart, G. F. (1994). Spinal cord NADPH-diaphorase histochemical staining but not nitric oxide synthase immunoreactivity increases following carrageenan-produced hindpaw inflammation in the rat. *Brain Res.* **668,** 204–210.

Trinchieri, G. (1998). Interleukin-12: A cytokine at the interface of inflammation and immunity. *Adv. Immunol.* **70,** 83–243.

Van de Loo, F. A. J., Arntz, O. J., Van Enckevort, F. H. J., Van Lent, P. L. E. M., and Van Den Berg, W. B. (1998). Reduced cartilage proteoglycan loss during zymosan-induced gonarthritis in NOS2-deficient mice and in anti-interleukin-1-treated wild-type mice with unabated joint inflammation. *Arthritis Rheum.* **41,** 634–646.

Vaupel, D. B., Kimes, A. S., and London, E. D. (1997). Further in vivo studies on attenuating morphine withdrawal: Isoform-selective nitric oxide synthase inhibitors differ in efficacy. *Eur. J. Pharmacol.* **324,** 11–20.

Verissimo de Mello, S. B., Novaes, G. S., Laurindo, I. M. M., Muscara, M. N., de Barros Maceil, F. M., and Cossermelli, W. (1997). Nitric oxide synthase inhibitor influences prostaglandin and interleukin-1 production in experimental arthritic joints. *Inflammation Res.* **46,** 72–77.

Villarete, L. H., and Remick, D. G. (1995). Nitric oxide regulation of IL-8 expression in human endothelial cells. *Biochem. Biophys. Res. Commun.* **211,** 671–676.

Vocci, F. J., Petty, S. K., and Dewey, W. L. (1978). Antinociceptive action of the butyryl derivatives of cyclic guanosine 3':5'-monophosphate. *J. Pharmacol. Exp. Ther.* **207,** 892–898.

Vyklicky, L. (1979). Techniques for the study of pain in animals. *In* ''Advances in Pain Research and Therapy'' (J. J. Bonica, ed.), Vol. 3, pp. 727–745. Raven, New York.

Weber, C., Erl, W., Pietsch, A., Strobel, M., Ziegler-Heitbrock, H. W., and Weber, P. C. (1994). Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. *Arterioscler. Thromb.* **14,** 1665–1673.

Wei, E. P., Moskowita, M. A., Boccalini, P., and Kontos, H. A. (1992). Calcitonin gene-related peptide mediates nitroglycerin and sodium nitroprusside-induced vasodilation in feline cerebral arterioles. *Circ. Res.* **70,** 1313–1319.

Weinberg, J. B., Granger, D. L., Pisetsky, D. S., Seldin, M. F., Misukonis, M. A., Mason, S. N., Pippen, A. M., Ruiz, P., Wood, E. R., and Gilkeson, G. S. (1994). The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: Increased nitric oxide production and nitric oxide synthase expression in MRL-*lpr/lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J. Exp. Med.* **179,** 651–660.

Weyand, C. M., and Goronzy, J. J. (1997). Pathogenesis of rheumatoid arthritis. *Adv. Rheumatol.* **81,** 29–55.

Wheeler, M. A., Smith, S. D., Saito, N., H. E., Foster, J., and Weiss, R. M. (1997). Effect of long-term oral L-arginine on the nitric oxide synthase pathway in the urine from patients with interstitial cystitis. *J. Urol.* **158,** 2045–2050.

Wheeler-Aceto, H., and Cowan, A. (1991). Standardization of the rat paw formalin test for the evaluation of analgesics. *Psychopharmacology* **104,** 35–44.

Wiesenfeld-Hallin, Z., Hao, J.-X., Xu, X.-J., and Hokfelt, T. (1993). Nitric oxide mediates ongoing discharges in dorsal root ganglion cells after peripheral nerve injury. *J. Neurophysiol.* **70,** 2350–2353.

Wilcox, G. L. (1988). Pharmacological studies of grooming and scratching behavior elicited by spinal substance P and excitatory amino acids. *Ann. N.Y. Acad. Sci.* **525,** 228–236.

Wilcox, G. L. (1991). Excitatory neurotransmitters and pain. *Proc. VIth World Congr. Pain,* 97–117.

Wilder, R. L., Allen, J. B., Wahl, L. M., Calandra, G. B., and Wahl, S. M. (1983). The pathogenesis of group A streptococcal cell wall-induced polyarthritis in the rat: Comparative studies in arthritis-resistant and -susceptible inbred rat strains. *Arthritis Rheum.* **26,** 1442–1451.

Wong, C.-S., Cherng, C.-H., and Tung, C.-S. (1998). Intrathecal administration of excitatory amino acid receptor antagonist or nitric oxide synthase inhibitor reduced autotomy behavior in rats. *Anesth. Analg.* **87,** 605–608.

Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997). IκB kinase-b: NF-κB activation and complex formation with IκB kinase-a and NIK. *Science* **278,** 866–869.

Wu, J., Lin, Q., McAdoo, D. J., and Willis, W. D. (1998a). Nitric oxide contributes to central sensitization following intradermal injection of capsaicin. *NeuroReport* **9,** 589–592.

Wu, J., Lin, Q., Lu, Y., Willis, W. D., and Westlund, K. N. (1998b). Changes in nitric oxide synthase isoforms in the spinal cord of rat following induction of chronic arthritis. *Exp. Brain Res.* **118,** 457–465.

Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269,** 4705–4708.

Xu, J. Y., and Tseng, L. F. (1993). Increase of nitric oxide by L-arginine potentiates β-endorphin- but not μ-, δ-, or κ-opioid agonist-induced antinociception in the mouse. *Eur. J. Pharmacol.* **236,** 137–142.

Xu, J. Y., and Tseng, L. F. (1995). Nitric oxide/cyclic guanosine monophosphate system in the spinal cord differentially modulates intracerebroventricularly administered morphine- and β-endorphin-induced antinociception in the mouse. *J. Pharmacol. Exp. Ther.* **274,** 8–16.

Xu, X. J., Ao, J. X., Aldskogius, H., Seiger, A., and Wiesenfeld-Hallin, Z. (1992). Chronic pain-related syndrome in rats after ischemic spinal cord lesion: A possible animal model for pain in patients with spinal cord injury. *Pain* **48,** 279–290.

Xu, Z., Tong, C., Pan, H. L., Cerda, S. E., and Eisenach, J. C. (1997). Intravenous morphine increases release of nitric oxide from spinal cord by an α-adrenergic and cholinergic mechanism. *J. Neurophysiol.* **78,** 2072–2078.

Yamaguchi, H., and Naito, H. (1996). Antinociceptive synergistic interaction between morphine and *N*-$^{\omega}$-nitro-L-arginine methyl ester on thermal nociceptive tests in the rat. *Can. J. Anaesth.* **43,** 975–981.

Ye, S. F., and Malik, A. B. (1997). *In vivo* inhibition of nuclear factor-κB activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. *J. Immunol.* **159,** 3976–3983.

Yoon, Y. W., Sung, B., and Chung, J. M. (1998). Nitric oxide mediates behavioral signs of neuropathic pain in an experimental rat model. *NeuroReport* **9,** 367–372.

Zahn, P. K., and Brennan, T. J. (1998). Lack of effect of intrathecally administered *N*-methyl-D-aspartate receptor antagonists in a rat model for postoperative pain. *Anesthesiology* **88,** 143–156.

Zhang, X., Verge, V., Wiesenfeld-Hallin, S., Ju, G., Bredt, D., Synder, S. H., and Hokfelt, T. (1993). Nitric oxide synthase-like immunoreactivity in lumbar dorsal root ganglia and spinal cord of rat and monkey and effect of peripheral axotomy. *J. Comp. Neurol.* **335,** 563–575.

CHAPTER 54

Nitric Oxide and Cancer

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CARCINOGENESIS IS A MULTISTEP PROCESS. IT STARTS WITH ACCUMULATION OF MUTATIONS CAUSED BY ENVIRONMENTAL OR HEREDITARY FACTORS THAT LEAD TO MALIGNANT CELL TRANSFORMATION, LOCAL INVASION, AND METASTASIS TO DISTANT SITES. THE MAIN TARGETS OF MUTAGENESIS ARE GENES THAT PROMOTE OR INHIBIT CELL GROWTH AND GENES THAT CONTROL DNA REPAIR AND APOPTOSIS.

THE ROLE OF NITRIC OXIDE (NO) IN CARCINOGENESIS, TUMOR PROGRESSION, AND CANCER THERAPY IS MULTIFACETED AND DEPENDENT ON A VARIETY OF CONDITIONS THAT EXIST IN THE CELL MILIEU. NO HAS BEEN SHOWN TO HAVE BOTH TUMORICIDAL AND TUMORIGENIC EFFECTS, AND MODULATION OF NO LEVELS IN TUMOR MICROCIRCULATION CAN AFFECT TUMOR HEMODYNAMICS AND CANCER THERAPY. TUMOR SURVIVAL DEPENDS ON ADEQUATE BLOOD FLOW TO SUPPLY NUTRIENTS AND OXYGEN AND TO MAINTAIN A SUITABLE PH. NO PRODUCED BY THE TUMOR CELLS MAINTAINS THESE OPTIMAL CONDITIONS, BUT IT ALSO REDUCES THEIR ABILITY TO GROW AND METASTASIZE BY INHIBITING DNA SYNTHESIS AND MITOCHONDRIAL RESPIRATION. HIGH NO LEVELS ARE GENERALLY TUMORICIDAL, AND THIS IS A THERAPEUTICAL APPROACH THAT CAN BE A USED FOR TUMOR CELLS THAT ARE SUSCEPTIBLE TO NO-MEDIATED APOPTOSIS. IT IS POSSIBLE THAT THE MAJORITY OF TUMOR CELLS CONTAINING HIGH NO LEVELS UNDERGO AUTOLYSIS, AND THE NO-RESISTANT ONES ARE THEN ABLE TO USE NO TO THEIR ADVANTAGE AND PROMOTE TUMOR PROGRESSION AND METASTASIS. THE ANTITUMOR ACTIVITY OF NO INCLUDES INHIBITION OF TUMOR CELL PROLIFERATION, PROMOTION, AND DIFFERENTIATION AND INCLUDES REDUCTION OF THE METASTATIC SPREAD IN CERTAIN TYPES OF CANCERS. THE ANTIMETASTATIC EFFECT OF NO OCCURS EITHER BY APOPTOSIS OR BY ALTERATIONS IN THE GENES THAT REGULATE METASTASIS. ANGIOGENESIS AND APOPTOSIS CAN ALSO BE REGULATED BY NO, WHICH HAS BEEN SHOWN TO BE BOTH AN INHIBITOR AND STIMULANT OF THESE PROCESSES.

THUS NO HAS THE UNIQUE ABILITY TO INITIATE AND/OR TO ARREST TUMOR FORMATION. ULTIMATELY, THE ROLE OF NO IN CANCER IS DETERMINED BY THE INTERACTION BETWEEN THE PROTECTIVE AND DESTRUCTIVE SIGNALING PATHWAYS IN A GIVEN CELL MILIEU.

Introduction

Nitric oxide (NO) is an essential molecule that mediates a number of cellular events. It has regulatory functions in the vascular and nervous systems, along with cytoprotective or cytotoxic actions that occur in inflammatory responses to infection, cancer, and transplant rejection. Nitric oxide is clearly involved in the regulation of physiological homeostasis, and owing to its paradoxical abilities to protect and/or damage tissues, NO is involved in a number of pathophysiological states as well.

The role of NO in carcinogenesis, tumor progression, and cancer therapy is multifaceted and dependent on the cell microenvironment. NO may influence the fate of the host,

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either positively or negatively, depending on the circumstances in the tumor milieu. NO has been shown to have both tumoricidal and tumorigenic effects, and modulation of NO levels in tumor microcirculation can affect tumor hemodynamics and anticancer therapy.

Therefore, to understand these multiple roles of NO in carcinogenesis, one must be fully aware of the complexity of NO generation and chemistry in biological systems as well as the multistaged development and diversity of cancers.

Biochemistry and Chemistry of Nitric Oxide

Nitric oxide is produced endogenously via the oxidation of L-arginine. This reaction is carried out by a family of enzymes known as the NO synthases (NOS), which comprises at least three isoforms. Two constitutively present isoforms, endothelial (eNOS) and neuronal (nNOS), are Ca^{2+} and calmodulin-dependent, and produce low levels of NO. The inducible isoform (iNOS) has calmodulin tightly bound, and there is no requirement for Ca^{2+} to maintain its high output activity.

Nitric oxide is chemically and biologically unique. It possesses an unpaired electron and therefore is formally a free radical species. In spite of the fact that NO is a radical, it is inherently stable as a monomer. Much of its physiological chemistry is dominated by its ability to react with other biological radical species such as molecular oxygen (O_2) and superoxide (O_2^-). Nitric oxide can also serve as a strong ligand for biological, coordinating metals. Nitric oxide is a small, lipophilic, and highly diffusible molecule. The rapid diffusion of NO away from the generating cell has a strong biological effect on surrounding cells as well as on the originating cell. An additive effect will result when NO is produced by a large number of cells at an inflammation site, for example, that would markedly increase local NO concentration.

The physiological chemistry of NO is extremely complex (Fig. 1). The reaction of NO with O_2 and O_2-derived species generates a variety of reactive nitrogen oxide species (RNOS), which account for much of the indirect activity of NO (Fig. 1). These RNOS, along with the well-studied reactive oxygen species (ROS) (Tables I and II), are capable of performing oxidation, nitrosation, and nitration chemistry producing a diverse number of cellular effects (DNA damage, lipid peroxidation, protein degradation, thiol modifica-

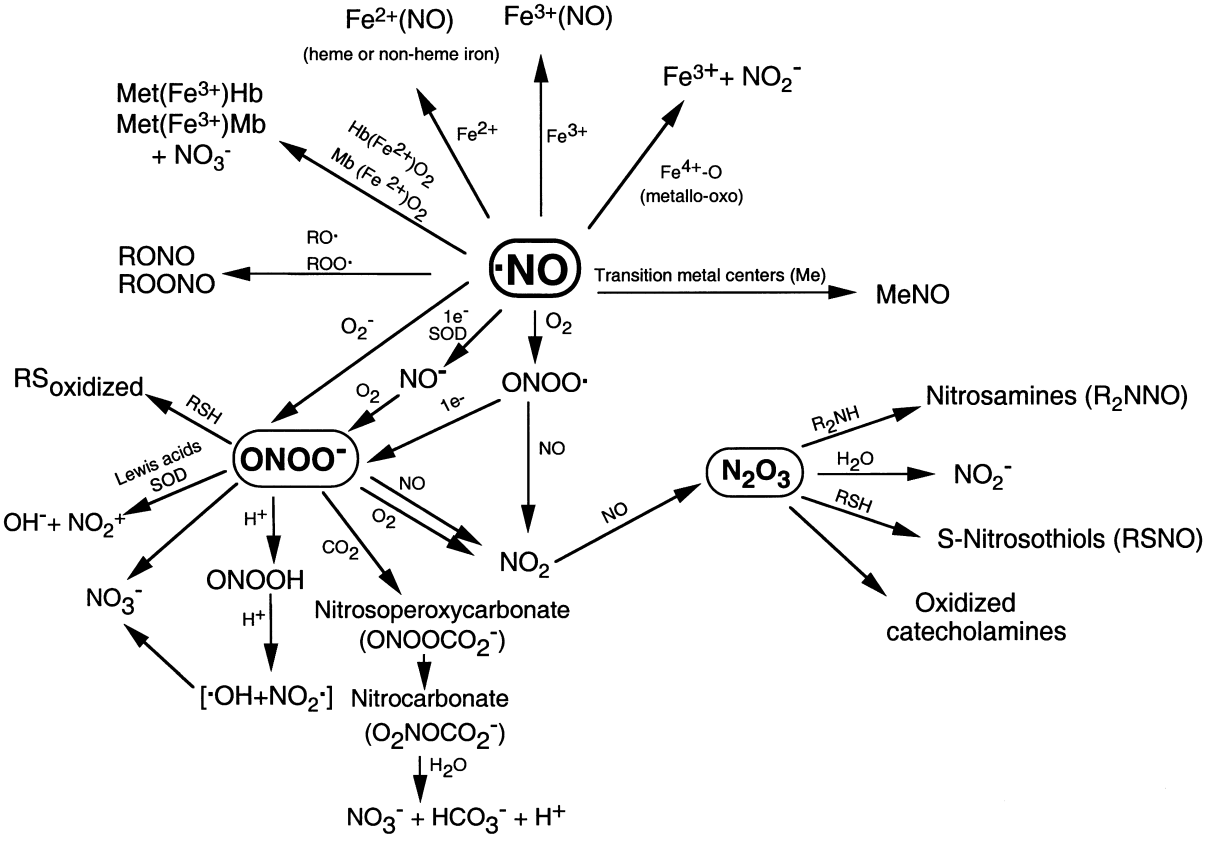


Figure 1 Diagrammatic representation of the products formed from direct and indirect reactions of nitric oxide. For reference regarding the chemistry and the nomenclature of the reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS), please see Tables I and II and Chapter 2 by J. M. Fukuto, J. Y. Cho, and C. H. Switzer, in this book.

Table I Reactive Oxygen Species (ROS)

Name	Formula
Alkoxyl radical	RO·
Hydroxyl radical	·OH
Hydroperoxyl radical	HO_2·
Hydrogen peroxide	H_2O_2
Hydrobromous acid	HOBr
Hypochlorous acid	HOCI
Ozone	O_3
Peroxyl radical	RO_2·
Singlet oxygen	$^1O_2$
Superoxide	$O_2^{\cdot-}$

tion, nitrosamine generation, nitrotyrosine formation, etc.). The occurrence of this type of chemistry can result in oxidative, nitrosative, and nitrative stresses in cells.

Indirect Effects of Nitric Oxide

One of the most widely studied RNOS is the product of the reaction of NO with $O_2^{\cdot-}$, peroxynitrite anion ($ONOO^-$) (Fig. 1, Table II). This species is capable of inducing all three forms of cellular stresses mentioned earlier. Under acidic conditions $ONOO^-$ is protonated ($pK_a = 6.8$) to form peroxynitrous acid, which decomposes rapidly to generate nitrate (NO_3^-). It is thought that reactive species such as nitrogen dioxide ($\cdot NO_2$), the highly reactive hydroxyl radical (HO·), and/or other highly oxidizing species can be generated on the decomposition pathway of $ONOO^-$ to NO_3^-. The generation of these reactive $ONOO^-$ decomposition intermediates may be responsible for the oxidizing nature of $ONOO^-$. In the presence of Lewis acids such as cupric ion, peroxynitrite can be cleaved heterolytically to generate hydroxide ion (OH^-) and nitronium ion (NO_2^+). This reaction has been proposed to be catalyzed by the antioxidant enzyme copper–zinc superoxide dismutase (Cu,Zn-SOD). If formed, NO_2^+ is a potent oxidant capable of nitrating protein tyrosine residues to form 3-nitrotyrosine. Indeed, the detection of nitrotyrosine has been used as a marker for $ONOO^-$ generation *in vivo* and *in vitro.* Peroxynitrite may also react with NO or O_2^- and form NO_2, which in turn, by reacting with NO, forms dinitrogen trioxide (N_2O_3), a strong nitrosating agent. Since $ONOO^-$ reacts further with its biosynthetic precursors, an excess of either NO or O_2^- may limit the lifetime of peroxynitrite. Also a number of factors that may interfere *in vivo* with the reaction of O_2^- and NO, such as SOD, oxyhemoglobin (HbO_2), or diffusion, may further limit peroxynitrite formation. The cellular sites most likely to produce peroxynitrite are mitochondria, where O_2^- is produced during aerobic respiration. Peroxynitrite is nucleophilic and can react with biological electrophiles. One of the most abundant biological electrophiles is carbon dioxide (CO_2). In fact, this reaction likely represents the predominant reaction pathway for biological $ONOO^-$. The adduct of $ONOO^-$ with CO_2, nitrosoperoxycarbonate ($ONOOCO_2^-$) is capable of decomposing to NO_3^- and nitrating tyrosine. Thus, reaction of $ONOO^-$ with CO_2 may represent a mechanism for degrading/activating $ONOO^-$ without the intermediacy of ONOOH.

Table II Reactive Nitrogen Oxide Species (RNOS)

Name	Formula
Alkyl peroxynitrites	ROONO
Dinitrogen trioxide	N_2O_3
Dinitrogen tetroxide	N_2O_4
Dinitrigen pentoxide	N_2O_5
Nitric oxide	NO·
Nitrite	NO_2^-
Nitrous acid	HNO_2
Nitrogen dioxide	NO_2·
Nitronium cation	NO_2^+
Nitrosonium ion	NO^+
Nitroxyl	HNO
Nitroxyl anion	NO^-
Nitrate radical	NO_3·
Nitrous oxide	N_2O
Peroxynitrite anion	$ONOO^-$
Peroxynitrite radical	ONOO·
Peroxynitrous acid	ONOOH

Reaction of NO with O_2 generates $\cdot NO_2$, which is a reactive radical and potent oxidant capable of inducing cytotoxicity. Nitrogen dioxide reacts readily with NO to form N_2O_3. This species is a potent nitrosating agent (i.e., it adds the elements of NO^+ to nucleophilic species) and may be biologically accessible under conditions of high NO production. Nitrosation of biological amines, to form nitrosamines, and nitrosation of thiols, to form *S*-nitrosothiols (RSNO), have been observed and may represent a crucial process in NO biology. Cellular consequences resulting from the reaction of NO with O_2- and O_2^--derived species are myriad and include potential DNA damage and cell death (Fig. 2).

As mentioned earlier, nitrosamine formation can result from the oxidation of NO to N_2O_3 followed by reaction with amine. Dinitrogen trioxide can also be generated from acidic nitrite (NO_2^-) and, in fact, is the preferred reagent for nitrosation in chemical synthesis. Nitrosamines are found in food, cigarette smoke, and can be generated via ingested nitrite, which forms N_2O_3 in the acidic conditions present in the stomach (Table XII). Activated immune cells also produce nitrosamines.

S-Nitrosothiols (RSNO) can be formed from the reaction of nitrosonium ion (NO^+), with thiols. Although NO^+ is, for the most part, biologically inaccessible, its chemical equivalents can be generated under physiological conditions. For example, N_2O_3 or select metal-bound nitrosyls are capable

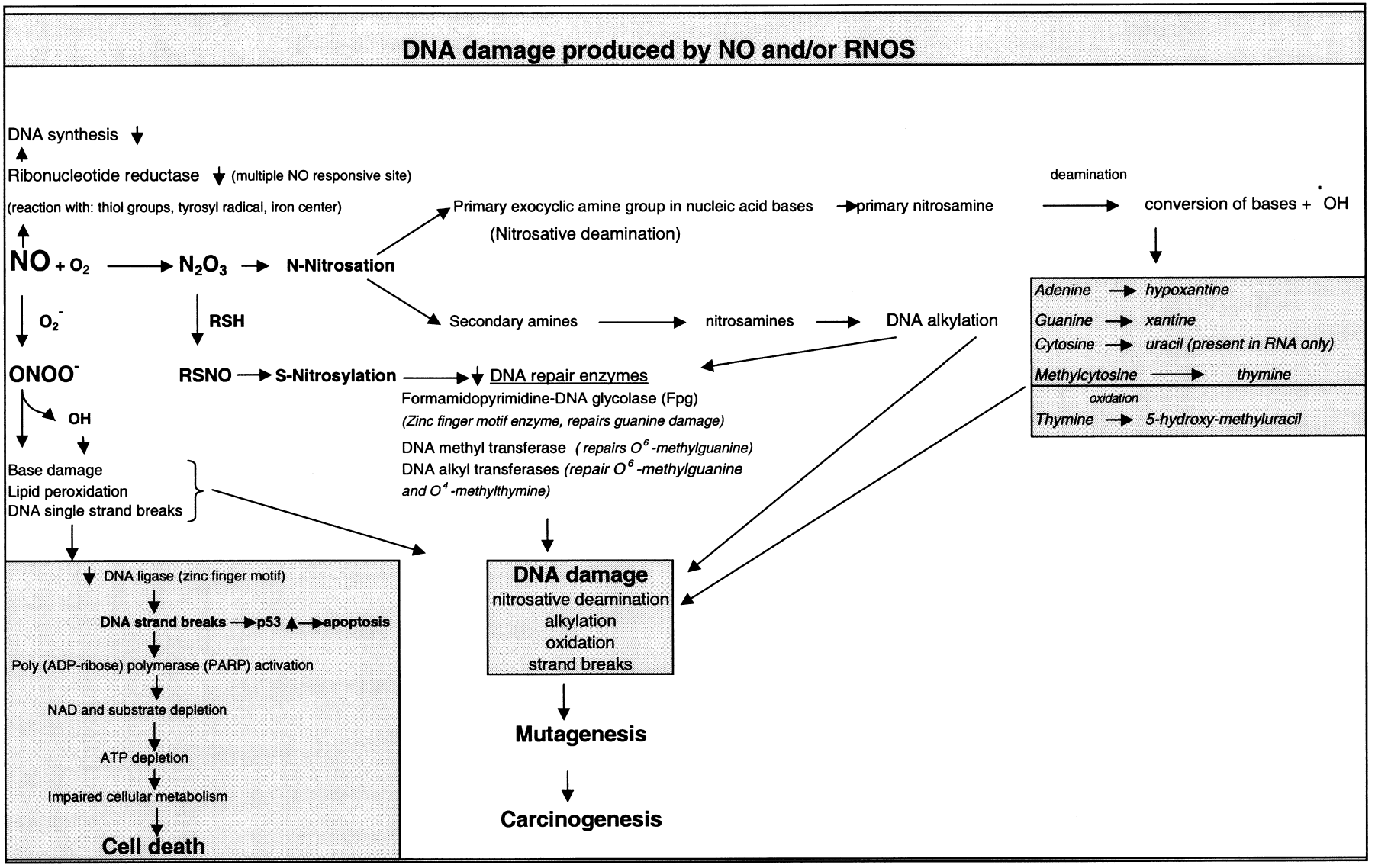


Figure 2 Effects of nitric oxide (NO) and RNOS on the nuclear DNA in mammalian cells.

of serving as a chemical equivalent to NO^+ and may participate in nitrosation chemistry. Nitrosation of thiols has multiple cellular effects ranging from modulation of signal transduction to carcinogenesis and cell death. S-Nitrosylation of enzymes can produce conformational changes that affect the enzyme–substrate interactions, and a number of enzymes that contain key sulfhydryl groups at their active sites are inhibited by S-nitrosylation (Table III).

The biochemical interrelationship of NO with RNOS and ROS is complex and, as expected, the cellular outcomes are highly dependent on the exact nature of these interactions. The reaction of NO with O_2^-, for example, may activate NO to produce a reactive and highly oxidizing species. Alternatively, the reaction of NO with O_2^- may decrease the biological activity of O_2^-, which may be cytotoxic on its own. Furthermore, reaction of NO with the highly toxic HO· would result in the generation of a much weaker oxidant, NO_2^-. Thus, it may be expected that NO can serve as either a cytoprotective or cytotoxic species, depending specifically on the cellular environment and the mechanism of toxicity. Indeed, it has been reported that NO can serve to protect cells from free radical damage as well as promote cellular demise.

CYTOTOXIC ROLE OF NITRIC OXIDE

The cytotoxicity of NO affects both cGMP-dependent and -independent mechanisms, and it contributes to the oxidative damage induced by ROS, alkylating agents, and free heavy metals ions. NO has the ability to release heme from some heme-containing proteins, such as the cytochrome P-450 family of enzymes. The released heme triggers the activation of heme oxygenase, which in turn hydrolyzes cellular heme complexes, and cytotoxic free iron is released inside the cell. NO, ROS, and RNOS can react with labile iron–sulfur centers to regulate the mitochondrial tricarboxylic acid cycle enzyme aconitase. This enzyme participates in fueling the electron transport chain, and its inhibition would lower cellular ATP production. Furthermore, cytosolic aconitase, a regulator of intracellular iron levels, is similarly modulated by NO and redox mechanisms. This enzyme is identical to iron regulatory protein-1 (IRP-1), formerly known as an iron-response element-binding protein (IRE-BP). IRP-1 regulates the expression of key proteins involved in iron homeostasis such as ferritin and transferrin. Low intracellular iron triggers the binding of IRP-1 to iron-responsive elements (IREs) in mRNAs encoding ferritin and transferrin receptor. Excess NO can also increase the IRP-1/IRE complex formation and increase iron uptake into the cell, while abolishing the aconitase activity of IRP-1. NO can also bind to transferrin receptors and cause iron loss from ferritin and further increase the levels of free cytotoxic intracellular iron. IRP-1 is also targeted by peroxynitrite, which irreversibly inhibits the aconitase activity and the binding of IRP-1 to IRE, thus decreasing the iron uptake into the cell. It is possible that IRP-1-like proteins may be redox signaling regulators, and depending on local conditions, they may transduce NO or metal signals into gene expression. The biological effects of NO or peroxynitrite action on iron homeostasis can lead either to cytotoxicity owing to elevated free intracellular iron or to cytostasis owing to decreased intracellular iron, respectively. Moreover, cellular damage produced by oxidative and nitrosative stress may lead to activation of cellular proteases and nucleases and further damage the cells. Depletion of energy sources by nitrosative damage to the mitochondrial electron transport chain complexes I (NADH:ubiquinone oxidoreductase) and II (succinate:ubiquinone oxidoreductase), and by activation of poly(ADP-ribosyl) polymerase (PARP), also known as poly(ADP-ribosyl) synthetase (PARS), which supports high-energy phosphate metabolism, results in extensive cell injury and death. Consequently, the NO-mediated cytotoxicity is both beneficial and detrimental to the host in the sense that it destroys tumors or pathogens but also causes inflammation, cytokine-induced hypotension, immunosuppression, and carcinogenesis (Table IV).

As shown earlier in this chapter, the majority of NO-mediated cytotoxic effects are indirectly produced via NO interaction with ROS. Hydrogen peroxide (H_2O_2) and NO may affect the cellular redox balance, and their interaction may potentiate each other's cytotoxic effects. Cells that are resistant to H_2O_2 seem to be unaffected by the toxic effects of NO. H_2O_2 cytotoxicity is enhanced by the fact that NO inhibits catalase activity. Likewise, decreased intracellular levels of glutathione, glutathione peroxidase, and metallothionein enhance the cytotoxicity of both, NO and H_2O_2.

Table III Enzymes Inhibited by S-Nitrosylation

Alcohol dehydrogenase
Aldehyde dehydrogenase
Aldolase
Calpain
Cathepsin B
Creatine kinase
Cysteine proteases
Cytochrome P-450 aromatase
DNA repair enzymes (see Fig. 2)
Ecto-5'-nucleotidase
Glutathione peroxidase
Glutathione reductase
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Methionine synthase
NADPH oxidase (assembly but not activity)
Ornithine decarboxylase (ODC)
Phosphotyrosine protein phosphatase
Protein kinase C
Zinc finger motif DNA-binding enzymes (DNA ligase, formamidopyrimidine DNA-glycolase)

Table IV Cytotoxic Effects of NO and/or RNOS[a]

Antioxidant depletion → ascorbate and glutathione scavenging

Modulation of action of ROS, RNOS, toxic metals, and alkylating agents

Nitrosation of amines → nitrosamines

Key enzymes inactivated by S-nitrosylation: see Table III
- Other proteins affected by S-nitrosylation include actin, albumin, activator protein 1 (AP-1), DNA methyltransferases, glutathione, G proteins, NF-κB, protein kinase C, and tissue plasminogen activator (tPA)
- Poly(ADP-ribosyl) synthetase/polymerase deregulation (PARS/PARP) → depletion of cellular energy sources
- NAD dehydrogenase → ↓ ADP ribosylation of GAPDH
- ↓ Alkyl transferases
- ↓ Iron–sulfur centers
  - NADH:ubiquinone oxidoreductase (mitochondrial electron transport chain complex I)
  - NADH:succinate oxidoreductase (mitochondrial electron transport chain complex II)
  - Regulates cytosolic aconitase/IRP-1 (iron regulator protein-1) and binding to IREs (iron response elements)
  - Mitochondrial *cis*-aconitase (tricarboxylic acid cycle), supports high energy phosphate metabolism
  - Ferrochelatase → ↓ heme
  - Ferritin
  - Ribonucleotide reductase → ↓ DNA synthesis
  - Cyclooxygenase 1, 2
  - Cytochrome P-450 → drug metabolism and therapeutic gain (reversible inhibition)
  - Cytochrome P-450 → heme release → heme oxygenase activation → heme hydrolysis → ↑ free iron (irreversible inhibition)
  - Ribonucleotide reductase → ↓ DNA synthesis
  - Nitric oxide synthase
  - Cyclic AMP responsive element-binding protein (CREB)
  - Cytochrome *c* oxidase → ↓ mitochondrial electron transport → respiration → energy depletion

Interacts with:
- Fe^{2+} and free thiols → dinitrosyl–iron complex → depletes cellular iron (transport form of NO)
- Transition metals → metal NO adducts
- ↑ Release of iron from ferritin and transferrin
- ↑ Free intracellular transition metal ions → activation of cellular proteases and nucleases → ↑ cell damage
- ↑ Free intracellular heavy metal ions
- ↑ Free intracellular calcium

DNA damage (see also Fig. 2)
- ↑ DNA deamination
- ↓ DNA binding proteins
- ↑ DNA/RNA strand breakage
- ↑ DNA alkylation
- ↓ DNA repair enzymes
- ↓ Ribonucleotide reductase → ↓ DNA synthesis
- ↑ DNA oxidation
- ↓ Zinc–sulfur centers (zinc fingers)

Other effects
- ↓ Membrane receptors
- ↓ Ion transporters
- ↑ Lipid peroxidation
- ↑ Inflammation
- ↑ Hypotension
- ↑ Immunosuppression
- ↑ Carcinogenesis

[a] Cytotoxic effects include direct and/or indirect reactions (oxidation, nitrosation, and nitration). GAPD, glyceraldehyde-3-phosphate dehydrogenase; ADP, adenosine diphosphate; ↑, increased activity; ↓, decreased activity.

CYTOPROTECTIVE ROLE OF NITRIC OXIDE

At the other end of the spectrum NO can act as an antioxidant or cytoprotector, and the resistance of certain cell types to NO-mediated cytotoxicity is due a complex and effective defense arsenal. Superoxide dismutase, catalase, glutathione peroxidase, metal storage proteins, heme, and hemoglobin, plus a number of free-radical scavengers such as ascorbate, tocopherol, and glutathione, are all part of the cellular defense system, and NO is capable of influencing the activity of some of them. For example, intracellular glutathione has high affinity for RNOS, and its depletion markedly enhances NO cytotoxicity. Activator protein 1 (AP-1), a NO-controlled transcription factor, is the mediator of glutathione *S*-transferase expression. Additionally, NO may have a protective role by diverting O_2^- away from iron–sulfur clusters toward sulfhydryl centers, preferred by $ONOO^-$.

NO is also able to rapidly bind metallo–oxo complexes (strong oxidants, containing high valent metal complexes, formed from oxidation of metals by ROS such as hydrogen peroxide), lower their valent state and therefore, reduce their cytotoxicity. Thus, by limiting the availability of iron, by preventing the cytotoxic effects of superoxide and hydrogen peroxide, NO plays another one of its cytoprotective roles. This may take place by inhibition of heme oxidation and attenuation of DNA and lipid peroxidation. Accordingly, NO-mediated antibacterial, antiparasitic, and tumoricidal effects are cytoprotective to the host during the normal acute immune response (Table V).

Direct Effects of Nitric Oxide

NO is able to react directly with metals and metal-containing proteins, with free radicals, and with lipid peroxidation products to produce a wide range of biological effects, some of them in physiological and others in pathophysiological conditions. The resulting biological actions of NO may be regulatory, cytoprotective, and/or cytotoxic, as shown earlier, and are illustrated in Tables IV, V, and VI and elsewhere in this book.

Nitric Oxide Effects on DNA

NO has been shown to damage DNA in bacteria and mammalian cells. Elevated NO, RNOS, and ROS are the cause of cellular nitrosative, oxidative, and nitrative stresses that can produce a number of nucleic acid modifications, such as cross-linking of purine and pyrimidine bases and single-strand breaks in RNA and DNA, leading to mutagenesis, cancer, or cell death (Fig. 2, Table IV). Additionally, RNOS can damage DNA by nitrosating an exocyclic amine group in the nucleic acid bases to form a primary nitrosamine, which on deamination results in conversion of bases and generation of the highly reactive hydroxyl radical. These products of nitrosative deamination are elevated in the cells treated with lipopolysaccharide (LPS), and inhibition of iNOS reverses their formation, suggesting that NO is a strong contributor to the oxidative and nitrosative DNA damage leading to mutagenesis and carcinogenesis. DNA is considerably more susceptible to the nitrosative damage during replication and transcription, when it is single stranded. A large number of point mutations that result in G:C to A:T transitions in p53 and retinoblastma antioncogenes have been found in brain tumors, lymphomas, leukemias, and small-cell lung carcinomas. Deaminations of 5-methylcytosine to thymidine (G:C to T:A transversions) are seen in liver, lung, breast, and stomach cancers. Mutations at A:T base pairs were shown in esophageal cancer.

Furthermore, NO can modulate the action of other carcinogens, and it has been shown that nitrosamines produced by activated macrophages or hepatocytes can be converted to alkylating agents, which in the presence of NO cause additional DNA damage and perturb the normal function of DNA repair apparatus. NO inhibits DNA alkyl transferase by S-nitrosation of critical sulfhydryl residues and thus potentiates the toxicity of the alkylating agents. NO also modifies structurally proteins containing zinc finger motifs, resulting in loss of protein function (Table III, Fig. 2). Many of these enzymes are involved in transcription, replication, restriction, or DNA repair, and the NO-mediated release of Zn^{2+} from their molecule leads to structural changes that permanently inactivate them. Ribonucleotide reductase represents a multiple NO responsive site, and the reactions of NO with tyrosyl radicals, iron centers, and/or thiol groups inhibit the activity of this enzyme. As a result, DNA synthesis is dramatically diminished, and this cytostatic effect of NO is useful in anticancer therapy. In addition, $ONOO^-$, $\cdot NO_2$, and $HO\cdot$ cause DNA single-strand breaks that trigger activation of the DNA repair enzyme PARP and result in NAD^+ substrate depletion, inhibition of glycolytic and electron transport pathways, and diminished ATP production. Depletion of cellular energy sources leads to a rapid cell destruction and death (Fig. 2).

Prior to investigating the role of NO in cancer it is important to review the biological and molecular changes occurring in normal cells that lead to cell transformation and malignancy, as well as the factors that contribute to these changes.

Biological and Molecular Aspects of Carcinogenesis

Carcinogenesis is a multistep process starting with accumulation of mutations in somatic cells that alter cell morphology and growth patterns, leading to cell transformation, local invasion, and metastasis to distant sites. Environmental (chemicals, radiation, viruses) or hereditary factors are the cause of mutations that can result in malignant transformation. The main targets of mutagenesis are the growth promoting genes, growth inhibitory genes, and genes that control DNA repair and apoptosis.

Growth Promoting Genes (Proto-oncogenes)

Proto-oncogenes promote normal cell growth and differentiation (Fig. 3). The cell cycle starts with binding of a growth factor to its receptor on the cell membrane. This triggers a chain of events that leads to activation of the cell surface receptors, the inner membrane signaling proteins, and the cytosolic signal transduction pathways. The result is induction of nuclear factors that regulate DNA transcription, translation, and cell division. Additionally, a group of proteins known as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs), modulate reentry of resting cells into the cell cycle and the progression through the cell-cycle phases. The genes encoding the proteins responsible for each of these steps can be targeted by mutagenesis. Proto-oncogenes transformation into oncogenes takes place via structural and regulatory changes that result in accumulation of multiple abnormal genes producing inappropriate amounts of growth-stimulating protein (Fig. 4).

Table V Cytoprotective Effects of Nitric Oxide[a]

Antibacterial
Antiparasitic
Tumoricidal
↓ DNA synthesis by inactivating ribonucleotide reductase → ↑ tumor sensitization in radiation therapy
↓ DNA damage by Fenton reaction products
↓ Oxidative damage by hydroxylation
↓ Lipid peroxidation by reaction with: Hydroxyperoxyl radical in atherosclerosis Hydroxyperoxyl radical in lypoxygenase (NO also binds the nonheme iron at the active site) ROS, copper ions
↓ Rapid binding of metallo-oxo complexes and reduction of high valent metal toxicity
↓ Iron availability by: ↓ Ferrochelatase (places iron in the porphyrinic ring) → ↓ Heme availability, ↓ NOS Modulating iron uptake in transferrin receptor Modulating iron storage in ferritin protein ↓ Iron regulatory protein-1 (IRP-1) ↑ Binding of IRP-1 to iron regulatory elements (IREs) ↓ Iron release from ferritin by preventing NADPH oxidase assembly
Peroxynitrite formation inhibits IRP-1 and binding of IRP-1 to IREs → ↓ iron uptake → ↓ oxidative stress
↓ Cytochrome *c* oxidase and electron flow in the mitochondria → ↓ ROS
Antioxidant (interacts with the cell defense mechanisms) Free radical scavengers (ascorbate, tocopherols, glutathione) Superoxide dismutase (SOD) ↓ Catalase Glutathione peroxidase Glutathione *S*-transferase (AP-1 mediated under NO control) Glucose-6-phosphate dehydrogenase (G6PD)
Prevents dexamethasone-induced apoptosis
↓ Leukocyte adhesion and platelet aggregation and adhesion
↑ Bcl-2 via a cGMP-dependent mechanism
Diverts superoxide away from iron–sulfur centers toward sulfhydryl molecules preferred by peroxynitrite
Protects against H_2O_2 toxicity by inhibiting aconitase
Protects against TNF-α and IFN-γ toxicity
Protects cytokine-depleted eosinophils in peripheral blood by a cGMP-dependent mechanism
Heme-containing proteins: ↑ Cyclooxygenases 1, 2 ↑ Soluble guanylate cyclase ↓ Catalase ↓ Cyclooxygenases 1, 2 ↓ Nitric oxide synthase ↓ Oxyhemoglobin → methemoglobin ↓ Oxymyoglobin → metmyoglobin ↓ Peroxidase
Reacts with thiols → *S*-nitrosothiols
↓ Neutrophil NADPH oxidase (limits superoxide production)
↓ Vascular tone (activates soluble guanylate cyclase and calcium activated potassium channels)
Activates protein kinase signaling in activated macrophages

[a] ↑, increased activity; ↓, decreased activity; ROS, reactive oxygen species; NOS, nitric oxide synthase; TNF-α, tumor necrosis factor α; IFN-γ, γ-interferon.

Oncogenes are cancer causing genes derived from proto-oncogenes. Oncogenes encode oncoproteins, which are no longer able to respond to growth factors or other external signals during the cell cycle. Mutations in the proto-oncogenes that encode growth factors, growth factors receptors, and signaling proteins may ultimately lead to continuous delivery of mitogenic signals to the cells and have been associated with various types of cancers.

Table VI Nitric Oxide Effects on Heme-Containing Proteins[a]

Activates
↑ Cyclooxygenases 1, 2
↑ Soluble guanylate cyclase
Inactivates
↓ Catalase
↓ Cyclooxygenases 1, 2
↓ Cytochrome P-450
↓ Cytochrome *c* oxidase
↓ Heme oxygenase
↓ Indoleamine 2,3-dioxygenase
↓ Nitric oxide synthase
↓ Oxyhemoglobin → methemoglobin (NO inactivation in circulation)
↓ Oxymyoglobin → metmyoglobin (NO inactivation in circulation)
↓ Peroxidase

[a] ↑, increased activity; ↓, decreased activity.

Nuclear regulatory proteins of growth-related genes are the product of a family of oncogenes (*myc, myb, jun,* and *fos*) that when mutated are associated with malignancy.

Growth Inhibitory Genes

Growth inhibitory genes (antioncogenes, tumor suppressor genes) regulate multiple cellular events, and their products have different intracellular locations. Some are present in the nucleus (p53, Rb, p16, BRCA-1, BRCA-2, WT-1). APC is cytosolic, NF-1 and -2 are located in the inner plasma membrane and cytoskeleton, respectively, and transforming growth factor β (TGF-β) receptors are situated on the cell surface. All antioncogenes generate products that regulate cell proliferation. Antioncogenes associated with cancers undergo mutations of both alleles (loss of heterozygosity) and are known as recessive cancer genes. These genes are activated by extracellular growth inhibitors, which trigger the activation of a signaling pathway similar to that of proto-oncogenes.

The p53 gene is a nuclear phosphoprotein that regulates multiple cellular processes, including DNA transcription, DNA repair, angiogenesis, tumor progression, invasiveness and metastasis, genomic stability, cell cycle, senescence, and cell death. This gene has a half-life of several minutes and is not required for normal cell proliferation. p53 loss of heterozygosity is found in the majority of colon, breast, and lung cancers, and also in leukemias, sarcomas, and neurogenic tumors. Exposure to mutagens leads to p53 stabilization and accumulation into the nucleus, where it binds to DNA and arrests the cell cycle in G_1 phase, allowing the cell time to repair the damaged DNA. If the repair mechanism fails, the p53 triggers the apoptotic mechanism to kill that cell.

Consequently, loss of normal p53 function allows the damaged DNA to be replicated and to permanently add the mutations to the genome. Cumulative addition of mutations to the genome, as a result of loss of p53 pathway activity, may ultimately lead to malignant transformation. Transforming proteins of DNA viruses (Table XIV) can also bind and sequester both normal p53 and Rb genes and further increase the risk of malignancy. Additionally, p53 has the ability to function both as an oncogene and antioncogene, owing to the fact that a single-allele mutation in p53 can bind and inactivate normal p53 protein. Functional inactivation of p53 occurs in the majority of human cancers and also in the tumor resistance to the DNA-damaging effects of chemotherapy and radiation therapy.

Mutations in the Rb gene, produce retinoblastoma in children. The Rb gene product, pRB, is an omnipresent nuclear phosphoprotein that regulates the cell cycle. Mutations in the Rb gene generally affect the regulation by the transcription factor-binding domain that leads to erratic cycling and malignant transformation.

DNA Repair Regulatory Genes

Genes that regulate the repair of damaged DNA and affect indirectly cell proliferation, include proto-oncogenes, antioncogenes, and apoptosis-regulating genes. The DNA repair regulatory genes are not oncogenic by themselves, but they allow replication errors in other genes to become permanent (Fig. 2).

Cyclins and Cyclin-Dependent Genes

Cyclins and CDKs mediate the return of resting cells to the cell cycle and ensure progression through the cell cycle phases (Fig. 3). CDKs are constitutively expressed and become activated by phosphorylation after binding to the cyclins induced during the cell cycle. CDKIs are grouped in two families: broad-spectrum inhibitors (p21, p27, p57) and specific inhibitors (p15, p16, p18, p19). Overexpression of cyclin D has been shown in cancers of the esophagus, breast, and liver and in some lymphomas. Amplification of the CDK4 gene is present in melanomas, sarcomas, and glioblastomas.

Mechanism of Carcinogenesis and Tumor Progression

Tumors contain two basic tissue components: parenchyma, which consists of proliferating neoplastic cells, and supportive stroma that includes extracellular matrix (ECM) components and blood vessels. There are several stages of cellular changes that lead to malignancy: (A) transformation of normal cells, (B) growth of the transformed cells, (C) angiogenesis, (D) local invasion, and (E) metastasis to distant sites.

Transformation of Normal Cells

Transformation of normal cells is the result of accumulation of mutations that produce activation of multiple onco-

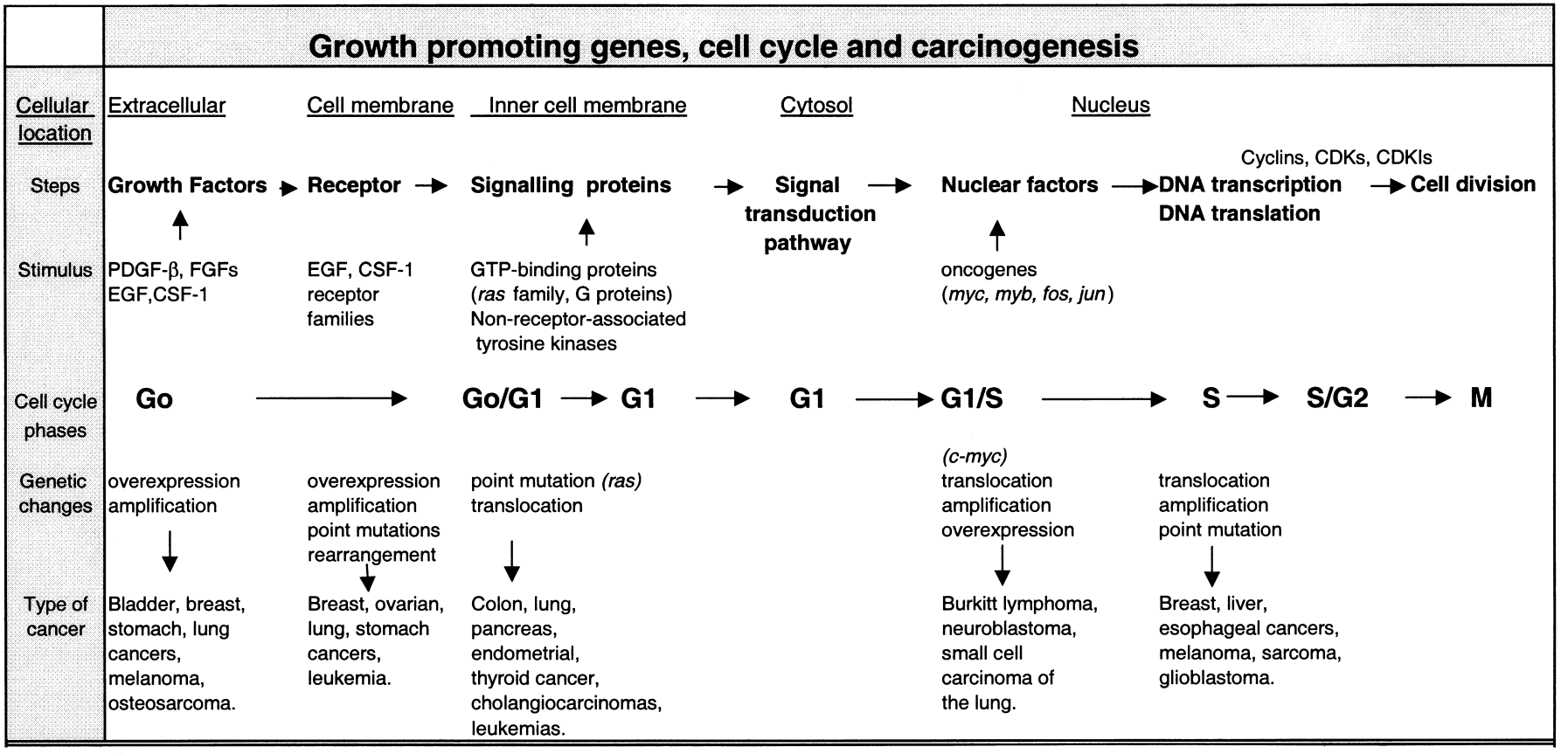


Figure 3 Schematic illustration of the sequence of events that take place in proto-oncogenes following growth factor stimulation, and the biological effects occurring after alterations in the growth factor signaling pathways during the cell cycle. PDGF = platelet-derived growth factor, FGFs = fibroblast growth factors, EGF = epithelial growth factor, CSF-1 = colony stimulating factor-1, CDKs = cyclin-dependent kinases, CDKIs = cyclin-dependent kinase inhibitors.

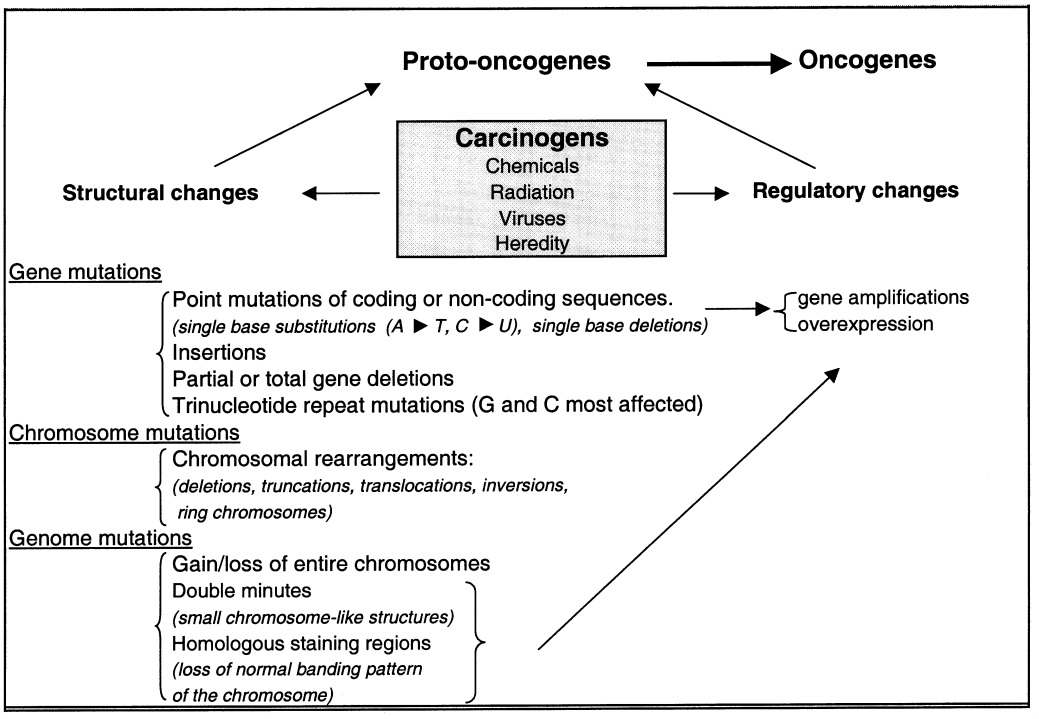


Figure 4 Proposed role of carcinogens in transformation of proto-oncogenes into oncogenes. A = adenine, T = thymine, U = uracil, C = cytosine, G = guanine.

genes and inactivation or loss of multiple antioncogenes and DNA repair and apoptosis genes. The genetic changes that lead to cell transformation may be as discreet as the point mutations, or as pronounced as the gain or loss of entire chromosomes (Figs. 2–4) and may provide important diagnostic information.

Growth of Transformed Cells

The growth of transformed cells is influenced by a number of tumor and host factors that ultimately determine tumor characteristics such as differentiation level, cell kinetics, progression, and cytological heterogeneity.

1. An important factor in determining the malignancy of tumors is cell differentiation level. Malignant tumor cells vary from well differentiated, moderately well differentiated to poorly differentiated, or undifferentiated (anaplastic). Well-differentiated tumor cells retain the morphology and most of the functions of their normal counterparts, whereas the anaplastic cells become morphofunctionally simplified and resemble each other regardless of their original source. As a result, a tumor grading system was designed to express the level of differentiation of tumor cells. Cancers are classified as grades I–IV with increasing anaplasia. The staging (I–IV) of cancers defines the size of the primary tumor and the level of spreading to regional lymph nodes as well as the presence or absence of metastases.

2. Cell cycle, growth fraction, and progressive growth are characteristics that define the tumor cell kinetics. The tumor cell cycle contains the same phases that are present in the normal cells, and the total cycle time is essentially the same. What differentiates the tumor from the normal tissues is the proportion of proliferating cells in the tumor (the growth fraction). In the early stages of tumor growth the majority of transformed cells are proliferating, whereas in later stages the cell proliferation is dramatically attenuated and cells are lost to shedding, differentiation, or reversal to G_0 phase. Thus, by the time the tumor is detected the majority of cells are no longer replicating and this constitutes one of the main reasons for the high failure rate of anticancer therapy. The imbalance between cell production and cell loss, which represents the progressive growth of a tumor, is high in rapidly growing tumors, such as leukemias and lymphomas. In low-growth tumors such as in colon cancers, the cell production slightly exceeds cell loss.

3. Tumor progression from preneoplastic lesions, to benign tumors, to invasive malignancies, to metastatic tumors, attests the increasing aggressivity of tumors over time. This is owed to formation of heterogeneous tumor subpopulations that are different in their rate of growth, invasiveness, metastatic dissemination, and responsiveness to hormones and drugs. Consequently, despite the fact that most cancers are monoclonal in origin, by the time they become clinically evident, their cell population is highly heterogeneous as a result of multiple mutations accumulated in different cells and their subsequent subclonal characteristics. Host immune activity may destroy some of the highly antigenic tumor cells and allow only slower growing cells to survive (Fig. 6). Ultimately, a growing tumor is populated with highly resistant

Table VII Cellular Events That Occur in Angiogenesis[a]

1. Angiogenic stimulus (TGF-α and -β, leptin, EGF, PDGF, hypoxia)
2. Basement membrane degradation
3. Secretion of angiogenic factors
4. Sprouting and migration into extracellular matrix
5. Endithelial cell proliferation (VEGF+VEGF-R2)
6. Endothelial cell maturation
7. Development of capillary lumen (VEGF+VEGF-R1)
8. Recruitment of pericytes and smooth muscle cells
9. Secretion of growth inhibitors (Ang 2/Tie-2)

[a] Abbreviations: VEGF, vascular endothelium growth factor; VEGF-R, vascular endothelium growth factor receptors; PDGF, platelet-derived growth factor; EGF, epithelial growth factor; TGF, transforming growth factor; Ang/Tie, angiopoetin/angiopoetin receptor.

cells that are able to survive, proliferate, invade, and metastasize to distant sites.

Angiogenesis

Angiogenesis, or formation of new blood vessels by sprouting of new capillaries from existing vessels, is required for both tumor growth and metastasis. Angiogenesis occurs both physiologically in pregnancy and during the menstrual cycle, and pathologically in atherosclerosis, diabetes, and carcinogenesis. This process is triggered by a number of angiogenic factors, which can either be released from tumors, or can be induced in the surrounding cells by the tumor cells. Angiogenesis is regulated by endothelial tyrosine kinase receptors (Tables VII and VIII). Tumor growth is supported by the normal host cells, which are induced to form new capillaries around the tumor to provide adequate blood supply for metastatic dissemination. Conceivably, host endothelial cells and tumor cells closely interact and influence each other in multiple ways.

Local Invasion

Local invasion is an exclusive characteristic of malignant cells, which have the ability to infiltrate and invade surrounding tissue. Invasion of ECM, is both an active process, involving the detachment of tumor cells from each other, attachment to ECM components, degradation of ECM, and migration, and a passive process due to cell growth. Serine and cysteine proteinases and matrix metalloproteinases (MMPs, also known as collagenases or gelatinases), are active during the early stages of tumor invasion. They are

Table VIII Angiogenic Factors[a]

Ligand	Receptor
Tumor-derived	(Endothelial tyrosine kinases)
VEGFs	VEGF-R1, -R2, -R3
Angiopoetins (Ang-1, Ang-2)	Tie-1, Tie-2
β-FGF	
PDGF	
EGF	
TGF-α and -β	
Tumor-induced	
Angiogenic factors (in infiltrated inflammatory cells)	
β-FGF, induced by tumor TGF-β	
TNF-α, induced by tumor TGF-β	
Antiangiogenic factors	
Thrombospondin-1 (can be also tumor-derived)	
Endostatin	
Angiostatin	
Vasculostatin	
Other angiogenic factors	
Neuronal guidance proteins	Neuropilin
Ephrin (Eph)	Eph B2, Eph B4
Leptin	Leptin receptors
Regulators of angiogenesis	
Integrins	
Thrombospondin-1 via p53 (see Table IX)	
Interferons	
Proteases (plasminogen activators, MMPs)	

[a] Abbreviations: VEGF, vascular endothelium growth factor; VEGF-R, vascular endothelium growth factor receptors; β-FGF, fibroblast growth factor; TNF-α, tumor necrosis factor; PDGF, platelet-derived growth factor; EGF, epithelial growth factor; TGF, transforming growth factor.

released either directly from the tumor cells or by the surrounding fibroblasts. The tumor proteolytic activity generates cleavage products of a number of ECM components, which together with some tissue-derived factors have chemotactic, angiogenic, and growth stimulating effects on tumor cells, and may play a role in organ-selective homing of tumor cells.

Metastasis

Metastasis is a multistep, interactive process between the malignant tumor cells and the host cells. Primary tumors and their metastases contain multiple cell types that are capable to resist the host immune attack and anticancer treatment by modulating their microenvironment to their advantage. As shown earlier, tumor biological diversity includes differences in growth pattern, cell deformability, surface receptors and adhesion molecules, immunogenicity, and karyotype. Other factors include the ability to form aggregates (emboli) with other tumor cells and/or lymphocytes and platelets to induce hemostasis and the ability to invade their surrounding tissue. Metastasis can occur by lymphatic, vascular, or direct dissemination. Lymphatic dissemination is conceivably the preferred route for carcinomas and sarcomas. Local lymph nodes are the first to be involved in metastasis, and also represent the first stop where tumor cells are exposed to the host immune attack. Enlargement of local lymph nodes is the result of both tumor cell proliferation and the reactive hyperplasia of the host tissue. Venous dissemination is used by sarcomas and some carcinomas penetrating the thinner walls, to follow the venous blood to the liver and lungs, a second stop in metastasis. Arterial dissemination, via pulmonary capillaries, involves mostly lung metastases. During vascular dissemination tumor cells are exposed to a variety of host factors such as local hemodynamics and the immune attack. The action of the activated macrophages and natural killer (NK) cells plays a key role in the destruction of circulating tumor cells. At this stage tumor cells tend to aggregate in homotypic (tumor cells only) or heterotypic (tumor cells and platelets or immune cells) emboli. Heterotypic aggregation with platelets enhances the tumor ability to survive and attach to the blood vessel wall and to penetrate it (extravasation), during a process that again involves the interaction between the host ECM and the tumor adhesion molecules, receptors, and proteinases. Conceivably the vast majority of tumor cells that enter circulation die and only a small, highly specialized subpopulation of cells originating in the primary tumor cells are capable of producing metastases. This cycle, if it is not interrupted by anticancer therapy, continues until the function of most of the host vital organs is compromised.

Organ tropism represents the site at which tumor cells extravasate. Adhesion molecules present on the tumor cell surface may prefer the ligands on the endothelium of certain organs, which may secrete either chemoattractants to recruit certain tumor cells or antiproteases that may prevent tumor metastasis.

Is Nitric Oxide Tumorigenic or Tumoricidal?

Tumoricidal or tumorigenic actions of NO are highly dependent on the conditions in the tumor microenvironment. A number of experimental and clinical examples confirm the duality of the cellular effects of NO and the significance of other factors independent of NO that may ultimately determine its biological role at any given time.

Tumorigenic Actions of Nitric Oxide

The nitric oxide concentration in tumors seems to be an important factor in carcinogenesis, and a number of studies have shown that high NO levels are tumoricidal, whereas low NO levels are tumorigenic (Figs. 2 and 5, Table IV). Some cancers are less susceptible to the toxic effects of NO, and in these types NO can actually stimulate their growth by promoting angiogenesis and tumor cell proliferation. Most experimental solid tumors were shown to have increased expression of iNOS, and with NO being both angiogenic and genotoxic, its increased production may contribute to carcinogenesis. Additionally, NO generated in tumors increases vascular permeability, which enhances nutritional supply and sustains the rapid growth of tumor cells. Inflammatory responses induced by various pathogens can accelerate tissue damage and mutagenesis, and NO may effectively sustain the growth of the transformed cells.

The cytostatic effects of NO seem to occur predominantly in tumor cells containing a functional wild type of p53 antioncogene. As shown earlier tumor cells with missing or mutated p53 show posttranslational modifications in p53, a preinvasive feature evident in cancers resistant to NO-mediated apoptosis such as, breast, esophageal, and bronchial cancers. Therefore, in the presence of low NO levels these tumors are capable of rapid growth. Elevated NO causes DNA damage and apoptosis in many cell types. A variety of genetic and physiological data indicate that p53 is an important negative feedback regulator of excessive NO production *in vivo.* NO induces p53 accumulation and as part of a feedback mechanism, it mediates the transcriptional transrepression of iNOS. p53-deficient mice, prone to spontaneous tumorigenesis, excrete 70% more nitrite and nitrate than wild-type p53 mice.

The role of NO and estrogens in breast cancer is still unclear, although the balance tends to lean toward a procarcinogenic action. In combination with a NO donor agent, a cathecol–estrogen was shown to synergistically induce DNA strand breakage. The DNA damage was much more pronounced than the one produced by each substance alone. These effects were inhibited by NO-trapping agents and by antioxidants. Both NO and cathecol–estrogen are formed in the human breast and uterine tissues, and their synergism generates potent ROS that may be involved in hormone-induced carcinogenesis. Similarly, cNOS has been shown to be present in various human tumor cell lines such as neuroblastoma, cervix carcinoma, and breast cancer cells, where it

correlates strongly with the expression of estrogen receptor. Selective iNOS inhibition of *in vivo* growth of murine mammary adenocarcinoma cells expressing iNOS, resulted in a significant reduction of tumor weight as compared to that of controls, suggesting a definite role for NO in tumorigenesis in this type of cancer. Inhibition of iNOS in a murine colon adenocarcinoma, containing an increased number of intratumoral macrophages as the main source of NO, did not affect tumor growth or plasma NO levels. All of these examples suggest that NO production in the tumor microenvironment plays a key role in promoting tumor growth in certain cancers, and that the host defense mechanism is often ineffective in inhibiting tumor growth in these types of cancer.

It is also possible that the majority of tumor cells containing high NO levels undergo autolysis, and only the NO resistant ones survive. These tumor cells are then able to generate and use NO to their advantage and promote tumor progression and metastasis. Such cells have been shown to exist in a number of human cancers of CNS, breast, gynecological (ovarian, mixed mesodermal, and endometrial) cancers, and in murine colon, or mammary adenocarcinomas. Furthermore, in some cancers only the poorly differentiated tumors produce NO, which is absent in the normal surrounding tissues. In other tumors both the infiltrating macrophages and the vascular cells become activated by the tumor-generated cytokines and produce low NO levels. In all these cancers NO is a facilitator of tumor growth by maintaining optimum blood flow and pH, by limiting leukocyte–endothelial cell interactions, and by increasing vascular permeability (Fig. 5). Inhibition of iNOS effectively reverses the tumorigenic effects of NO in this type of cancers.

Tumoricidal Actions of NO

The antitumor activity of NO includes inhibition of tumor cell proliferation, promotion of cell differentiation, and reduction of the metastatic spread (Fig. 5 and Table V). Tumoricidal effects of NO generated by macrophages was demonstrated soon after the identification of endothelium-derived relaxing factor (EDRF) as NO. Macrophages activated with LPS and γ-interferon (IFN-γ) were shown to cause specific injuries to the iron–sulfur clusters present in complex I and II enzymes of the mitochondrial electron transport chain, and to inhibit DNA synthesis and mitochondrial respiration. These effects were inhibited by superoxide or by reduced myoglobin, agents that inactivate NO. High NO levels are generally tumoricidal and this is of particular significance as a therapeutical approach that can be used in tumors susceptible to NO-mediated apoptosis. For example, iNOS overexpression in murine melanoma cells was shown to inhibit tumor growth and survival, both *in vitro* and *in vivo*. Relaxin, a peptide hormone produced in the ovary, inhibits growth and promotes differentiation of breast adenocarcinoma cells and also stimulates NO production. These observations suggest that the cytostatic effect of relaxin on breast tumor cells may be mediated by the increased NO production. Similarly prolonged administration of sodium nitroprusside significantly decreased the incidence of gastric cancers, without affecting the morphology of the lesions. This inhibitory effect is presumed to be due to the suppression of cell proliferation in the gastric epithelial cells by NO. Bacillus Calmette-Guerin (BCG) has been used for years in the treatment of urinary bladder cancer. There is both *in vivo*

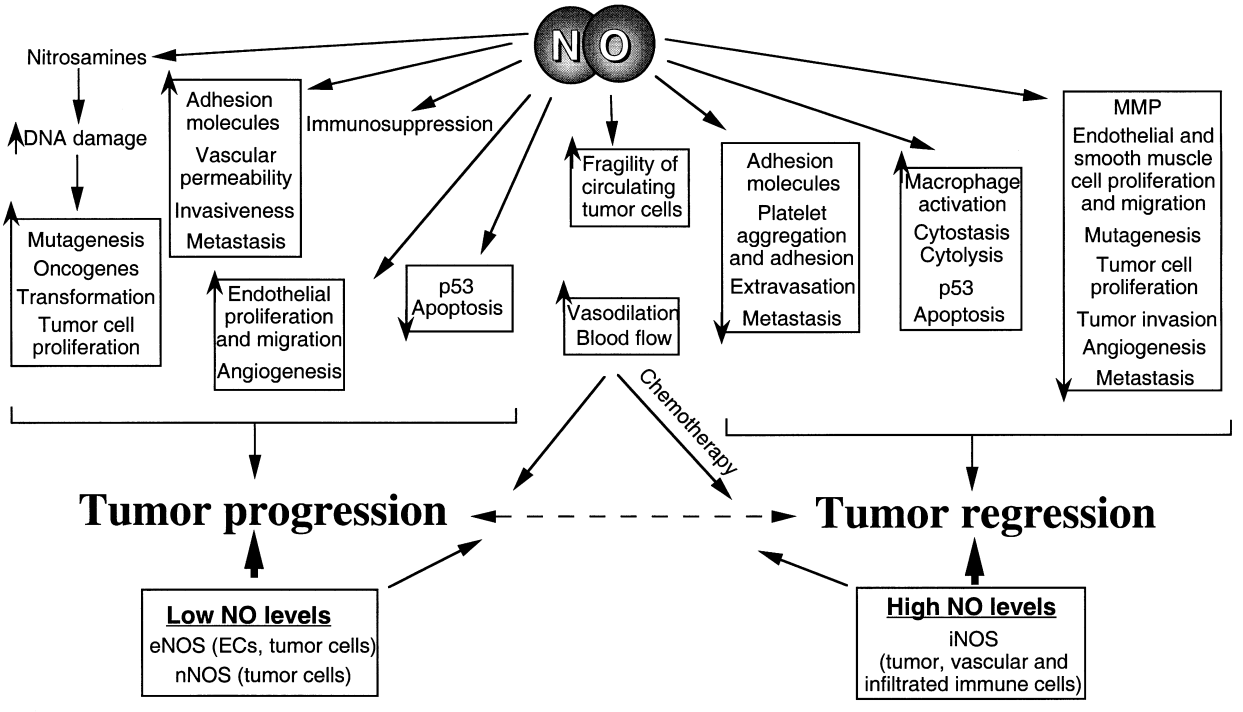


Figure 5 Cellular events mediated by NO and tumor development.

and *in vitro* evidence indicating that elevated NO generated following the treatment with BCG exerts cytotoxic effects on the urinary bladder tumor cells. These findings suggest that NO synthesis during the immune-mediated response to BCG may be an important factor in BCG-mediated anticancer therapy.

The main effector cells responsible for the early antitumor defense are NK cells and macrophages (Fig. 6). Depletion of NK cells or iNOS inhibition decreased the tumoricidal activity of tumor-associated macrophages in a melanoma cell line. Additionally iNOS inhibition accelerated the growth of inoculated melanoma cells and this effect was reversed by administration of IFN-γ. The cytostatic and cytotoxic activity of macrophages is in part attributed to the production of NO as a result of NOS induction. The intensity of iNOS expression in alveolar macrophages was shown to be positively correlated with the levels of exhaled NO in patients with primary lung cancer as compared to control subjects.

In addition to NO, its precursors L-arginine and N^G-hydroxy-L-arginine (NOHA), along with polyamines, may participate in the regulation of carcinogenesis. L-Arginine is a substrate for both NOS and arginase, one of the urea cycle enzymes that has been found in hepatocytes and in a number of other cell types. Arginase can be present constitutively (arginase I) or it can be induced (arginase II) by LPS, cAMP, and some cytokines to convert L-arginine into L-ornithine and urea. L-Ornithine is the substrate for ornithine decarboxylase (ODC) a short-lived enzyme which decarboxylates ornithine to produce the diamine putrescine, the precursor of the polyamines spermidine and spermine, molecules that play a critical role in cell proliferation. ODC is the rate-limiting enzyme in the synthesis of polyamines, and more recently it was shown that both arginase and ODC can be modulated by NOS reaction products. Increased arginase expression and elevated levels of putrescine have been found in tumor cells and inhibition of one or both urea cycle enzymes (arginase and ODC) inhibited tumor development. Thus, two sequential products of NOS activity, NOHA and NO, inhibit two sequential enzymes in the urea cycle. NOHA is a potent inhibitor of arginase, whereas NO inhibits ODC activity by S-nitrosylation (Table III). Additionally, polyamines have both a proliferative effect on tumor cells and an angiogenic effect on vascular cells. Therefore, modulation of the polyamine pathway may be of critical importance in cancer.

Nitric Oxide and Chronic Inflammation

NO has long been shown to inhibit the growth of bacteria, fungi, and parasites by reacting with key sulfhydryl groups in glycolytic enzymes, ferredoxins, or hydrogenases, and consequently it plays a role in host defense mechanisms. This is important considering that chronic exposure to high levels of NO, ROS, and RNOS, commonly present at chronic infection and inflammation sites, may subsequently lead to carcinogenesis. For example, gastric cancers often occur in patients suffering from chronic atrophic gastritis. *Helicobacter pylori* infected patients, may have an increased risk of developing gastric cancer due to bacterial-induced chronic inflammation and also due to ingestion of dietary nitrosamine precursors in the acidic nitrosating conditions in the stomach. Furthermore, chronic heartburn, known medically as gastroesophageal reflux disease (GERD), can considerably increase the risk of developing adenocarcinoma of the esophagus, one of the deadliest forms of cancer. Similarly, colon cancer is frequently present in patients with ulcerative cholitis. Hepatocellular carcinoma develops in patients suffering from chronic hepatitis and cirrhosis. Gallbladder carcinoma may occur in patients with gallstones and chronic cholecystitis. Chronic infection with liver fluke is accompanied by prolonged elevation of NO in the plasma, saliva, and urine of infected patients or experimental animals and it strongly correlates with the development of cholangiocarcinoma. Additionally, nitrosamines formed endogenously from dietary precursors are potential risk factors for the development of cholangiocarcinoma and hepatocellular carcinoma in patients infected with liver flukes and cirrhosis, respectively. There is a strong correlation between infection with hepatitis C virus, NOS induction, upregulation of p21 (see earlier), and the development of hepatocellular carcinoma.

Nitric Oxide and Metastasis

Metastatic dissemination represents the main cause of death in cancer patients, as an ongoing war ensues between the invading tumor cells, the host defense arsenal, and the anticancer therapy. Resistance to therapy is caused by the aforementioned factors (see earlier) that facilitate the survival and growth of tumor cells. Moreover, other determinants including late detection of primary tumor and the spreading of metastases beyond the local lymph nodes, tumor inaccessibility to surgery or to conventional anticancer therapy, are all due to the tumor cytological heterogeneity. As mentioned earlier, tumors contain multiple cell subpopulation clones of the original transformed cell that are capable to survive the host immune attack and are able to resist treatment by modulating their milieu to their advantage. Tumor resistance to drugs is a significant contributor to the high failure rate of current anticancer therapy.

Prometastatic Role of Nitric Oxide

Tumor progression and metastasis can be impacted positively or negatively by the presence of NO, and studies propose a dual role for NO in tumor biology. High NO concentrations possess tumoricidal actions, whereas lower NO concentrations supposedly promote tumor growth. A number of human cancers including breast cancer, colorectal adenocarcinomas, squamous cell carcinomas, glioblastomas, and meningiomas invariably express iNOS mRNA.

eNOS and iNOS activity is augmented in invasive breast tumors, as compared to normal or benign tissue. In human breast tumors NOS expression invariably correlates with

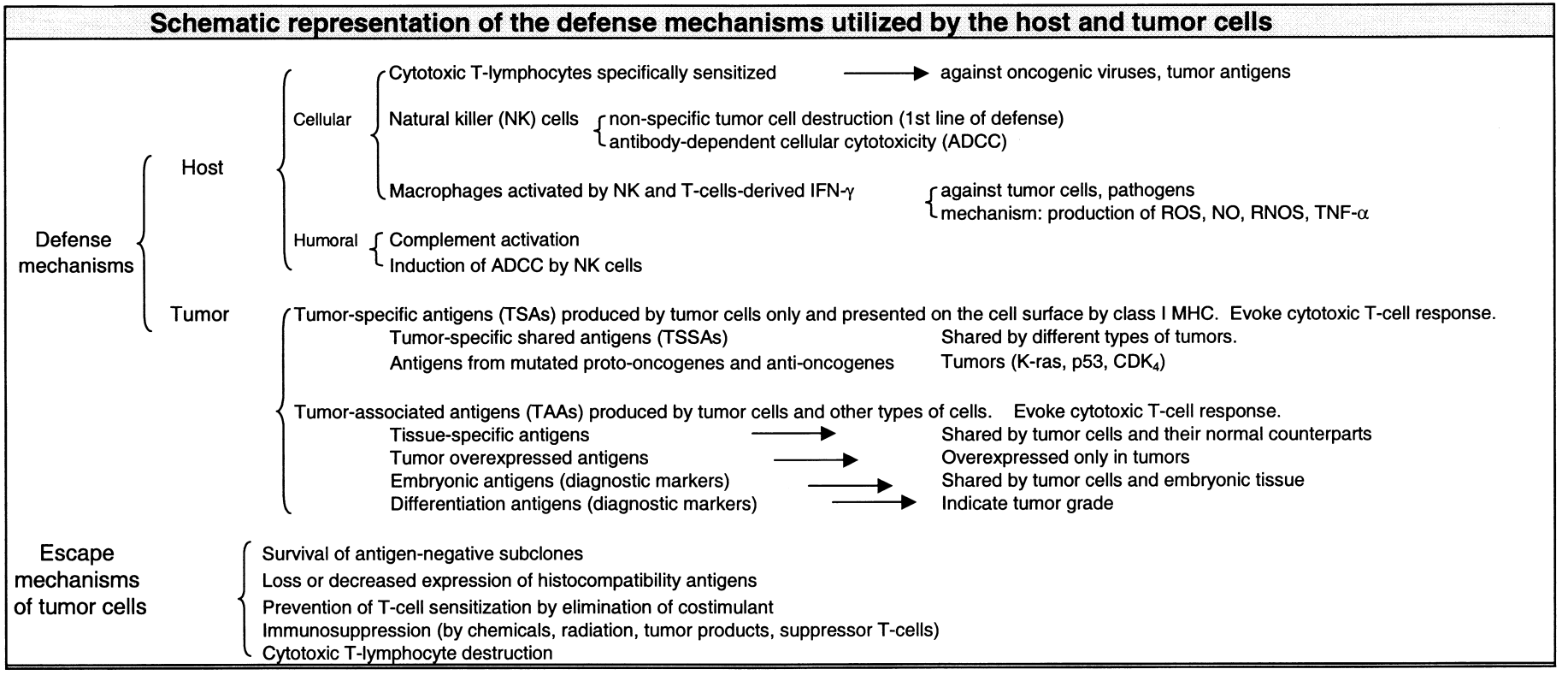


Figure 6 Schematic representation of the defense mechanisms utilized by the host and tumor cells.

tumor grade. For example, in invasive ductal carcinomas NO biosynthesis was significantly enhanced in grade III compared to grade II tumors, whereas NOS activity in cells harvested from primary and metastatic sites of patients with ovarian cancer was higher in the more differentiated tumors. Histological examination of the samples revealed an elevated macrophage content accompanied by high NOS activity. This further underlines the complexity of the relationship between tumor progression, NOS expression, and the immune response, indicating that the differentiated status of the tumor is only one of the multiple factors responsible for cancer progression. NO is presumed to be involved in the abnormal vascularization associated with tumorigenesis. There is a strong correlation between the presence of NOS and axillary lymph node metastasis and also between NOS and the absence of the antimetastatic gene nm23 markers, indicating a role for NO in the increased blood flow and metastasis. Breast cancer has the ability to metastasize rapidly and tumor vascularization is one of the factors contributing to increased metastasis (Fig. 5, Table IX). Increased expression of NOS has been observed in human colon, ovary, breast, and central nervous system tumors, with iNOS mainly located in the immune cells, endothelium, and tumor cells, indicating a pathophysiological role in tumor-associated NO production. 3-Nitrotyrosine, a marker for peroxynitrite formation, was detected in a population subset of tissue mononuclear cells, and vascular endothelial growth factor (VEGF) was detected in adenomas expressing iNOS, suggesting that NO may be a contributor to colon cancer progression. Additionally, NO was associated with tumor growth, angiogenesis, and metastasis in patients with solid tumors of the head and neck, which positively correlated with the levels of total NOS, iNOS, cGMP, and microvessel densities. Lymph node metastases contained higher NO levels and microvessel density than lymph nodes without metastatic involvement. Inhibition of NO production reduced tumor growth and angiogenesis, suggesting a key role for NO in tumor metastasis in these patients. The total NOS activity in lung adenocarcinoma specimens was significantly higher than in the normal lung tissue or in other types of lung cancer, suggesting that NO may play a role in the growth, progression, and metastatic spread of certain lung tumors. Exogenous NO or iNOS expression induced in human fibrosarcoma tumor cells by cd15/endothelial–leukocyte adhesion molecule-1 (ELAM-1) adhesion system, enhanced tumor cell adhesion to endothelial cells, increased endothelial permeability, and facilitated tumor cell invasion. Ultraviolet A (UVA)-irradiated human squamous cell carcinoma cells were shown to release NO, peroxynitrite, nitrosocompounds, ammonia, and hydroxylamine. Formation and release of these nitrogen products was time and concentration-dependently stimulated by UVA and inhibited by *N*-methyl-L-arginine (NMA). These cells have a higher NOS and soluble guanylate cyclase activity than normal keratinocytes, and this may be a reason for the poor prognosis of patients with squamous cell carcinoma.

ANTIMETASTATIC ROLE OF NO

Tumor heterogeneity allows a specialized tumor cell subpopulation to survive and metastasize, and it was found that some of these cells are unable to express iNOS. In a melanoma cell line, the cells expressing low levels of iNOS produce slow-growing tumors, the cells that produce higher NO levels undergo autolysis and cause the cytolysis of surround-

Table IX Role of Nitric Oxide in Angiogenesis[a]

Antiangiogenic effects	Angiogenic effects
↓ Expression of VEGF, VEGF-R, PDGF	↓ p53 → ↓ thrombospondin-1
↓ Expression of MMP	↑ IL-8 expression
↓ Smooth muscle cell migration	↑ Vascular permeability
↓ DNA synthesis	↑ DNA synthesis
↓ Cell proliferation (endothelium and smooth muscle cells)	↑ bFGF release Stimulates endothelial cell proliferation Inhibits smooth muscle cell proliferation
↓ Capillary density	↑ Cell proliferation
↓ Collagen synthesis	↑ Cell migration
↑ p53 → ↑ thrombospondin-1	↑ Capillary density
	↑ Capillary growth
	↑ Blood flow in solid tumors
	↑ Maintain optimum pH in solid tumors
	↑ Tumor oxygenation

[a] ↑, increased activity; ↓, decreased activity. Abbreviations: VEGF, vascular endothelium growth factor; VEGF-R, vascular endothelium growth factor receptors; PDGF, platelet-derived growth factor; β-FGF, fibroblast growth factor; IL-8, interleukin-8; MMP, matrix metalloproteinase.

ing cells, whereas the cells unable to express iNOS establish rapid liver metastases. LPS and cytokine treatment results in iNOS induction and high NO level production only in nonmetastatic cells. Increased NO production by the tumor cells or by the host immune cells, or transfection of the iNOS gene to the tumor cells or to the host immune cells, leads to tumor suppression. This antimetastatic effect of NO, occurs either by apoptosis, or by alterations in the genes that regulate metastasis (cell survival, invasion, and angiogenesis genes) (Fig. 5).

Activation of macrophages and vascular cells or administration of NO donor agents, inhibited tumor growth and metastasis in mouse lymphomas, B-16 melanoma, or Lewis lung carcinoma. Pretreatment of macrophages with specific iNOS inhibitors reversed the tumoricidal effects of NO. The highly metastatic K-1735 murine melanoma tumor cells seem to be more sensitive to exogenous NO than the cells in the primary tumor, and their ability to metastasize was dramatically reduced by NO. *In vitro* transfection of these cells to express the active iNOS gene resulted in NO-mediated apoptosis, and iNOS inhibitors prevented apoptotic tumor cell death. *In vivo,* the untransfected cells produced rapidly growing tumors in nude mice, whereas the transfected ones did not. Even in immune-deficient mice, the melanoma cells were not metastatic in the presence of an active iNOS gene. Repeated induction of iNOS expression by cytokines was shown to cause regression of liver metastases in a murine sarcoma cell line *in vivo.*

NO can interfere with other steps of the metastatic process such as expression of adhesion molecules, arrest of tumor emboli, and their extravasation, which are hindered by the vasodilator effect of NO and its ability to inhibit platelet aggregation and adhesion to capillary endothelium. The increased fragility of the circulating tumor cells in the presence of NO and their damage in microvasculature is another mode in which NO prevents metastasis.

It is clear that elevated NO is required to produce tumor cell death and the use of a new therapeutic approach, in which multiple systemic administrations of lipopeptide-containing liposomes and IFN-γ, eradicated established liver metastases of reticulum cell sarcoma in mice. Tumor regression correlated with the increased iNOS expression and NO levels.

However, metastatic cells have developed a number of mechanisms that allows them to evade NO-mediated cytotoxicity. Some tumors produce osteopontin, an inhibitor of NO production in the activated host macrophages or endothelial cells. Metastatic cells from a murine colon carcinoma and K-1735 mouse melanoma, exhibit lower NOS activity than the nonmetastatic cells, suggesting a differential pattern of iNOS induction in response to combinations of cytokines [tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IFN-γ, which are found in circulation], and LPS. There is a direct correlation between NO production and cytotoxicity, and addition of iNOS inhibitors reverses these effects. Thus, the fate of circulating tumor cells is dependent on the presence of iNOS activating factors and the responsiveness of the tumor cells to the cytotoxic effects of NO. Hypoxia, oxidative, and nitrosative stress existent in the solid tumor microenvironment, are additional modulators of NO production and effects in biological systems.

Nitric Oxide and Angiogenesis

Angiogenesis, can also be regulated by NO, which has been shown to be both an inhibitor and stimulant of angiogenesis (Table IX, Fig. 5). NO selectively inhibits the expression of MMP a collagenase that promotes both angiogenesis and metastasis, but it does not affect the levels of its natural inhibitor, tissue inhibitor of metalloprotease-1 (TIMP-1), a process that is negatively affected by iNOS inhibitors. Additionally, NO inhibits vascular endothelium and smooth muscle cell proliferation and prevents migration of vascular smooth muscle cells, thus inhibiting the early stages of angiogenesis and tumor development. Elevated NO suppresses the expression of angiogenesis genes at the transcriptional level. VEGF, VEGF receptor transcripts, and platelet-derived growth factor (PDGF), which are both constitutively present and hypoxia-induced (remember, tumor microenvironment is hypoxic), were inhibited by NO and addition of iNOS inhibitors reversed these effects.

NO also stimulates angiogenesis, and its effects can be direct or indirect. In solid tumors, low levels of NO maintain optimum blood flow, pH, and increase tumor oxygenation. Additionally, NO enhances vascular permeability, stimulates proliferation, and migration of endothelial cells, therefore directly supporting tumor growth and neovascularization. Activated human monocytes, which produce low levels of NO, also have an angiogenic effect. Indirectly, NO induces the release of basic fibroblast growth factor (bFGF), which is cytotoxic to vascular smooth muscle cells but stimulates the proliferation of endothelial cells. Also, low levels of NO can induce IL-8 gene expression and indirectly have an angiogenic effect. Substance P releases endothelial NO, which acts as an autocrine regulator of angiogenesis.

Nitric Oxide and Cell Death

As shown earlier, NO can exert its tumoricidal effects by inducing apoptosis or necrosis, two biological processes characterized by multiple biochemical and morphological changes that are described in Table X and Fig. 7. Endogenous or exogenously administered NO has been shown to mediate cell death in normal, transformed, and neoplastic cells. LPS and cytokine treatment of murine reticulum cell sarcoma and mastocytoma cell lines, or normal L-929 fibroblasts, exhibited increased NO-mediated apoptosis, an effect that was attenuated by iNOS inhibition. NO delivered from activated macrophages cocultured with murine melanoma, and other tumor cell lines, induced apoptosis in the target cells. Prolonged activation was also shown to produce NO-mediated apoptosis in macrophages. DNA fragmentation was present in both tumor cells generating low levels of NO and in the non-NO-producing bystander cells. Higher and prolonged NO production caused necrosis in both tumor and surrounding cells.

Table X Characteristics of Cell Death

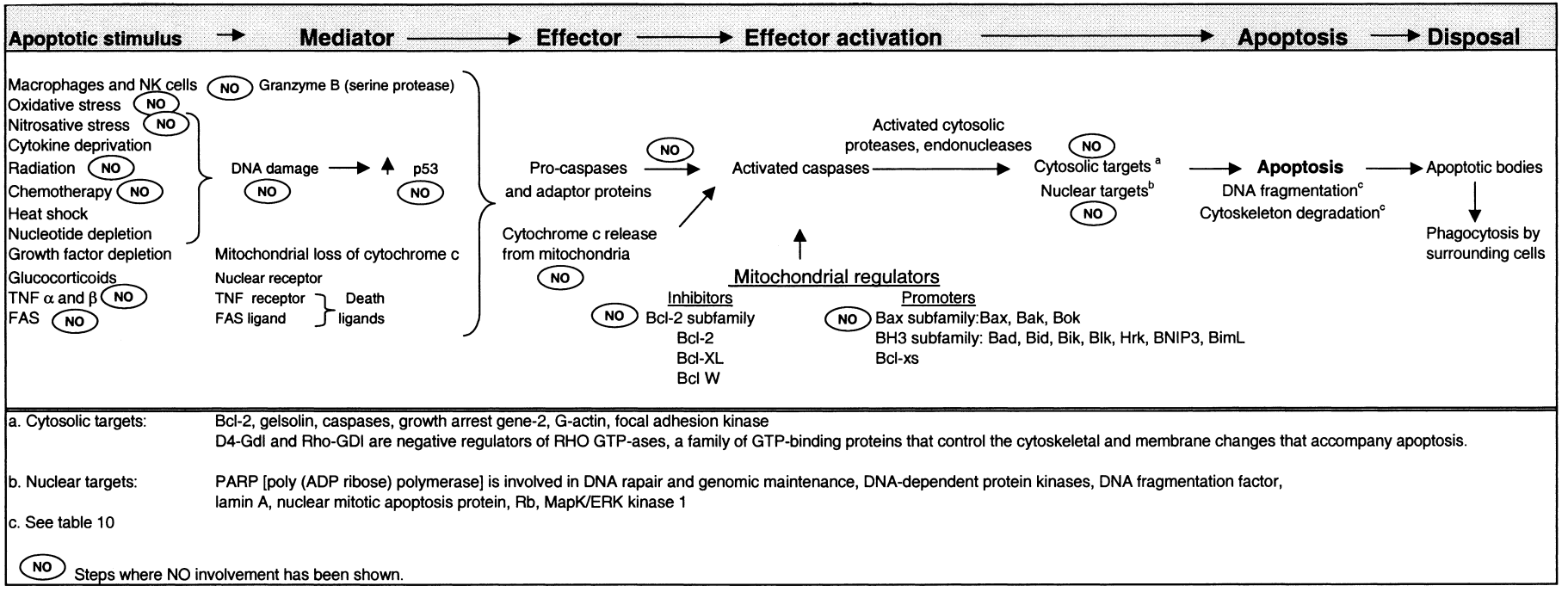
Types of cell death	Biochemical changes	Morphological changes
Apoptosis or programmed cell death: Active, regulated process, induced by alterations in physiological conditions. Requires energy, nucleic acid, and protein synthesis and activation.	Caspase activation Protease and endonuclease activation Cleavage of nuclear and cytoskeletal proteins Protein cross-linking (by transglutaminase activation) DNA fragmentation Intranucleosomal DNA cleavage into oligonucleosomes Plasma membrane alterations Expression of cell death markers on cell surface Redistribution of phosphatidylserine, thrombospondin, and glycoprotein in plasma membrane Phagocytic recognition by adjacent cells Selective cell disposal	Cell shrinkage Loss of plasma membrane asymmetry and attachment Cytoplasm condensation Condensation of chromatin to the margins of nuclear membrane Cell surface blebbing Intranucleosomal DNA fragmentation by a specific endonuclease Distinct laddering and banding on gel electrophoresis Cell detachment Cell fragmentation into apoptotic bodies Phagocytic elimination without inflammatory damage to other cells No inflammatory response Loss of single cells
Necrosis: Passive unregulated process triggered by nonphysiological stimuli. Does not require energy, nucleic acid, or protein synthesis.	Plasma membrane and mitochondrial dysfunction Deregulation of ion homeostasis Release of cytoplasmic contents (proteases, nucleases) Uncontrolled DNA degradation Inflammatory reaction in the surrounding cells	Cell and organelle swelling Lyosomal leakage Microvesicle formation Early rupture of plasma membrane Cytoplasm leakage and cell lysis Clumping of chromatin Smearing of DNA on gel electrophoresis Significant inflammatory response Phagocytosis by macrophages Loss of cell groups

Exogenously administered NO donor agents were also capable of producing apoptotic features in a variety of normal and tumor cell lines. Low NO donor agent concentrations produced different degrees of apoptotic changes in the target cells, whereas higher concentrations of NO donor agent induced cellular necrosis. Different types of NO donors with dissimilar NO release kinetics showed distinct apoptosis-inducing effects. The most rapid and potent apoptotic NO donor compound was *S*-nitrosoglutathione (GSNO). As shown earlier in this chapter, the majority of NO-mediated cytotoxic effects are indirectly produced via interaction of NO with ROS. Peroxynitrite, for example, at low concentrations is apoptotic and at high concentrations is necrotic.

The mechanism of NO-mediated apoptosis is still unclear (Fig. 7; Tables IV and XI), but there is evidence suggesting NO involvement in upregulation of genes that regulate cell death or survival (p53, Fas, caspases, and Bcl-2 family). Expression of p53 has been correlated with increased NO levels and apoptosis in a number of cell types, in a time- and dose-dependent manner. For example, murine melanoma cells, rat normal and cancer cells, and human normal and cancer cells show increased wild type of p53 protein accumulation in the nucleus in the presence of elevated NO levels, suggesting that in these cells NO-mediated apoptosis may be the result of NO-induced DNA damage that triggered the p53 expression. Cells with mutated or missing wild-type p53 do not seem to undergo apoptotic changes. Mutations in conserved codons of p53 are frequently seen in cancers of the brain, liver, breast, and also in the radon- or smoking-induced lung cancers. Another mechanism by which NO induces apoptosis is by reacting with cytochrome *c* oxidase, thus diverting the electrons to oxygen to form O_2^- and H_2O_2 (Table IV, Fig. 7). The increased H_2O_2 causes DNA damage, which upregulates p53 expression and results in apoptotic cell death.

As in other biological processes, NO has a dual effect, and therefore it can induce apoptosis or protect the cells from it (Fig. 7, Table XI). In most normal cells (rat ovary, thymocytes, hepatocytes) apoptosis can be prevented by NO, whereas in other normal cells (neurons, macrophages), and tumor cells (sarcoma, mastocytoma, melanoma) NO induces apoptosis. This is caused either by an increased p53 expression in response to DNA fragmentation, or by activation of protein kinases A and C, which seem to be part of the NO-induced apoptotic signaling pathway. Indeed, increased p53 expression by low concentrations of NO, suggest that NO may induce apoptosis, whereas high NO levels prevent apoptosis by inhibiting p53 expression. One possible explanation is the reaction of RNOS with zinc finger proteins in DNA that can either cause necrotic cell death, or malignant transformation (Fig. 2).

Transfection of Bcl-2, a cell-survival gene, suppressed apoptosis in cells exposed to NO at concentrations that induce apoptosis in nontransfected cells. NO mediated p53 expression, PARP cleavage, and apoptosis were blocked by Bcl-2 overexpression that resulted in malignancy, supposedly



a. Cytosolic targets: Bcl-2, gelsolin, caspases, growth arrest gene-2, G-actin, focal adhesion kinase
D4-GdI and Rho-GDI are negative regulators of RHO GTP-ases, a family of GTP-binding proteins that control the cytoskeletal and membrane changes that accompany apoptosis.

b. Nuclear targets: PARP [poly (ADP ribose) polymerase] is involved in DNA rapair and genomic maintenance, DNA-dependent protein kinases, DNA fragmentation factor, lamin A, nuclear mitotic apoptosis protein, Rb, MapK/ERK kinase 1

c. See table 10

NO Steps where NO involvement has been shown.

Figure 7 The apoptotic cascade. Schematic illustration of the steps involved in the apoptosis of mammalian cells.

Table XI Effects of NO and RNOS on Apoptosis[a]

Proapoptotic effects
↑ Protein kinases A and C
↑ Protein tyrosine nitration → disruption of cell redox status
↑ DNA damage
↑ PARP → PARP cleavage
↑ Intracellular acidification
↑ Intracellular accumulation of calcium and iron ions → cytotoxicity
↑ FAS
↑ p53
↑ Mutagenesis
↓ DNA synthesis
↓ Protein synthesis
↓ ATP levels
↓ Cell energy
↓ Cell respiration
↓ Catalase, cytochrome *c* oxidase → H_2O_2 cytotoxicity
↓ Glutathione, glutathione peroxidase, metallothionein → cytotoxicity
↓ Bcl-2 (prolonged exposure to high NO levels)
Antiapoptotic effects
↓ Cytochrome *c* release from mitochondria
↑ Bcl-2 (short, low-level NO exposure)
↓ p53 (prolonged exposure to high NO levels)
↑ Mutagenesis
↑ Carcinogenesis

[a] ↑, increased activity; ↓, decreased activity.

due to increased resistance to NO-mediated cytotoxicity. Conversely, downregulation of Bcl-2 lowers tumorigenicity and predisposes the cell to NO-mediated apoptosis. As with other biological systems, in this case too, NO has a dual effect, since it was shown that a short pretreatment of B lymphocytes with low levels of NO could effectively inhibit apoptosis due to sustained Bcl-2 expression. Increased NO levels, however, cause Bcl-2 suppression therefore allowing apoptotic events to take place. Thus, there appears to be a concentration-dependent differential effect elicited by NO on Bcl-2 expression (Table XI).

Additionally, other cellular mechanisms may intervene in NO-mediated cell protection against apoptosis. In a series of cGMP-dependent processes, NO was shown to interrupt the apoptotic cascade in hepatocytes, splenocytes, eosinophils, and lymphocytes, or to inhibit caspase activation and function. Through these mechanisms NO prevents the decrease in Bcl-2 expression and protein, as well as the release of cytochrome *c* from mitochondria. In a cGMP-independent manner, NO prevents apoptosis by interacting with heme-containing proteins such as cyclooxygenases and heme oxygenase-1 or via redox mechanisms that attenuate cell injury. It is possible that NO and O_2^- are apoptotic only when they are not in equal steady-state concentrations. At equal steady-state concentrations they produce peroxynitrite, which in the presence of intracellular glutathione may be detoxified to nitrate. All of these examples demonstrate the ability of NO to initiate and/or to arrest cell death. Ultimately, the role of NO in apoptosis is determined by the interaction between the protective and destructive signaling pathways in a given cell milieu.

Environmental Carcinogens

Carcinogenesis is a multistep process that includes initiation, promotion, conversion, cell transformation, progression, invasion, and metastasis. Carcinogenesis involves the action of hereditary and/or environmental factors such as, carcinogenic chemicals, radiation, and oncogenic viruses. Some of the phases of carcinogenesis were discussed earlier, and in this section we will describe only the initiation and promotion steps, as well as their contribution to neoplastic transformation.

Carcinogenic Chemicals

Chemical carcinogens can be produced naturally or artificially (Table XII). Initiation—exposure of cells to the appropriate dose of the carcinogen (initiator), that permanently damages DNA—is followed by exposure to promoters, which can reversibly produce tumors from initiated cells. Alkylating agents and acylating agents, are direct acting initiators that form covalent adducts between the carcinogens and the target cell DNA, RNA, or proteins. The indirect-acting initiators (procarcinogens) require metabolic conversion to become carcinogens. Most of the procarcinogens are metabolized in the liver by the cytochrome P-450 monoxygenases. Detoxification or inactivation of chemical carcinogens is important in evaluation of their biological effects. Because malignant transformation is the result of mutations occurring in oncogenes, antioncogenes, DNA repair, or apoptosis regulating genes, the primary site of action of carcinogens is the DNA. Failure to repair DNA damage is the starting point of initiation, and cell replication renders the mutation permanent (Fig. 2).

Promotion potentiates the carcinogenicity of initiators. Promoters (Table XII) are not mutagenic but are strong inducers of cell proliferation. Forced to proliferate, the initiated clone undergoes additional mutations that ultimately lead to malignancy.

Radiation-Induced Carcinogenesis

Radiation (Table XIII), represented by the UV light and ionizing and particulate radiation, can induce neoplastic transformation in cells. UV light is the main cause of skin cancers. In xeroderma pigmentosum, the inherited inability to repair UV-induced DNA damage results in skin cancer. Ionizing electromagnetic radiation leads to lymphoid malignancies in patients diagnosed with ataxia-telangiectasia. Particulate radiation is responsible for a variety of cancers, which are the consequence of the cumulative effect of radiation exposure over long periods of time.

Viral Carcinogens

A number of DNA and RNA viruses are oncogenic (Table XIV). Some DNA oncogenic viruses are known to cause cancer in animals, whereas others have been implicated in

Table XII Chemical Carcinogens

Chemical carcinogen type	Tumor type
Initiators	
Direct acting (carcinogens)	
Alkylating agents	
Anticancer drugs	Lymphoid neoplasms
Immunosuppressants	Leukemias
Acylating agents	
Indirect acting (procarcinogens)	
Aromatic polycyclic hydrocarbons (cigarette smoke, broiled, smoked meats)	Sarcomas, skin, lung, bladder carcinomas
Drugs	
Heterocyclic hydrocarbons	
Azo dyes (food coloring)	Hepatocellular and bladder carcinoma
Aromatic amines	Hepatocellular and bladder carcinoma
Aromatic amides	
Plant and microbial products (betel nuts, safrole, griseofulvin, aflatoxin)	Hepatocellular carcinoma
Insecticides, fungicides, herbicides	Lymphomas, sarcomas
Metals (chromium, nickel, arsenic)	Lung, liver, skin cancers
Asbestos	Mesothelioma, bronchial, gastrointestinal, prostate, nasal carcinoma
Nitrosamines, amides	Gastrointestinal carcinoma
Promoters	
Hormones (estrogens)	Liver, endometrial, vaginal cancers
Drugs	
Phorbol esters	
Okadaic acid	
Artificial sweeteners	

human cancers. Transforming DNA viruses form stable associations with the genome of the host cell. RNA oncogenic viruses (retroviruses) are known to cause cancers in animals. Only one human retrovirus is presently known, human T-cell leukemia virus type 1 (HTLV-1), which is associated with a form of T-cell leukemia and lymphoma described in certain parts of the world.

Host Defense

Antitumor activity of the host can be cellular and humoral, and it was shown that immunodeficient animals or immunosuppressed transplant and AIDS patients exhibit an increased frequency of lymphomas. Nevertheless, the majority of cancers occur in persons that are not immunodeficient,

Table XIII Radiation-Induced Carcinogenesis

Radiation type	Cellular effects	Tumor type
Radiation		
Ultraviolet (UV) light [UVA (320–400 nm), UVB (280–320 nm), UVC (200–280 nm)]	↓ cell division, inactivation of DNA repair enzymes, mutagenicity (ras, p53), cytotoxicity	Squamous cell carcinoma, basal cell carcinoma, malignant melanoma
Ionizing electromagnetic (X rays, Gamma rays)		
Particulate (alpha particles, beta particles, protons, neutrons)	DNA lesions, DNA cross-links, DNA–protein cross-links, oxidation, degradation of bases, damage of sugar–phosphate bonds, single- and double-stranded DNA breaks, ↓ DNA repair genes, ↑ free radicals, ↑ oxidative stress, ↑ NF-κB, ↑ gene expression	Skin cancers, cancers of the breast, lung, colon, thyroid, and salivary glands

Table XIV Viral Carcinogens

DNA oncogenic (transforming) viruses	RNA oncogenic viruses (retroviruses)
Animal viruses	Animal retroviruses (slow transforming viruses)
Small DNA polyoma virus	Feline sarcoma virus (FSV)
Adenoviruses	Simian sarcoma virus (SSV)
Bovine papillomavirus	Rodent leukemia virus (RLV)
Simian virus-40 (SV-40)	
Human viruses	Human viruses
Human papillomaviruses (HPV)	Human T-cell leukemia virus type 1 (HTLV-1)
Epstein-Barr herpes virus (EBHV)	Target: T cells, $CD4^+$ subset
Hepatitis B virus (HBV)	Latency: Long, up to 30 years
Kaposi sarcoma herpes virus (KSHV)	

indicating the presence of immune-evading mechanisms either in the tumor cells or in their environment that allows the host defense mechanisms to fail (Fig. 6). As a result, a number of techniques have been developed, such as immunotherapy and gene therapy to replace or enhance immune activity in cancer patients. Adoptive cellular therapy, for example, is based on the generation of LAK (lymphokine-activated killer) cells from the treatment of peripheral lymphocytes, or tumor-infiltrating lymphocytes with IL-2 in culture, and reinfusion of these cells into patients with advanced metastases. Cytokine therapy activates specific and nonspecific host defense mechanisms and amplifies immune recognition. Furthermore, a number of antibody-based therapeutic methods are used to deliver toxins to tumor cells. Therefore, understanding all the various processes that result in carcinogenesis, is important in identification of molecular targets toward which the use of combined chemotherapy, radiation therapy, immunotherapy, and gene therapy may be effective.

Nitric Oxide and Anticancer Therapy

Radiation Therapy

The toxic effect of radiation to the tumors and normal tissue is the result of ROS and RNOS generation. Hydroxyl radical is the most reactive species *in vivo* that contributes to peroxidation of lipids and nucleic acids following radiation therapy. Reperfusion reoxygenation damage to tumor vasculature between radiation fractions, and the toxicity to normal tissue are probably due to formation of RNOS. The role of NO in radiation therapy was first mentioned in 1957, when it was found to be a radiosensitizer in bacteria. More recently, NO was found to be a hypoxic radiosensitizer in mammalian tumors, possibly by substituting for oxygen in the rapid reactions with carbon-centered radicals formed in DNA by ionizing radiation. The newly formed *C*-nitroso adducts produce stabilization of radiation-induced DNA damage. Generally the hypoxic milieu of solid tumors restricts the effects of radiation therapy. NO-mediated radiosensitization prevents DNA repair in tumor cells, thereby increasing the effectiveness of this therapeutical approach.

There are a number of changes that occur in normal tissue following irradiation. First there is endothelial damage, which leads to microvascular disruption and altered blood flow. These changes are accompanied by capillary thrombi formation, endothelial swelling, increased vascular permeability, and interstitial edema. Later, aberrant endothelial proliferation and capillary sclerosis completely halts the blood flow and leads to tissue fibrosis. Atherosclerosis may develop in larger vessels due to endothelial dysfunction, erratic proliferation of the cells in the vascular wall, and increased platelet aggregation. The vascular remodeling produced by radiation-induced endothelial damage is noticeably similar to that induced by NO, RNOS, and ROS. Elevated NO causes free radical damage to lipid membranes and DNA. Peroxynitrite diffusion to more distant sites and its decomposition to $OH^{\cdot}$ causes additional damage. Lipid peroxidation activates phospholipase A_2 (PLA_2) and increases TNF-α and essential fatty acid consumption. Radiation-induced TNF-α triggers iNOS induction in macrophages and vascular cells, and the increased NO production causes further damage subsequent to each radiation fraction. This type of radiation-induced damage is preventable by thiols, which scavenge free radicals, reduce tissue fibrosis by attenuating the accumulation of macrophages and mast cells, and thereby, reverse endothelial damage.

Tissue undergoing remodeling after radiation injury is populated by mast cells, which release a number of substances such as, histamine, heparin, proteases, hydroxylases, peroxidase, and SOD. Some of these substances impair both the NO production and its effects, thus reducing the cytotoxicity of macrophages and fibroblasts in the irradiated area. During tissue repair, the angiogenic effect of macrophage-derived TNF-α, is reduced in the presence of the mast cells, an effect that also attenuates the NO production. In the absence of mast cells, however, TNF-α induces NO accumulation, which in turn by inhibiting cell proliferation, reduces angiogenesis and tumor growth.

In intermediate stages of radiation-induced damage, the normally quiescent endothelial cells start an abnormal pattern of dedifferentiation and proliferation to repair the damaged vessels that may lead to chronic and occlusive fibrosis. Radiation therapy was shown to cause a redistribution and

partial synchronization of the endothelial cell cycle in both tumor and normal vasculature. This may influence endothelial cell sensitivity to the subsequent radiation doses and affect cellular metabolism. For example, production of angiotensin-converting enzyme (ACE), prostacyclin (PGI_2), and tissue plasminogen activator (tPA) was shown to be altered by radiation and to consequently affect tissue hemodynamics, thrombogenicity, and fibrinolysis. Radiosensitivity is cell-cycle dependent, a process known as age response, and endothelial cells are more radioresistant in S phase and more radiosensitive in G_1 phase. Due to age response, smaller vessels are considerably more radiosensitive than larger ones and because tumor vessels contain more cycling cells, the consensus is that radiation may be more damaging to those vessels. In reality, normal cells are also proliferating during tissue repair or growth, and radiation damages them as well. For example, there is increased capillary permeability of the blood–brain barrier, after irradiation treatment. In addition, there is neuronal damage due to increased levels of glutamate produced during the radiation-induced ischemia. The glutamate activation of NOS through the *N*-methyl-D-aspartate (NMDA) receptors, increases calcium flux into the neurons to cytotoxic levels (Table IV). NO in the presence of O_2^- produces $ONOO^-$, which may diffuse further and damage the surrounding neurons. Irradiation of the heart causes acute cardiac function depression, by quenching endothelial NO by the radiation-produced O_2^- and peroxynitrite formation. Late postirradiation effects include cardiomyopathy and premature coronary stenosis. Irradiation of gastrointestinal (GI) tract results in GI dysfunction, whereas prostate gland irradiation produces impotence. These effects are the result of neuronal damage and nNOS dysfunction in nitrergic nerves innervating these organs. Additionally, there is vascular damage that has as a consequence reduction of constitutively produced NO.

In certain types of cancers elevated NO enhances the radiation-induced damage to the tumor, and as a result a number of NO donor agents have been used to directly increase tumor perfusion during radiation therapy. For example, nitrosothiols are good inhibitors of platelet aggregation at concentrations that do not affect the perfusion pressure. NO complexes with nucleophiles (NONOates) improve the tumor blood flow because they can release NO at acidic pH in the hypoxic tumor microenvironment. The NONOates may also inhibit the tumor cell proliferation as we have shown in human and murine colon carcinoma cell lines. There are a number of other compounds that may indirectly augment NO levels in tumors. For example, ACE inhibitors, stimulate endothelial NO release, and superoxide scavengers increase the availability of NO, while lowering the $ONOO^-$ and $HO\cdot$ levels. This would improve tissue perfusion and reduce the tissue damage by free radicals. Another indirect mode of elevating tumor NO levels is iNOS induction by TNF-α and IL-2, which will result in increased tumoricidal activity and decreased tumor cell proliferation. However, the cardiotoxic side effects to this approach greatly diminish its therapeutic use.

Tumor progression is prevented in certain cancers by drugs that lower the levels of NO in the tumor. These drugs are either selective NOS isoforms inhibitors, or drugs that limit the availability of certain NOS cofactors. Methotrexate, for example, blocks NO production in tumors by inhibiting the tetrahydrobiopterin synthesis. Steroids may counteract the inflammatory response to radiation by inhibiting PKC activity and consequently the induction of iNOS. Ketoconazole lowers the NO levels by preventing TNF-α induction. The mechanism of nicotinamide effect in cancer treatment is still unclear and there are reports that show a direct vasodilator effect, which is independent of NO release. Also nicotinamide inhibits NOS mRNA induction and downregulates TNF-α. As a result, there is a reduction of radiation-induced edema in the presence of nicotinamide. The tumoricidal effects of 5-fluorouracil (5-FU) are increased following radiation when the NO presence increases blood flow in the tumor and the surrounding tissue.

Thus the presence of NO in irradiated tumors has the ability to modulate the free radical-mediated cell damage in both normal and tumor tissues. At physiological NO levels microvascular perfusion is adequately maintained. Lowering the levels of NO in the presence of cytotoxic drugs causes ischemia and leads to tumor and normal tissue necrosis while greatly diminishing the effectiveness of radiation therapy. Conversely, the consequences of high levels of NO during radiation therapy include enhanced tumor and normal tissue damage, increased vascular permeability, and edema.

Chemotherapy

NO plays an important role in the modulation of blood flow and it may alter the toxic effects of some chemotherapeutic drugs in tumors or in normal tissue. Some chemotherapy drugs such as bleomycin, vinblasine, vincristine, and colchicine damage the vascular endothelium. Bleomycin-induced lung damage is probably due to the iNOS induction in alveolar macrophages. The cytokines TNF-α and IL-2 cause the induction of iNOS in the vascular cells, leading to profound hypotension, one of the major side effects of cytokine treatment. The symptoms that accompany vascular damage in cancer therapy include increased vascular permeability, extravasation of plasma proteins and circulating cells, edema, pain, tissue damage and destruction, disseminated intravascular coagulation, hypotension, shock, cachexia, and death. The occurrence of these symptoms is known as multiple organ failure or multiple organ dysfunction syndrome.

The information provided by such findings was used to design tumor reducing treatments, though in many cases the tumoricidal effects produced by immune cell activation were accompanied by serious side effects. For example the use of IL-2 to treat renal cancers had to be discontinued due to severe hypotension, capillary damage, and increased vascular permeability. NO seemed to be the main cause in the hypotensive effect and the endothelial damage produced during IL-2 treatment. Coadministration of NOS inhibi-

tors, or NO scavengers, greatly ameliorated the side effects of IL-2 treatment without compromising its therapeutic efficacy.

Both primary and metastatic tumors are histologically heterogeneous, containing infiltrates with host lymphocytes, macrophages, neutrophils, fibroblasts, and endothelial cells. iNOS can be activated in either tumor or infiltrated cells or in both. It is important to know that tumor regression does not require all cells in the tumor to produce augmented NO levels. The tumor microenvironment contains numerous cytokines and other factors that can both activate or inhibit the production of NO. Among the cytokines produced by stimulation of various cells, IFNs (α, β, and γ) have the potential to directly or indirectly modulate tumor growth through multiple mechanisms. Directly, the interferons inhibit cell proliferation and stimulate differentiation. Indirectly, they can activate the immune and vascular cells to produce NO. In addition, they are antiangiogenic because of the inhibitory action on the angiogenesis genes. IFNs are thus highly effective antitumor agents, although they damage normal tissue and cause severe side effects that render them useless in anticancer therapy. Unlike IL-2, the tumoricidal action of IFN is due to the elevated NO that resulted from iNOS induction. Consequently, the only therapeutic approach in this case is gene therapy. Transfection of the murine IFN-β gene, for example, completely inhibited tumor progression and metastasis produced in nude mice by the highly metastatic human melanoma, colon carcinoma, and renal cell carcinoma. Additionally, the IFN-β generating tumor cells cause both autolysis and cytolysis of surrounding cells in solid tumors, without any systemic side effects. Therefore, this localized, nonspecific, tumorigenic effect is exclusively due to the high local levels of NO.

The previously described symptoms greatly resemble the ones produced by bacterial and fungal proteases, or by bacterial cell wall components such as the negatively charged endotoxin or LPS in gram-negative bacteria and teichoic acid in gram-positive bacteria. There are a number of cellular events that take place at the inflammation site of bacterial infection and in cancerous tissues, and bradykinin (Bkn) and NO are some of the key players in this scenario. These events are responsible for the fluid accumulation in ascitic and pleural carcinomas, or for the increased vascular permeability in solid tumors. As mentioned earlier, MMPs from tumor cells play an important role in the metastatic process. Elevation of NO, Bkn, kallikrein, and MMP IV (also known as a metastatic factor) enlarges the endothelial gap opening, enhances extravasation of immune and tumor cells as well as that of plasma proteins, and promotes collagen IV degradation in interstitial space. The increased pressure in the interstitial tissue allows the reverse transfer of macromolecules, tumor cells, or bacteria into the bloodstream and their dissemination to distant organs. The angiogenic effect of NO further enhances metastatic spread. Consequently, only a cocktail of iNOS, kinin, and kallikrein inhibitors may potentially eradicate microbial infection, septicemia, or cancer.

Considering that systemic chemotherapy often results in damage to normal tissue, different modalities of drug delivery to the tumors have been tested. For instance, administration of phospholipid liposomes containing macrophage activators successfully eradicated liver metastases in mice, without significant systemic side effects. Likewise, the use of highly phagocytic organ specific cells, or transfection of iNOS or INF-γ genes to tumor cells, or transfection of tumor cells with a retrovirus containing the iNOS gene, resulted in tumor cell destruction with dramatic attenuation of undesired side effects. Therefore, all these examples suggest that high, localized NO delivery or production has the most tumorigenic effect, with the least damage to normal tissue.

Administration of cytotoxic drugs results in extensive tumor cell lysis, a process that releases the ''injury mediators'' including transition metal ions, discussed earlier, that damage normal tissues in the vicinity of cancer cells. For example, patients receiving chemotherapy for leukemia have been shown to have high levels of free iron in the plasma. Iron ions, in combination with some of the released ''injury mediators'' such as NO, H_2O_2, and O_2^-, may be toxic to both tumor and normal tissue, and contribute to the unwanted side effects of chemotherapy. Ideally, agents that selectively modulate individual NOS isozymes, and distinguish the normal tissue from the diseased one, as well as specific free radical scavengers in combination with radiation or chemotoxic drugs may be efficacious and able to improve the therapeutic gain.

Additional research is indispensable to fully understand the multiple roles played by NO in tumor inhibition or tumor progression and to improve the delivery of drugs, vectors, and effector cells to solid tumors. Furthermore, identification of NO-resistant and NO sensitive types of tumors, improvement of tumor suppression methods, characterization of all additional pathways involved in tumorigenesis, and development of selective drug combinations that have a high tumoricidal effect with minimum damage to normal tissues, are areas that require immediate attention.

Additional Reading

Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol.* **271** (*Cell Physiol.* **40**), C1424–C1437.

Buga, G. M., Wei, L. H., Bauer, P. M., Fukuto, J. M., and Ignarro, L. J. (1998). N^G-Hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms. *Am. J. Physiol.* **275** (*Regulatory Integrative Comp. Physiol.* **44**), R1256–R1264.

Correa, P., and Miller, M. J. S. (1998). Carcinogenesis, apoptosis and cell proliferation. *Br. Med. Bull.* **54,** 151–162.

Cotran, R. S., Kumar, V., and Colliuns, T. (1999). Neoplasia. *In* ''Robins' Pathologic Bases of Disease'' (R. S. Cotran, V. Kumar, and T. Collins, eds.), pp. 260–327. Saunders, Philadelphia, London, Toronto, Montreal, Sydney, and Tokyo.

Donehower, L. A. (1996). Effects of p53 mutation on tumor progression: Recent insights from mouse tumor models. *Biochim. Biophys. Acta* **1242,** 171–176.

Fukumura, D., and Jain, R. K. (1998). Role of nitric oxide in angiogenesis and microcirculation in tumors. *Cancer Metastasis Rev.* **17,** 77–89.

Fukuto, J. M. (1995). Chemistry of nitric oxide: Biologically relevant aspects. *Adv. Pharmacol.* **34,** 1–14.

Gallo, O., Masini, E., Morbidelli, L., Franchi, A., Fini-Storchi, I., Vergari, W. A., and Ziche, M. (1998). Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J. Natl. Cancer Inst.* **90,** 587–596.

Gross, S. S., and Wolin, M. S. (1995). Nitric oxide: Pathophysiological mechanisms. *Annu. Rev. Physiol.* **57,** 737–769.

Halliwell, B., Gutteridge, J. M. C., and Cross, C. E. (1992). Free radicals, antioxidants and human disease: Where are we now? *J. Lab. Clin. Med.* **119,** 598–620.

Howard-Flanders, P. (1957). Effect of nitric oxide on the radiosensitivity of bacteria. *Nature* **364,** 577.

Kim, Y.-M., Bombeck, C. A., and Billiar, T. R. (1999). Nitric oxide as a bifunctional regulator of apoptosis. *Circ. Res.* **84,** 253–256.

Kroncke, K.-D., Fehsel, K., and Kolb-Bachofen, V. (1997). Nitric oxide: Cytotoxicity versus cytoprotection—How, why, when, and where? *Nitric Oxide* **1,** 107–120.

Lala, P. K., and Orucevic, A. (1998). Role of nitric oxide in tumor progression: Lessons from experimental tumors. *Cancer Metastasis Rev.* **17,** 91–106.

Lala, P. K. (1998). Significance of nitric oxide in carcinogenesis, tumor progression and cancer therapy. *Cancer Metastasis Rev.* **17,** 1–6.

Maeda, H., Akaike, T., Wu, J., Noguchi, Y., and Sakata, Y. (1996). Bradykinin and nitric oxide in infectious disease and cancer. *Immunopharmacology* **33,** 222–230.

Ohshima, H., and Bartsch, H. (1994). Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mutat. Res.* **305,** 253–264.

Sagar, S. M., Singh, G., Hodson, D. I., and Whitton, A. C. (1995). Laboratory–clinic interface. Nitric oxide and anti-cancer therapy. *Cancer Treatment Rev.* **21,** 159–181.

Stamler, J. S. (1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78,** 931–936.

Stuehr, D. J., and Nathan, C. F. (1989). Nitric oxide a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169,** 1543–1555.

Thomsen, L. L., and Miles, D. W. (1998). Role of nitric oxide in tumor progression: Lessons from human tumors. *Cancer Metastasis Rev.* **17,** 107–118.

Uren, A. G., and Vaux, D. L. (1996). Molecular and clinical aspects of apoptosis. *Pharmacol. Ther.* **72,** 37–50.

Wink, D. A., and Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radicals Biol. Med.* **25,** 434–456.

Wink, D. A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M. W., and Mitchell, J. B. (1998). The multifaceted roles of nitric oxide in cancer. *Carcinogenesis* **19,** 711–721.

Xie, K., and Fidler, I. J. (1998). Therapy of cancer metastasis by activation of the inducible nitric oxide synthase. *Cancer Metastasis Rev.* **17,** 55–75.

Xie, K., Dong, Z., and Fidler, I. J. (1996). Activation of nitric oxide synthase gene for inhibition of cancer metastasis. *J. Leukocyte Biol.* **59,** 797–803.

CHAPTER 55

Vascular Nitric Oxide in Health and Disease

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NITRIC OXIDE HAS EFFECTS ON VASCULAR TONE, CELLULAR ADHESION, GROWTH AND REMODELING, INFLAMMATION, AND PERMEABILITY. STUDIES IN ANIMALS HAVE DEMONSTRATED THAT THESE PROCESSES ARE INVOLVED IN THE NORMAL HOMEOSTASIS OF THE CARDIOVASCULAR SYSTEM AND, IF ABNORMAL, CAN CONTRIBUTE TO DISEASE STATES. THERE IS NOW ABUNDANT EVIDENCE THAT NITRIC OXIDE BIOSYNTHESIS ALSO PLAYS AN IMPORTANT ROLE IN THE REGULATION OF HUMAN VASCULATURE. INHIBITION OF NITRIC OXIDE SYNTHASES INCREASES VASCULAR TONE AND BLOOD PRESSURE IN HUMANS, AND DEFECTS OF BASAL AND AGONIST-STIMULATED RELEASE OF NITRIC OXIDE ARE FOUND IN A VARIETY OF VASCULAR DISEASES OR IN THE PRESENCE OF RISK FACTORS FOR DISEASE. CHANGES IN NITRIC OXIDE-MEDIATED VASCULAR PROTECTION MAY ARISE FROM COMMON GENETIC VARIATION IN NITRIC OXIDE SYNTHASE GENES, ENVIRONMENTAL INFLUENCES ON ENZYME EXPRESSION OR ACTIVITY, OR A COMBINATION OF GENETIC AND ENVIRONMENTAL INTERACTIONS. OVERPRODUCTION OF NITRIC OXIDE WITHIN THE VESSEL WALL ALSO CONTRIBUTES TO DISEASE. THIS CHAPTER CONSIDERS THE ROLES AND EFFECTS OF NITRIC OXIDE IN HUMAN VASCULATURE AND HIGHLIGHTS OPPORTUNITIES FOR THERAPEUTIC INTERVENTION.

Endothelium-Dependent Relaxation in Humans

Endothelial nitric oxide synthase (eNOS) is found in endothelial cells throughout the cardiovascular tree. Human arteries, arterioles, veins, and venules all show nitric oxide-mediated relaxation in response to agonists such as acetylcholine or bradykinin (Calver *et al.,* 1993). Stimuli that increase nitric oxide mediated vasodilation in human blood vessels are shown in Fig. 1. The evidence that nitric oxide is a regulator of vascular tone in humans is provided by studies in which eNOS has been blocked by N^G-monomethyl-L-arginine (L-NMMA) and by the direct detection of NO using a porphyrinic microsensor (Vallance *et al.,* 1995).

Arteries

Epicardial coronary arteries relax in response to acetylcholine, bradykinin, substance P, or increased shear stress, and the relaxation is mediated largely by nitric oxide. The brachial artery behaves similarly. Although a major part of the relaxant response to acetylcholine in human conduit arteries is mediated by NO, the overall response to this agonist is complex. Acetylcholine produces direct vasoconstriction through activation of receptors on smooth muscle cells and can alter the output of norepinephrine from sympathetic nerve terminals (Calver *et al.,* 1993). Thus an altered response to acetylcholine is not synonymous with altered NO generation. Coronary arteries seem to have some basal nitric oxide dilator tone, because infusion of L-NMMA reduces

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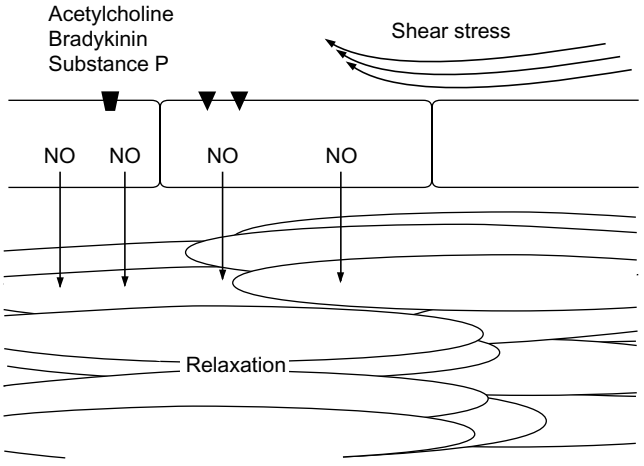


Figure 1 Endothelium generates nitric oxide to relax the underlying smooth muscle. Agonists such as acetylcholine, bradykinin, and substance P can increase nitric oxide production through interaction with receptors on the endothelial cell. The endothelium is also capable of picking up physical signals in the lumen of the vessel such as increased shear stress. Shear stress is probably detected by specialist ion channels in the endothelial cell membrane. Chemical probes, such as acetylcholine, or physical stimuli, such as increased shear stress associated with flow, are used to probe the NO pathway *in vivo.* Alternatively, NO synthesis can be inhibited with L-NMMA.

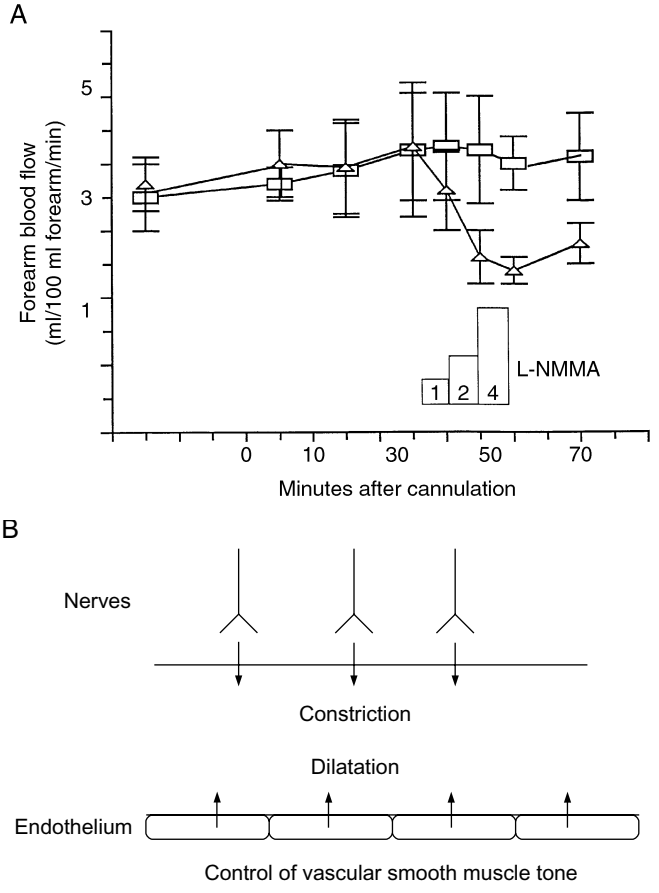


Figure 2 (A) Infusion of L-NMMA into the brachial artery of one arm leads to a dose-dependent fall in resting forearm blood flow. Blood flow in the other arm stays constant. This indicates that there is a basal generation of nitric oxide in resistance vessels, which maintains these vessels in a state of active vasodilation. (B) The NO component of the control of vascular tone seems to be approximately equal in magnitude and opposite in effect to that of the sympathetic nervous system. Together the NO pathway and the sympathetic nervous system provide a local (NO) and a centrally regulated (sympathetic) system for the rapid adjustment of vascular tone.

resting diameter by about 5% (Lefroy *et al.,* 1993). In contrast, there appears to be little basal nitric oxide dilator tone in the brachial or radial arteries of healthy volunteers. Human conduit arteries show prominent dilation in response to increases in flow (shear stress), and this is blocked by L-NMMA (Joannides *et al.,* 1995). The physiological significance of flow-mediated dilation is not known, but it may help to keep shear stress constant (for review, see MacAllister and Vallance, 1996).

Arterioles

Human resistance vessels (small arteries and arterioles) show marked relaxation to acetylcholine, bradykinin, and substance P (Calver *et al.,* 1993). However, unlike the relaxant responses seen in conduit arteries and veins, in the resistance vessels only part of the response to these agonists is blocked by L-NMMA or other inhibitors of NOS. Some of the residual response may be mediated by endothelium-dependent hyperpolarization, but some may even be endothelium-independent. However, resistance vessels show the most obvious basal NO dilator tone. L-NMMA reduces resting blood flow by about 40 to 50% (Vallance *et al.,* 1989a), and this effect has been observed in skeletal muscle, coronary, renal, and cerebral beds in humans. The reduction in flow in response to L-NMMA represents a near doubling of vascular resistance and indicates that basal NO-mediated dilator tone is approximately equal in magnitude and opposite in effect to the constrictor tone provided by the sympathetic nervous system (Fig. 2).

Veins

Like arteries and arterioles, venous endothelium expresses NOS. However, unlike in the arterial vessels, this NOS appears quiescent under basal conditions (Vallance *et al.,* 1989b). L-NMMA does not seem to cause venoconstriction in humans and does not alter the constrictor responses either to exogenous norepinephrine or to activation of the sympathetic nervous system. However, acetylcholine, bradykinin, and various other agonists produce endothelium-dependent relaxation of human veins, and this can be blocked totally by L-NMMA. Furthermore, the veins are exquisitively sensitive to NO donor agents. Thus the lack of basal NO dilation in veins seems to be due to lack of basal activity of NOS rather than deficient expression of the enzyme or inability of the smooth muscle to respond. The reasons for the arteriovenous difference in basal NOS activity are unclear. It does not relate solely to the environment in which the vessels are studied, since the arteriovenous dis-

tinction persists *ex vivo* if the vessels are studied under identical conditions in organ baths. Similar to the responses seen in conduit arteries, in veins the response to acetylcholine is a mixture of endothelium-dependent, NO-mediated relaxation and direct constrictor effects on smooth muscle.

Venules

There have been few studies of human venules. However, the limited data available suggest that venules do not generate NO basally but show pronounced endothelium-dependent relaxation to bradykinin, which is almost entirely due to NO (Riezebos *et al.,* 1994).

Integrated Responses

Systemic inhibition of NOS in humans increases total peripheral resistance and blood pressure (Fig. 3). However the rise in blood pressure is partially offset by a fall in cardiac output (Stamler *et al.,* 1994; Haynes *et al.,* 1993). The mechanism underlying the fall in cardiac output is not known, but it is due to both bradycardia and a fall in stroke volume, and may be a reflex compensatory response. Systemic administration of L-NMMA causes a fall in renal blood flow, and this vascular bed seems to be particularly sensitive to NOS inhibition; indeed, very low doses of NOS inhibitors produce a selective fall in renal blood flow and cause sodium retention. In addition to producing arteriolar vasoconstriction, loss of NO seems to decrease vascular compliance, and this leads to an increase in pulse pressure.

Effects on Circulating Cells

It has been difficult to study the effects of endogenous NO on circulating cells in humans. Studies using human endothelial cells or vessels *in vitro* clearly show an antiplatelet and antiadhesive action of endothelium-derived NO. However, L-NMMA does not alter platelet aggregation in humans (Vallance *et al.,* 1992a); rather, it produces a small fall in the bleeding time (Simon *et al.,* 1995). This latter effect may be due to effects of platelets but might be secondary to changes in vascular tone. There is also evidence that provision of excess L-arginine might affect monocycle function in humans (Adams *et al.,* 1997), but the mechanisms are unclear.

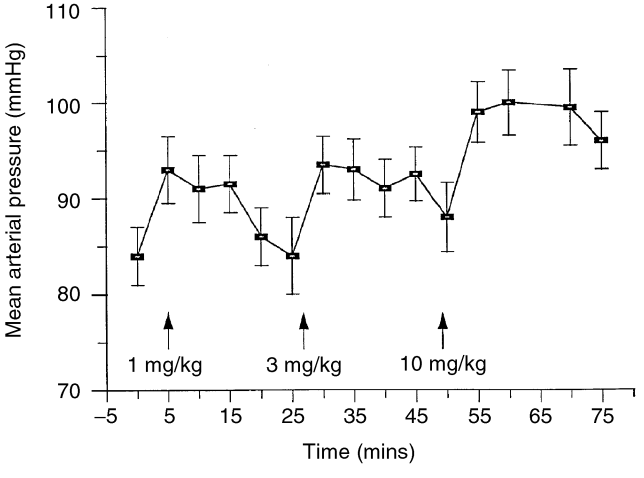


Figure 3 Injection of L-NMMA into healthy volunteers causes a dose-dependent increase in blood pressure. The increase in blood pressure is relatively modest compared to the very large increase in systemic vascular resistance that occurs. The increase in blood pressure is partially offset by a decrease in cardiac output.

Assessment of Nitric Oxide in Humans

The L-arginine:NO pathway has been assessed in humans at molecular, biochemical, and functional levels. Each approach has strengths and weaknesses and provides different information on the pathway.

Molecular Studies

In order to probe for the presence of mRNA or to identify NOS protein, it is necessary to remove cells or tissues. For studies of human vasculature this has meant that experiments have had to rely either on cultured cells or on vascular specimens obtained at operation or post mortem. Cellular studies *in vitro* have been of great use, but they may not be directly applicable to the situation *in vivo* or in disease states. The difficulty in obtaining properly matched samples taken in a uniform manner has meant that there have been few studies of the molecular expression or regulation of NOS in intact human blood vessels. Biopsies of human hand veins from healthy volunteers have shown the presence of mRNA for eNOS but not neuronal NOS (nNOS) or inducible NOS (iNOS), and this correlates with the functional responses seen in these vessels (Bhagat and Vallance, 1999).

Biochemical Studies

Biochemical studies of NOS in humans (excepting studies on cultured human cells) have relied on direct enzyme assays in tissue samples, the measurement of products of NOS, or assessment of cyclic GMP levels. Enzyme assays have been useful; however, again, they are dependent on the availability of tissues, and the assays measure the total amount of active enzyme in the sample (measured under near optimal conditions), rather than the level of activity of the enzyme when present in its usual environment *in vivo.* Measurements of products of NOS have included assessment of NO itself, assessment of more stable breakdown products of NO (nitrite or nitrate), measurement of nitrosothiols, or even measurement of citrulline.

Measurement of NO is fraught with problems due to the very short half-life of NO and its rapid destruction on contact with hemoglobin. Although measurement of nitrite and nitrate is more reliable, nitrate is also present in the diet, and so dietary intake may confound interpretation of results

(Bayliss and Vallance, 1998). The significance of circulating nitrosothiols is uncertain, and the levels cannot be taken as an index of NO generation. Citrulline is formed by a variety of biochemical pathways, and the plasma concentration of this amino acid is not a marker of NOS activity. Measures of cGMP are useful, but of course this intracellular messenger is also elevated in response to other mediators including atrial natriuretic peptide (ANP).

It is clear therefore that biochemical assessment of NO in humans *in vivo* is far from straightforward. Measurement of plasma nitrite and nitrate probably provides the simplest and most accessible measure, provided diet is controlled. The technique can be improved further by administering [^{15}N]arginine and measuring the generation of [^{15}N]nitrate, since with this approach, at least it is clear that the nitrate derives from the L-arginine:NO pathway (Hibbs *et al.,* 1992; Forte *et al.,* 1997). However, even with a reliable measure of total body NO generation, it is not known how much of the NO comes from the cardiovascular system and how much derives from other cellular sources of NO.

Functional Studies

Nitric oxide is a free radical with a short half-life in biological systems. This has confounded biochemical assessment in humans *in vivo,* but it is also clear that minor changes in the half-life of NO might lead to significant alterations in the amount of active NO reaching target enzymes. Thus while the generation of NO might remain constant, the biological effects of the pathway could vary with minor changes in the stability of the NO generated. An alternative approach to study the L-arginine:NO pathway is to rely on functional assessments of its effects in the cardiovascular system. This has advantages in that it measures the overall consequences of NOS activity, but it should be remembered that this is not necessarily the same as either enzyme activity or amount of NO generated. Nonetheless, most studies of NO in human vasculature have taken a functional approach. Techniques include measurement of blood vessel tone or diameter and assessment of blood flow or blood pressure in a variety of vascular beds. The basal activity of the NO pathway has been studied by blocking NOS with L-NMMA or other inhibitors. The ability of the system to increase NO generation has been studied by measuring responses to agonists (e.g., acetylcholine) or physical stimuli (e.g., shear stress).

Physiology

Endothelial NOS

Basal generation of nitric oxide maintains the arterial circulation in a state of tonic vasodilation. There is evidence that there is increased nitric oxide-mediated vasodilation in response to increased flow or shear stress. The physiological significance of this is discussed in detail elsewhere in this book. One situation in which this might be important is the response to acute changes in intravascular volume. If a healthy individual is rapidly volume loaded with saline, blood pressure does not change (Calver *et al.,* 1992a). This is because there is arteriolar and venous dilation. At least part of the arterial response is depenent on NO generation, suggesting that in the absence of an effective NO pathway, volume expansion might lead to hypertension.

Certain types of exercise increase NO-mediated dilation in the human forearm (Dyke *et al.,* 1995; Kingwell *et al.,* 1997). There appears to be an acute effect of exercise, and it is possible that this is mediated by increased flow secondary to enhanced cardiac output. Chronic exercise training might also increase endothelium-dependent vasodilation in humans, but the results are conflicting. Studies from animals and cultured cells suggest that such an effect might be mediated by induction of expression of eNOS by chronic increase in shear stress or mediated by alterations in the subcellular localization of eNOS (Garcia-Cardena *et al.,* 1998). Ischemia also causes vasodilation in human skeletal muscle and other beds, but this effect is probably not mediated by NOS activity.

The L-arginine:NO pathway is regulated in part by sex steroids. Estrogens can induce expression of eNOS in a variety of systems (Kauser and Rubanyi, 1997) and increase endothelium-dependent relaxation. There is evidence that flow-mediated dilation of the brachial artery alters throughout the menstrual cycle and that NO-mediated dilation is increased in normal pregnancy (Williams *et al.,* 1997). Pregnancy is associated with vasodilation of resistance vessels, an increase in arteriovenous shunts, and increased circulating blood volume. Studies in pregnant women have shown an increase constrictor response to L-NMMA and a decrease in constrictor response to norepinephrine in the hand circulation during pregnancy (Williams *et al.,* 1997), (Fig. 4). It is likely that this effect is dependent on estrogens, but it is also possible that increased shear stress contributes. Finally, other hormones, including insulin, might also affect endothelial NO, and there is evidence that insulin causes NO-dependent vasodilation in the human forearm (Steinberg *et al.,* 1994).

Neuronal NOS?

The role of nNOS in regulating vascular tone in humans is not clear (except in the genital tract; see elsewhere in this volume) (Fig. 5). In the cerebral circulation NO generated from nNOS is thought to be important in the process of vasoneuronal coupling, the mechanism by which neuronal activation leads to an appropriate increase in blood flow to the active brain region. However, studies with L-NMMA in healthy volunteers have failed to detect a significant effect of NO on vasoneuronal coupling. These studies do not rule out an effect of nNOS in the brain, however, since L-NMMA may not cross the blood–brain barrier sufficiently to inhibit the enzyme. In contrast to results of studies in animals, in humans NO seems to play a relatively minor role in the

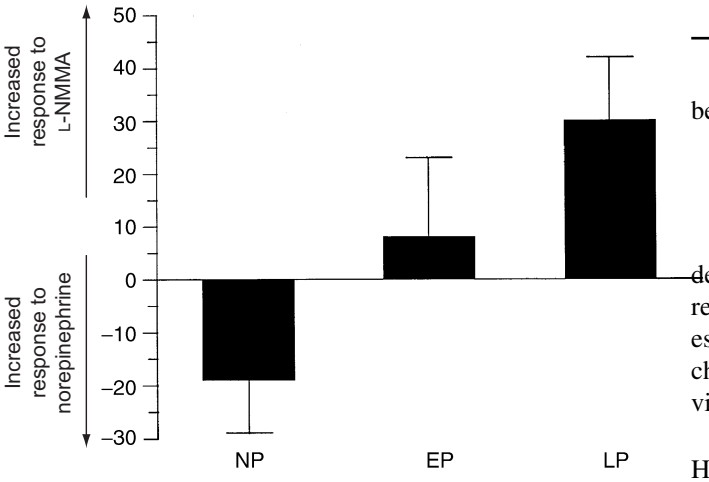


Figure 4 L-NMMA produces a greater fall in blood pressure in pregnancy than it does in the nonpregnant state. Studies were undertaken in 10 nonpregnant women (NP), 10 women in early pregnancy (EP), and 10 women in late pregnancy (LP). Vasoconstrictor responses to L-NMMA and to norepinephrine were compared in the forearm arterial circulation. In the nonpregnant state the doses of norepinephrine selected produced a greater fall in forearm blood flow than the doses of L-NMMA used. In early pregnancy the norepinephrine and L-NMMA were approximately equally effective; in late pregnancy the L-NMMA was more effective than the norepinephrine. (From Williams *et al.,* 1997.)

cerebrovascular dilator response to carbon dioxide (White *et al.,* 1998).

In the periphery, local warming causes vasodilation in humans, and in the hand circulation, this appears to be NO-mediated, at least in part (Williams *et al.,* 1995). It is not known whether the effect is mediated by eNOS or is secondary to local neurogenic stimulation and activation of nNOS. Mental stress causes a large increase in blood flow in the human forearm, and it had been assumed that this is due to withdrawal of sympathetic tone. However, it has been shown that L-NMMA blunts the response, but again it is not clear whether the NO originates from endothelium or nerves (Dietz *et al.,* 1994, 1997).

Pathophysiology

Increases and decreases in NO-mediated dilation have been implicated in vascular disease in humans.

Decreased NO-Mediated Dilation

Impaired responses to ''endothelium-dependent'' agonists, decreased flow-mediated dilation, or decreased constrictor responses to L-NMMA have been detected in patients with essential hypertension, diabetes (type I or type II), or hypercholesterolemia, in active or passive smokers, and in individuals with elevated circulating levels of homocysteine.

HYPERCHOLESTEROLEMIA

The most consistent results have been obtained in patients with hypercholesterolemia, in whom there seems little doubt that there is impairment of endothelium-dependent dilation (for review, see Maxwell *et al.,* 1998). Interestingly, the basal NO-mediated dilation seems to be preserved, but the dilator response to ''endothelium-dependent'' agonists or flow is impaired. The defect has been seen in conduit arteries (including the coronary vessels) and in resistant beds. Normally, epicardial coronary arteries dilate to low-dose acetylcholine, whereas in patients with raised cholesterol levels, the vessels constrict. A similar pattern of responses is seen in patients with overt atheromatous disease. The functional significance of these changes is not yet known, but impaired endothelium-dependent relaxation would be expected to render the vessels more susceptible to vasospasm or vasoconstriction in response to activated platelets, and might be associated with enhanced platelet and white cell adhesion. Together these changes may predispose to acute vaso-occlusive events, and studies in animals suggest that, if chronic, such changes may enhance atherogenesis. The potential significance of the changes in endothelium-dependent dilation in the resistance vessels is less well understood, but altered endothelial function might lead to dysregulation of the distribution of blood flow within a vascular bed, which

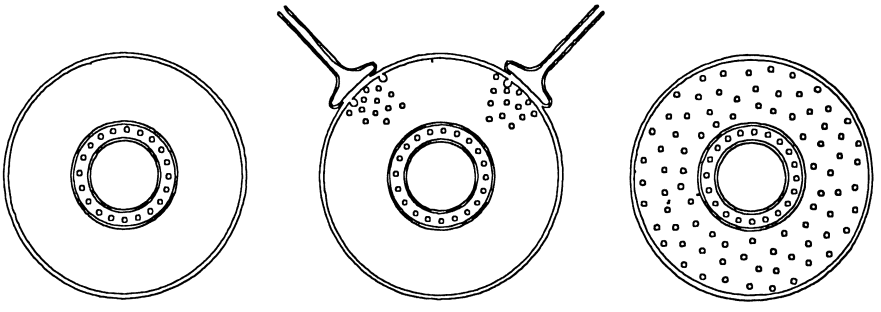


Figure 5 There are three potential enzymatic sources of nitric oxide within the blood vessel wall. eNOS in the endothelium probably accounts for the majority of physiological regulation of peripheral vascular tone. In some vessels, nerves in the adventita may also release nitric oxide generated by the activity of nNOS. This is probably important in the brain and also in the process of penile erection. In conditions of gross inflammation nitric oxide might be produced throughout the blood vessel wall through *de novo* expression of inducible isoform of nitric oxide synthase.

might cause regional ischemia. Interestingly, the defect seen in patients with hypercholesterolemia is reversed by exogenous L-arginine and possibly by the NOS cofactor tetrahydrobiopterin. This is discussed in greater detail later.

HYPERTENSION

Initial studies in patients with hypertension suggested that responses to endothelium-dependent dilators is diminished in the forearm arterial bed (Panza *et al.,* 1990; Linder *et al.,* 1990). Subsequently it was shown that the constriction to L-NMMA is also decreased in hypertensive subjects and that the higher the pressure, the smaller the response to NOS inhibition (Calver *et al.,* 1992b). Together these studies indicated a defect of both basal and stimulated NO-mediated dilation in hypertension. However, results have not been uniformly consistent (Cockcroft *et al.,* 1994). Some studies have failed to detect differences in responses to endothelium-dependent agonists or L-NMMA between hypertensive and normotensive subjects. Furthermore, it has been reported that flow-mediated dilation was preserved to normal levels in hypertensives. More recently, studies with [^{15}N]arginine have demonstrated that hypertension is associated with significantly reduced rates of production of NO (Forte *et al.,* 1997). In addition, a retrospective analysis of many hundreds of individuals who have taken part in studies of flow-mediated dilation of the brachial artery has identified the level of blood pressure as the major determinant of the response. Thus, although there are some discrepancies within the literature, the bulk of the available evidence supports the notion that there is a functional defect in NO-mediated dilation in many individuals with raised blood pressure, that there is an inverse relationship between the level of blood pressure and NO-mediated dilation, and that the defect is likely to be due to a true deficit in NO generation.

Data on the reversibility of the endothelial dysfunction in hypertension are also somewhat contradictory. However, several studies have shown that lowering blood pressure restores the response to L-NMMA toward normal (Calver *et al.,* 1994; Lyons *et al.,* 1994), suggesting that the defect is a consequence of the raised pressure rather than a primary cause. There is no convincing evidence than any one class of antihypertensive agents is any more or less likely than any other to restore endothelial function. Patients with secondary hypertension also seem to have impaired endothelium-dependent responses, and one study has suggested a genetic component to the defect, because unaffected normotensive offspring also seemed to have an impairment of normal endothelial function (Taddei *et al.,* 1996).

DIABETES

Patients with type II diabetes have impaired endothelium-dependent dilation to agonists and to flow. The situation with type I diabetes is less clear-cut, but it seems as though responses to L-NMMA are decreased (Calver *et al.,* 1992c; Elliott *et al.,* 1993) and might be most markedly abnormal in those individuals who have evidence of proteinuria. Unlike the situation in hypertension, in diabetes there is increasing evidence to suggest that the generation of NO might be normal, and that the defect might be due either to increased destruction of NO or to decreased sensitivity of the smooth muscle effector systems to NO. One possibility is that increased generation of oxygen free radicals leads to more rapid degradation of the NO. Several studies have also suggested that insulin resistance is associated with endothelial dysfunction; indeed, it has been hypothesized that endothelial dysfunction might be the primary cause of insulin resistance and contribute to both metabolic and vascular effects of the syndrome (for review, see Cleland *et al.,* 1998).

MECHANISMS OF CHANGES

The mechanisms of alterations of NO-mediated dilation are likely to vary between disease states and between individuals. The gene for eNOS shows polymorphic variation (Fig. 6), and several of these variants have been associated with altered cardiovascular risk. However, it is not yet known how or whether the variants alter expression or activity of eNOS in humans *in vivo.*

In diabetes, increased generation of oxygen radicals (''oxidative stress'') seems to be a likely mechanism of impaired endothelium-dependent dilation, and this might also be important in individuals with overt atheroma. Although acute systemic inflammation can be associated with increased NO generation and vasodilation (see later), there is also evidence that some types of inflammatory responses may lead to impaired endothelium-dependent relaxation. The cytokine tumor necrosis factor α can induce profound endothelial dysfunction in humans (Bhagat and Vallance, 1997), and this might also be mediated by increased generation of oxygen free radicals that decrease the bioavailability of NO.

In hypercholesterolemia there is some evidence to suggest that a defect in tetrahydrobiopterin can contribute to the impairment of endothelium-dependent relaxation. There is also strong evidence to suggest that L-arginine can restore normal endothelial function. This is an unexpected observation since the K_m for arginine as a substrate for NOS is of the

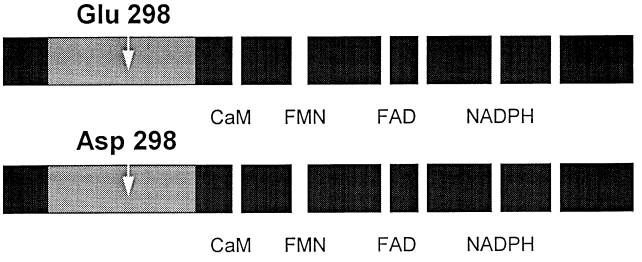


Figure 6 Several common variants of the eNOS gene occur in the population (polymorphic variations). Some of these are nonfunctional, some occur in the promoter region, and one encodes an amino acid substitution (Glu for Asp at position 298). In the United Kingdom about 10% of individuals are homozygous for the Glu variant, and about 50% are homozygous for the Asp variant. The Glu variant has been suggested to be associated with increased risk of coronary artery disease and other vascular problems. Association between other polymorphic variations and cardiovascular diseases have also been reported.

order of 1–2 μ*M,* the circulating concentration of arginine is of the order of 50–100 μ*M,* and the intracellular concentration of this amino acid may reach 1 m*M.* Thus arginine should not be rate limiting for NOS under normal circumstances. Indeed, this is the case in healthy individuals, in whom provision of excess arginine does not increase NO production through the mechanism of provision of excess substrate. The changes that occur in the presence of hypercholesterolemia that result in arginine apparently becoming rate limiting for stimulated NO-mediated dilation are not known. One possibility is that, owing to subcellular compartmentalization of NOS, there is a true local deficit in arginine in this condition. Another possibility is that accumulation of endogenous methylarginines, which inhibit NOS, alters the apparent K_m for arginine (Fig. 7). Endogenous methylarginines are found in increasing concentrations in individuals with hypercholesterolemia (Leiper and Vallance, 1999), and the plasma levels seem to correlate with endothelial dysfunction and with intimal thickening in the carotid artery. Such compounds also accumulate in patients with renal disease, another situation in which endothelial function seems to be impaired, and the defect is reversed by L-arginine (Vallance *et al.,* 1992b).

CONSEQUENCES OF CHANGES

Decreased functional activity of the L-arginine:NO pathway, whether this is due to decreased NO generation, increased destruction of NO, or decreased sensitivity of targets for NO, would be expected to alter vascular reactivity as well as platelet and white cell function. Ultimately, the changes may promote atherogenesis and/or enhance the likelihood of vessel occlusion. Interestingly, not all conditions associated with apparent loss of NO-mediated dilation cause hypertension, yet loss of NO would be expected to raise blood pressure. However, the major effect of NO is on vascular resistance rather than blood pressure, and it is clear that significant changes in resistance can occur before blood pressure is affected. Furthermore, hypertension is more prevalent in patients with other conditions associated with endothelial dysfunction, and indeed patients with diabetes or hypercholesterolemia tend to have higher blood pressures than age- and sex-matched healthy control subjects. The other important consequence of loss of NO-mediated dilation might be to decrease vascular compliance. This would have profound long-term hemodynamic consequences and would cause a ''stiffer'' vasculature.

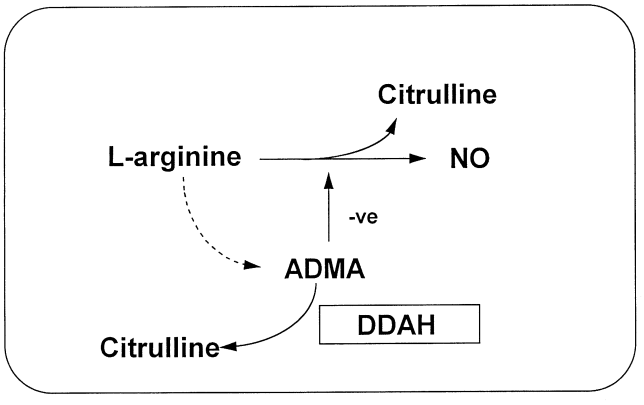


Figure 7 L-Arginine is converted to nitric oxide by the action of nitric oxide synthase. However, it can also be methylated in the guanidino nitrogens to produce L-NMMA and dimethylarginines. One of the dimethylarginines, asymmetric dimethylarginine (ADMA), is an inhibitor of NOS. This compound circulates in the plasma, is excreted in the urine, and is concentrated within cells. Certain cells also express the enzyme dimethylarginine–dimethylaminohydrolase (DDAH), which metabolizes ADMA back to citrulline, thereby inactivating the inhibitor. There is increasing interest in the possibility that endogenous ADMA might regulate the amount of NO synthesized in health or disease. -ve, inhibition of NOS activity.

Increased NO-Mediated Dilation

Increased NO production would be expected to cause vasodilation and might also disrupt normal regulation of vascular tone. There is abundant evidence from studies in animals and *in vitro* that certain inflammatory stimuli cause vasodilation by stimulating increased NO generation (for review, see Vallance and Charles, 1998). There is increasing evidence that the same occurs in humans *in vivo,* although mechanisms underlying the increased NO generation may differ between rodents and humans.

SEPTIC SHOCK AND SYSTEMIC INFLAMMATORY RESPONSE SYNDROME

Most patients with overwhelming acute systemic infection have hypotension, and the fall in blood pressure is due largely to a fall in systemic vascular resistance. At local levels this vasodilation might also impair appropriate distribution of oxygenated blood within tissues. There is biochemical evidence for increased generation of NO in patients with septic shock, in whom plasma concentrations of nitrite and nitrate are of the order of 50–100% higher than those seen in healthy control subjects (Evans *et al.,* 1993). Patients treated with the cytokine IL-2 also develop a septic shock-type systemic vasodilator response, and this is associated with a rise in the circulating concentrations of nitrate. Furthermore, in this situation, studies with [^{15}N]arginine have been undertaken, and it has been clearly shown that the nitrate derives from arginine (Hibbs *et al.,* 1992). The vasodilation and hypotension in septic shock or IL-2 induced shock are both reversed by L-NMMA, indicating that increased NO-mediated dilation contributes to the cardiovascular collapse. Administration of L-NMMA to patients with septic shock leads to arterial vasoconstriction in systemic and pulmonary beds and also causes venoconstriction, indicating that in this situation the veins also begin to generate significant amounts of ''basal'' NO (Petros *et al.,* 1991, 1994). It remains to be determined whether the overproduction of NO that occurs also contributes to impaired tissue oxygen consumption through effects on mitochondrial enzymes.

In rodents the mechanism of the increased vascular NO generation in response to bacterial endotoxin or certain proinflammatory cytokines is well established. The inflam-

matory mediators induce expression of iNOS in vascular smooth muscle, and this results in the generation of large amounts of NO. It is not yet clear whether the same mechanism occurs in humans (Fig. 8). Certainly human vascular cells can be induced to express iNOS, but they do so less readily than do rodent cells. In a model in humans in which human superficial veins were incubated *in situ* with proinflammatory cytokines, NO generation was induced, and this was sufficient to blunt the constrictor response to norepinephrine and abolish sympathetic-induced venoconstriction (Bhagat and Vallance, 1999). In this model, however, no iNOS was detected, and the NO seemed to be derived from eNOS. The cytokines did induce expression of GTP cyclohydrolase-1, expression of which is rate limiting for the generation of tetrahydrobiopterin, and it is possible that overproduction of this cofactor for NOS is important in the genesis of the response. It has been known for many years that pterin synthesis is increased during infection or inflammatory states, and it is possible that induction of GTP cyclohydrolase-1 is an important component of the overall increase in vascular NO generation seen in a wide range of inflammatory states (Bhagat and Vallance, 1999).

Acute Local Inflammation

The role of NO in mediating the vasodilation that characterizes acute localized inflammatory responses in humans has not yet been established. However, endothelium-derived NO mediates part of the dilator response to histamine and bradykinin, two mediators that are important in the vasodilator response of acute inflammation.

Therapeutic Implications

Strategies for boosting endogenous NO may be useful for preventing vascular complications of hypertension, diabetes, or hypercholesterolemia. On the other hand, decreasing abnormally high production of NO might have benefit for the treatment of conditions associated with excessive inflammatory vasodilation.

Boosting NO-Mediated Dilation

NO donor agents have been in clinical use for over 100 years and form the mainstay of treatment for patients with angina. Newer drugs that liberate NO differentially in different tissues may offer theoretical advantages over the older drugs, and one class of compounds, the nitrosothiols, appear to show significant antiplatelet effects in humans at doses that do not cause much vasodilation (de Belder *et al.,* 1994). This is unlike the classic NO donor agents that show prominent vasodilator actions (mainly venodilator) and little antiplatelet effect. It remains to be determined whether these or other properties of novel NO donor agents translate into clinical benefit.

Supplementation with L-arginine improves certain aspects of NO-mediated dilation in patients with hypercholesterolemia. The precise mechanism (i.e., one which explains why arginine should be rate limiting for NO generation) is unclear; however, numerous studies have now confirmed the initial observation, and clinical trials even suggest that arginine supplementation may improve hard clinical end points, such as walking distance, in patients with peripheral atherosclerosis. It will now be important to undertake properly powered clinical studies to determine the magnitude and clinical usefulness of effects of arginine supplementation in this and other conditions. If it does prove to be effective, arginine supplementation would appear to offer an inexpensive and freely available therapeutic option. Studies with tetrahydrobiopterin also seem to show some improvement of endothelium-dependent relaxation in certain patient groups, but the tetrahydrobiopterin has to be given intravenously. However, therapeutic strategies aimed at boosting levels of this cofactor might be useful.

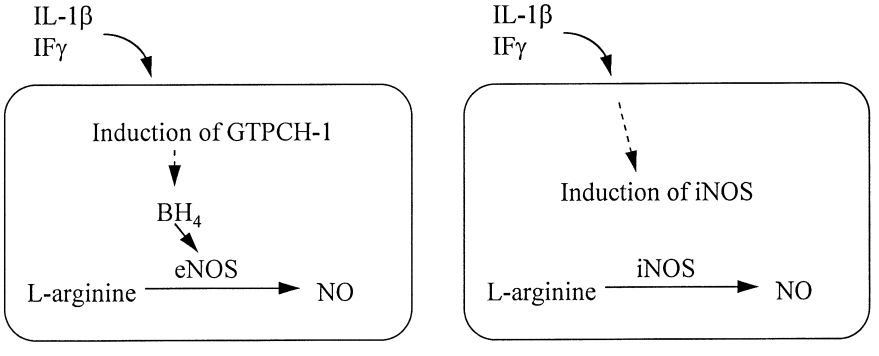


Figure 8 Certain proinflammatory cytokines such as interleukin-1β (IL-1β) and γ-interferon (IFN-γ) may increase NO generation either by inducing expression of iNOS or by inducing expression of GTP cyclohydrolase 1 (GTPCH-1). Induction of GTPCH-1 leads to generation of tetrahydrobiopterin (BH_4), an essential cofactor for all isoforms of NOS. Induction of GTPCH-1 may enhance NO output from eNOS, or from iNOS if this isoform is expressed. Studies in human endothelial cells and human veins *in vivo* suggest that induction of GTPCH-1 and subsequent activation of eNOS may be an important mechanism in human vessels.

In addition to using NO donor agents or providing excess arginine, several other therapeutic possibilities have emerged. These include gene transfer of eNOS, agents to stimulate guanylate cyclase, and agents that decrease superoxide production or increase its destruction. There are now well-established models in humans in which such therapeutic approaches could be tested.

Blocking NO-Mediated Dilation

It is still unclear whether blocking overproduction of NO will produce beneficial effects in conditions of inflammatory vasodilation. Studies in animals have been contradictory, but it does appear as though complete or near complete inhibition of NOS is likely to be harmful. Studies in animals have indicated that in some situations selective inhibition of iNOS may offer therapeutic advantages over nonselective inhibitors such as L-NMMA that inhibit all three isoforms of NOS. However, even with selective inhibitors it seems as though it will be important to aim for a modest degree of inhibition only. Ultimately, the test of inhibitors will come in clinical trials, in which hard clinical end points can be assessed.

References

Adams, M. R., McCredie, R., Jessup, W., Robinson, J., Sullivan, D., and Celermajer, D. S. (1997). Oral L-arginine improves endothelium-dependent dilation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. *Atherosclerosis* **129,** 261–269.

Baylis, C., and Vallance, P. (1998). Measurement of nitrite and nitrate levels in plasma and urine—What does this measure tell us about the activity of the endogenous nitric oxide system? *Curr. Opin. Nephrol. Hypertens.* **7,** 59–62.

Bhagat, K., and Vallance, P. (1997). Inflammatory cytokines impair endothelium-dependent dilation in human veins *in vivo*. *Circulation* **96,** 3042–3047.

Bhagat, K., and Vallance, P. (1999). Effects of cytokines on nitric oxide pathways in human vasculature. *Curr. Opin. Nephrol. Hypertens.* **8,** 89–96.

Calver, A., Collier, J., Green, D., and Vallance, P. (1992a). Effect of acute plasma volume expansion on peripheral arteriolar tone in healthy subjects. *Clin. Sci.* **83,** 541–547.

Calver, A., Collier, J., Moncada, S., and Vallance, P. (1992b). Effect of local intra-arterial K_m-monomethyl-L-arginine in patients with hypertension: The nitric oxide dilator mechanism appears abnormal. *J. Hypertens.* **10,** 1025–1031.

Calver, A., Collier, J., and Vallance, P. (1992c). Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J. Clin. Invest.* **90,** 2548–2554.

Calver, A., Collier, J., and Vallance, P. (1993). Nitric oxide and cardiovascular control. *Exp. Physiol.* **78,** 303–326.

Calver, A., Collier, J., and Vallance, P. (1994). Forearm blood flow responses to a nitric oxide synthase inhibitor in patients with treated essential hypertension. *Cardiovasc. Res.* **28,** 1720–1725.

Cleland, S. J., Petrie, J. R., Ueda, S., Elliott, H. L., and Connell, J. M. (1998). Insulin as a vascular hormone: Implications for the pathophysiology of cardiovascular disease. *Clin. Exp. Pharmacol. Physiol.* **25,** 175–184.

Cockcroft, J. R., Chowienczyk, P. J., Benjamin, N., and Ritter, J. M. (1994). Preserved endothelium-dependent vasodilation in patients with essential hypertension. *N. Engl. J. Med.* **330,** 1036–1040.

de Belder, A. J., MacAllister, R., Radomski, M. W., Moncada, S., and Vallance, P. (1994). Effects of *S*-nitroso-glutathione in the human forearm circulation: Evidence for selective inhibition of platelet activation. *Cardiovasc. Res.* **28,** 691–694.

Dietz, N. M., Rivera, J. M., Eggener, S. E., Fix, R. T., Warner, D. O., and Joyner, M. J. (1994). Nitric oxide contributes to the rise in forearm blood flow during mental stress in humans. *J. Physiol.* **480,** 361–368.

Dietz, N. M., Engelke, K. A., Samuel, T. T., Fix, R. T, and Joyner, M. J. (1997). Evidence for nitric oxide-mediated sympathetic forearm vasodilation in humans. *J. Physiol.* **498,** 531–540.

Dunnill, M. G., Mallett, R. B., Hawkins, P. N., Vallance, P., and Marsden, R. A. (1993). Severe dominant dystrophic epidermolysis bullosa complicated by systemic amyloidosis. *Br. J. Dermatol.* **128,** 708–709.

Dyke, C. K., Proctor, D. N., Dietz, N. M., and Joyner, M. J. (1995). Role of nitric oxide in exercise hyperemia during prolonged rhythmic handgripping in humans. *J. Physiol.* **488,** 259–265.

Elliott, T. G., Cockcroft, J. R., Groop, P. H., Viberti, G. C., and Ritter, J. M. (1993). Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients: Blunted vasoconstriction in patients with microalbuminuria. *Clin. Sci.* **85,** 687–693.

Evans, T., Carpenter, A., Kinderman, H., and Cohen, J. (1993). Evidence of increased nitric oxide production in patients with the sepsis syndrome. *Circ. Shock* **41,** 77–81.

Forte, P., Copeland, M., Smith, L. M., Milne, E., Sutherland, J., and Banjamin, N. (1997). Basal nitric oxide synthesis in essential hypertension. *Lancet* **349,** 837–842.

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392,** 821–824.

Haynes, W. G., Noon, J. P., Walker, B. R., and Webb, D. J. (1993). Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J. Hypertens.* **11,** 1375–1380.

Hibbs, J. B., Jr., Westenfelder, C., Taintor, R., Vavrin, Z., Kablitz, C., Baronowski, R. L., Ward, J. H., Menlove, R. L., McMurray, M. P., and Kishner, J. P. (1992). Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. *J. Clin. Invest.* **89,** 867–877.

Joannides, R., Haefeli, W. E., Linder, L., Richard, V., Bakkali, E. H., Thuillez, C., and Luscher, T. F. (1995). Nitric oxide is responsible for flow-dependent dilation of human peripheral conduit arteries *in vivo*. *Circulation* **91,** 1314–1319.

Kauser, K., and Rubanyi, G. M. (1997). Potential cellular signaling mechanisms mediating upregulation of endothelial nitric oxide production by estrogen. *J. Vasc. Res.* **34,** 229–236.

Kingswell, B. A., Sherrard, B., Jennings, G. L., and Dart, A. M. (1997). Four weeks of cycle training increases basal production of nitric oxide from the forearm. *Am. J. Physiol.* **272,** H1070–H1077.

Lefroy, D. C., Crake, T., Uren, N. G., Davies, G. J., and Maseri, A. (1993). Effect of inhibition of nitric oxide synthesis on epicardial coronary artery caliber and coronary blood flow in humans. *Circulation* **88,** 43–54.

Leiper, J., and Vallance, P. (1999). Biological significance of endogenous methylarginines that inhibit nitric oxide syntheses. *Cardiovasc. Res.* **43,** 542–548.

Linder, L., Kiowski, W., Buhler, F. R., and Luscher, T. F. (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation *in vivo*. Blunted response in essential hypertension. *Circulation* **81,** 1762–1767.

Lyons, D., Webster, J., and Benjamin, N. (1994). The effect of antihypertensive therapy on responsiveness to local intra-arterial K_m-monomethyl-L-arginine in patients with essential hypertension. *J. Hypertens.* **12,** 1047–1052.

MacAllister, R. J., and Vallance, P. (1996). Systemic vascular adaptation to increases in blood volume: The role of the blood-vessel wall. *Nephrol. Dial. Transpl.* **11,** 231–234.

Maxwell, A. J., Tsao, P. S., and Cooke, J. P. (1998). Modulation of the nitric oxide synthase pathway in atherosclerosis. *Exp. Physiol.* **83,** 573–584.

Panza, J. A., Quyyumi, A. A., Brush, J. E., Jr., and Epstein, S. E. (1990).

Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N. Engl. J. Med.* **323,** 22–27.

Petros, A., Bennett, D., and Vallance, P. (1991). Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* **338,** 1557–1558.

Petros, A., Lamb, G., Leone, A., Moncada, S., Bennett, D., and Vallance, P. (1994). Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovasc. Res.* **28,** 34–39.

Riezebos, J., Watts, I. S., and Vallance, P. (1994). Endothelin receptors mediating functional responses in human small arteries and veins. *Br. J. Pharmacol.* **111,** 609–615.

Simon, D. I., Stamler, J. S., Loh, E., Loscalzo, J., Francis, S. A., and Creager, M. A. (1995). Effect of nitric oxide synthase inhibition on bleeding time in humans. *J. Cardiovasc. Pharmacol.* **26,** 339–342.

Stamler, J. S., Loh, E., Roddy, M. A., Currie, K. E., and Creager, M. A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. *Circulation* **89,** 2035–2040.

Steinberg, H. O., Brechtel, G., Johnson, A., Fineberg, N., and Baron, A. (1994). Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Invest.* **94,** 1172–1179.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Sudano, I., and Salvetti, A. (1996). Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. *Circulation* **94,** 1298–1303.

Vallance, P., and Charles, I. (1998). Nitric oxide in sepsis: Of mice and men. *Sepsis* **1,** 93–100.

Vallance, P., Collier, J., and Moncada, S. (1989a). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* **2,** 997–1000.

Vallance, P., Collier, J., and Moncada, S. (1989b). Nitric oxide synthesized from L-arginine mediates endothelium dependent dilation in human veins *in vivo*. *Cardiovasc. Res.* **23,** 1053–1057.

Vallance, P., Benjamin, N., and Collier, J. (1992a). The effect of endothelium-derived nitric oxide on *ex vivo* whole blood platelet aggregation in man. *Euro. J. Clin. Pharmacol.* **42,** 37–41.

Vallance, P., Leone, A., Calver, A., Collier, J., and Moncada, S. (1992b). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* **339,** 572–575.

Vallance, P., Patton, S., Bhagat, K., MacAllister, R., Radomski, M., Moncada, S., and Malinski, T. (1995). Direct measurement of nitric oxide in human beings. *Lancet* **346,** 153–154.

White, R. P., Deane, C., Vallance, P., and Markus, H. S. (1998). Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia. *Stroke* **29,** 467–472.

Williams, D. J., Moosavi, A. H., and Imms, F. J. (1995). Nitric oxide contributes to local heat induced vasodilation in man. *J. Physiol.* **483,** 126–127.

Williams, D. J., Vallance, P., Neild, G. H., Spencer, J. A., and Imms, F. J. (1997). Nitric oxide-mediated vasodilation in human pregnancy. *Am. J. Physiol.* **272,** H748–H752.

CHAPTER 56

Clinical Therapy with Inhaled Nitric Oxide in Respiratory Diseases

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MANY INSIGHTS INTO THE MECHANISMS OF ACTION OF NITRIC OXIDE (NO) HAVE BEEN RAPIDLY APPLIED TO TREAT PATIENTS. SINCE THE REPORTED APPLICATIONS OF INHALED NO IN THE LABORATORY (FROSTELL *et al.,* 1991) AND IN ADULT PATIENTS WITH PRIMARY PULMONARY HYPERTENSION (PEPKE-ZABA *et al.,* 1991), HUNDREDS OF STUDIES HAVE BEEN CONDUCTED TO DETERMINE THE CLINICAL APPLICABILITY OF INHALED NO. IN SELECTED GROUPS OF SEVERELY ILL AND HYPOXIC CHILDREN AND ADULTS, INHALED NO IMPROVES ARTERIAL OXYGENATION AND SELECTIVELY REDUCES PULMONARY ARTERIAL HYPERTENSION (PAH). NO INHALATION THERAPY, IN COMBINATION WITH CONVENTIONAL (NEONATAL INHALED NITRIC OXIDE STUDY GROUP, 1997A; ROBERTS *et al.,* 1997) OR HIGH-FREQUENCY OSCILLATORY VENTILATION (KINSELLA *et al.,* 1997), CAN SIGNIFICANTLY IMPROVE ARTERIAL OXYGENATION AND REDUCE THE NEED FOR EXTRACORPOREAL MEMBRANE OXYGENATION (ECMO), AN EXPENSIVE AND INVASIVE SUPPORT PROCEDURE IN NEWBORN PATIENTS WITH HYPOXIC RESPIRATORY FAILURE. HOWEVER, IT REMAINS UNCERTAIN WHETHER NO INHALATION IMPROVES SURVIVAL RATES IN ADULTS WITH SEVERE ACUTE LUNG INJURY.

NEW APPLICATIONS FOR NO INHALATION HAVE BEEN DISCOVERED. STUDIES INDICATE THAT INHALED NO MAY DECREASE ISCHEMIA–REPERFUSION INJURY (BACHA *et al.,* 1996) AND MAY BE USEFUL TO TREAT THROMBOTIC DISORDERS (ADRIE *et al.,* 1996; NONG *et al.,* 1997). BY INCREASING THE O_2 AFFINITY OF SICKLE CELL HEMOGLOBIN, INHALED NO MAY PREVENT OR TREAT SICKLE CELL CRISIS. THIS CHAPTER WILL REVIEW THESE DIVERSE CLINICAL APPLICATIONS FOR INHALED NO THERAPY.

Background

Nitric oxide (NO) is a small, easily diffusible molecule that can be administered simply by inhalation. Because avid binding of NO to hemoglobin limits the action of NO in the systemic circulation, inhaled NO produces selective pulmonary vasodilation, a long-sought action that should be useful in the treatment of many lung diseases. Many studies have concluded convincingly that inhaled NO is a selective pulmonary vasodilator, in a variety of both animal models and clinical conditions.

The administration of inhaled NO often improves systemic oxygenation during acute lung injury. Commonly used intravenously administered vasodilators diffusely release hypoxic pulmonary vasoconstriction within the lungs and can worsen oxygenation. Inhaled nitric oxide, by being delivered to areas of the lungs that are best ventilated and then by being rapidly bound to hemoglobin and inactivated in

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the circulation, can selectively vasodilate ventilated lung regions. Regions of the lungs that are not ventilated are not exposed to inhaled NO. Oxygenation improves via a reduction in relative blood flow to nonventilated regions.

Borland and co-workers administered inhaled NO to patients and volunteers to determine the diffusing capacity of the lung (Borland and Higenbottam, 1989). They found that a single breath of nitric oxide could be administered safely. Because NO is also an atmospheric pollutant (Alberts, 1994), human toxicity studies (von Nieding *et al.,* 1975) and exposure recommendations (Centers for Disease Control, 1988) had been previously reported and provided the foundation for initial clinical studies. Today, substantiated indications for inhaled nitric oxide (Table I) include hypoxic respiratory failure of the newborn (Clark, 1999; Hoffman *et al.,* 1997; Kinsella *et al.,* 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997a; Roberts *et al.,* 1997) and the assessment of pulmonary vascular reactivity in patients with pulmonary hypertension (Fishman *et al.,* 1998). Inhaled NO has also been used in the treatment of acute respiratory distress syndrome (ARDS), lung and cardiac transplants, congenital and acquired heart disease, and chronic pulmonary hypertension, and it has been used to produce desirable direct effects on blood elements, specifically for the treatment of acute chest syndrome in sickle cell disease.

Neonatal Respiratory Failure

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome characterized by sustained pulmonary hypertension and severe hypoxemia, resulting in cyanosis that is unresponsive to oxygen therapy. Persistent pulmonary hypertension of the newborn may be due to a variety of etiologies (Roberts, 1993). Diagnostic confirmation of PPHN includes echocardiographic observation of a right-to-left shunt through the ductus arteriosus or foramen ovale, due to increased pulmonary vascular resistance (PVR), in the absence of congenital heart disease. Conventional treatment strategies include breathing high inspired O_2 concentrations (FiO_2), mechanical ventilation with high-frequency oscillatory ventilation, hyperventilation and infusion of bicarbonate to produce alkalosis, inhalation treatments with bovine surfactant, and intravenous vasodilator therapy. Where available, extracorporeal membrane oxygenation is used to treat severe hypoxemia. The anticoagulation and cannulation of large vessels required for ECMO, however, is associated with hemorrhagic complications. In adults receiving ECMO, bleeding complications have been reported to occur in 88% of patients (Anderson *et al.,* 1993). Bleeding at a rate of over 1.3 liters per day is common (Pesenti *et al.,* 1988). In neonates, intracranial hemorrhage occurs at a rate of approximately 15% of patients receiving ECMO [ECMO registry of the Extracorporeal Life Support Organization (ELSO), 1996].

Table I Clinical Indications for Inhaled Nitric Oxide

Substantiated
Hypoxic respiratory failure and persistent pulmonary hypertension of the newborn (PPHN)
Assessment of pulmonary vascular reactivity in patients with pulmonary hypertension
Investigational
Acute respiratory distress syndrome (ARDS)
Lung and cardiac transplantation
Congenital and acquired heart disease
Chronic pulmonary hypertension
Ischemia–reperfusion injury
Antiplatelet effects
Acute chest syndrome in sickle cell disease
Bronchodilation

In 1992, Roberts *et al.* and Kinsella *et al.* reported that 80 parts per million (ppm) (Roberts *et al.,* 1992) or 6–20 ppm (Kinsella *et al.,* 1992) inhaled NO improved oxygenation in newborns with hypoxic respiratory failure and PPHN. Several controlled, randomized multicenter trials of the effects of inhaled NO in near-term and term hypoxic newborn patients were reported in 1997 (Fig. 1) (Hoffman *et al.,* 1997; Kinsella *et al.,* 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997a,b; Roberts *et al.,* 1997). In the majority of patients with PPHN and hypoxic respiratory failure, NO improved oxygenation and decreased the requirement for ECMO. The decisions to initiate ECMO were made by the clinical team on the basis of center-specific ECMO entry criteria and without knowledge of assignment of the patient to the treatment group or placebo.

It is worthwhile to review several important studies. The Neonatal Inhaled Nitric Oxide Study (NINOS) group of investigators studied 230 infants of at least 34 weeks gestational age with hypoxemic respiratory failure of various etiologies (Neonatal Inhaled Nitric Oxide Study Group, 1997a). They were randomized to receive 100% oxygen or oxygen plus inhaled NO. The use of ECMO was reduced significantly from 54% in the control group to 39% in the NO group. The study did not find a change in mortality that was statistically significant. Roberts and co-workers (1997) randomized 58 infants with severe hypoxemia and PPHN to receive nitrogen or 80 ppm NO.

Oxygenation doubled in 53% of the children receiving inhaled NO versus 7% of controls. This initial effect was sustained in 75% of infants with initial improvement and resulted in a significant reduction in ECMO use from 71% in control patients to 40% in newborns receiving inhaled NO. Kinsella *et al.* (1997) reported that NO inhalation and high-frequency oscillatory ventilation were an effective combination that may increase the rate of responsiveness to inhaled NO. As opposed to studies in adults, in which the ventilatory strategies called for a reduction in lung recruitment (see later), the use of high frequency oscillation in combination with inhaled NO is important for maintaining alveoli open and possibly reducing barotrauma. In follow-up studies of children who received inhaled NO treatment for PPHN, neurodevelopmental scores and growth rates, as well

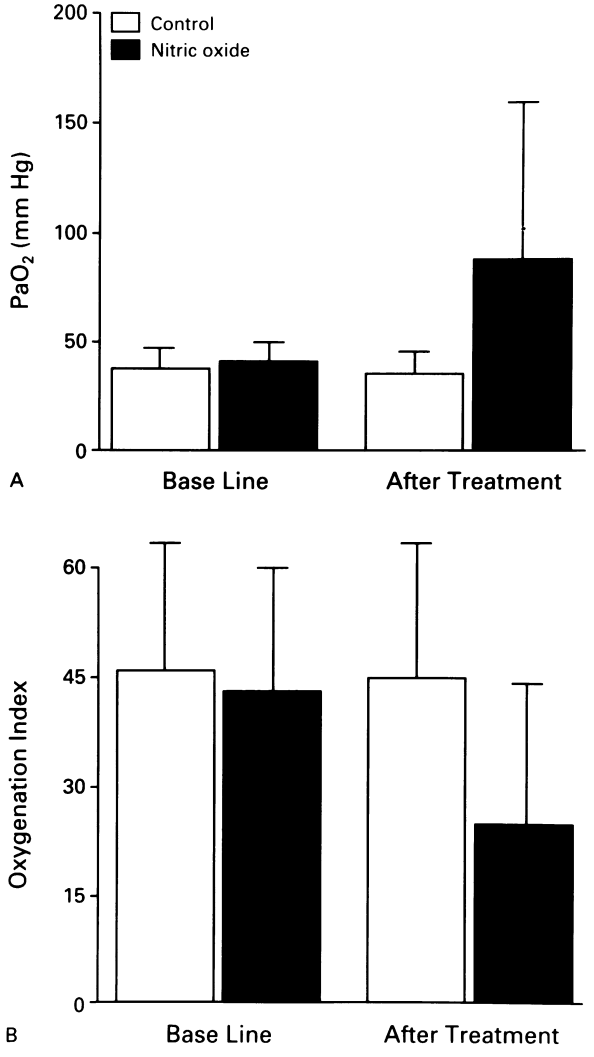


Figure 1 Short-term effects of inhaled NO on systemic oxygenation in infants with severe hypoxemia and persistent pulmonary hypertension of the newborn. Treatment with nitric oxide inhalation (80 ppm at FiO_2 0.9 for 20 min, $n = 30$), but not with placebo inhalation (control, nitrogen at FiO_2 0.9, $n = 28$), after randomized assignment significantly ($p < 0.001$) improved postductal PaO_2 (A) and oxygenation index (B) compared with baseline. Values are means ± SD. Reprinted with permission from Roberts *et al.* (1997).

as the frequency of airway disease and need for supplemental O_2, were comparable to conventionally ventilated or ECMO-treated patients (Rosenberg *et al.,* 1997). In summary, inhaled NO improves oxygenation in many newborns and significantly reduces the need for ECMO (Neonatal Inhaled Nitric Oxide Study Group, 1997a; Roberts *et al.,* 1997). These findings have more recently been confirmed in a third multicenter randomized trial (Clark *et al.,* 2000).

The use of inhaled NO to treat PPHN deserves special emphasis. Persistent pulmonary hypertension of the newborn is a relatively uniform disease characterized by severe pulmonary vasoconstriction. By relieving pulmonary vasoconstriction, inhaled NO directly treats one of the major pathological derangements of the disease. Consequently, the efficacy of NO in PPHN has been substantiated in multicenter, randomized, placebo-controlled trials.

Inhaled NO reduces the necessity for ECMO in newborns with PPHN and infants with hypoxic respiratory failure of various etiologies. This improvement in management will justify its continued use, because of the increased expense of ECMO and its morbidity secondary to its invasive nature and the necessity for systemic anticoagulation. Nevertheless, ECMO remains a necessary lifesaving therapy because inhaled NO is not effective in all patients. Such patients should be treated in a facility providing the possibility of ECMO therapy.

Respiratory distress syndrome (RDS), or hyaline membrane disease of the premature newborn, is characterized by a deficiency or dysfunction of surfactant and is often associated with acute PAH (Kinsella *et al.,* 1994). After promising preliminary studies of inhaled NO in the premature newborn with RDS (Abman *et al.,* 1993; Peliowski *et al.,* 1995), Skimming and associates (1997) studied the effect of inhaled NO at 5 and 20 ppm in preterm neonates (without systemic hypotension or congenital malformations and mechanically ventilated at $FiO_2 > 0.5$). They demonstrated that arterial oxygenation improved and that systemic arterial blood pressure was unaffected during a 15-min NO inhalation trial. The conclusions of this study were extremely limited, however, since pulmonary artery pressure was not measured, and only 7% of the initially evaluated premature infants were included in the study. There is concern that an inhibitory effect of NO on platelet aggregation might contribute to the occurrence of intracranial hemorrhages in premature infants (Meurs *et al.,* 1997). An increased rate of bleeding complications has been noted in term infants (Neonatal Inhaled Nitric Oxide Study Group, 1997b), but insufficient data have been published to confirm the safety of inhaled NO therapy in premature newborns. In neonatal lung diseases characterized by abnormal lung development, such as congenital diaphragmatic hernia (Neonatal Inhaled Nitric Oxide Study Group, 1997b), inhaled NO appears to be less effective or without benefit.

Acute Respiratory Distress Syndrome

Pulmonary Vascular Resistance

Rossaint *et al.* (1993) demonstrated that inhaled NO produced selective pulmonary vasodilation in patients with severe ARDS (Fig. 2). This was later confirmed by larger studies (Dellinger *et al.,* 1998; Manktelow *et al.,* 1997). In some patients, NO-induced pulmonary vasodilation was sufficient to improve right ventricular performance (Rossaint *et al.,* 1995a). In children with ARDS, inhaled NO (20 ppm) decreased mean pulmonary artery pressure (MPAP) by 25% and increased cardiac index by 14% (Abman *et al.,* 1994). Inhaled NO also effectively relieved the pulmonary hypertension associated with the use of permissive hypercapnia in patients with ARDS (Puybasset *et al.,* 1994).

Inhaled NO (40 ppm) decreased pulmonary capillary pressure (Benzing and Geiger, 1994) and pulmonary trans-

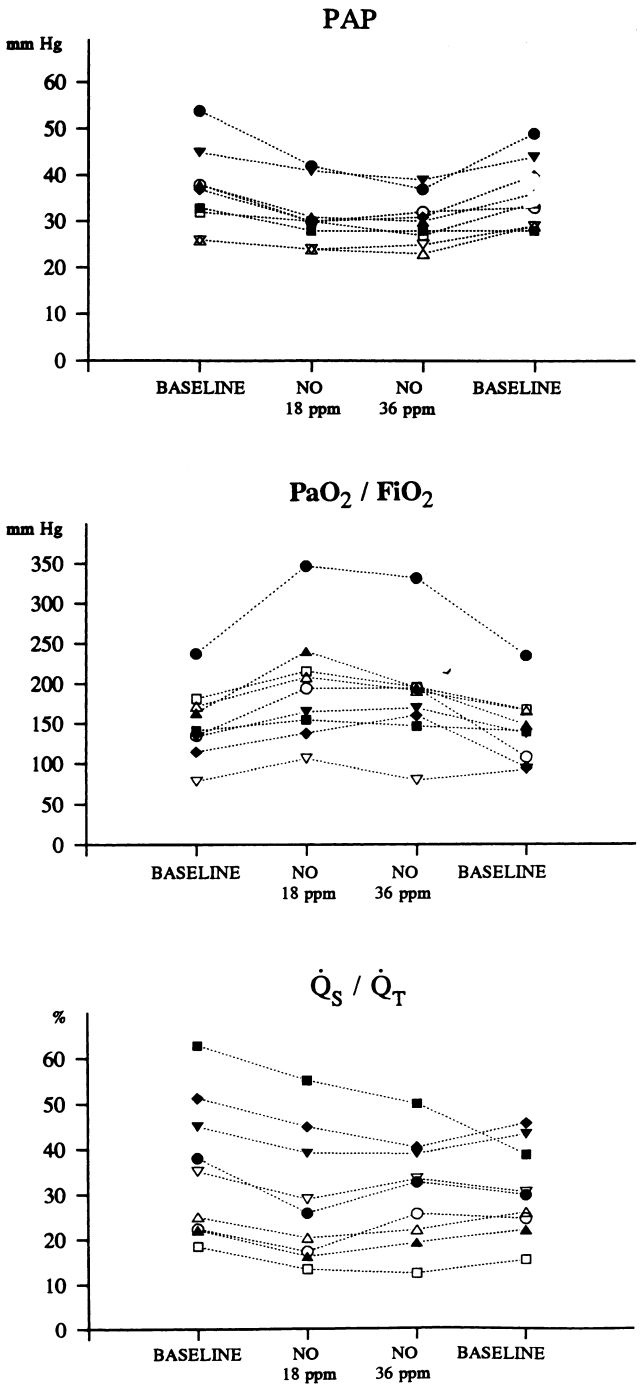


Figure 2 Mean pulmonary artery pressure (PAP), arterial oxygenation efficiency (PaO_2/FiO_2), and venous admixture ($\dot{Q}_S/\dot{Q}_T$) during inhalation of 18 and 36 ppm nitric oxide in nine patients with adult respiratory distress syndrome. Solid symbols represent patients treated with extracorporeal membrane oxygenation. Reproduced with permission from Rossaint *et al.* (1993).

vascular albumin flux (Benzing *et al.,* 1995) in patients with acute lung injury, partly due to reducing pulmonary venous resistance (Benzing and Geiger, 1994). Reductions of pulmonary venous and pulmonary capillary pressure should promote the resolution of pulmonary edema.

Arterial Oxygenation

Severe hypoxemia is characteristic of ARDS. Common current strategies of management include lung recruitment by high levels of positive end-expiratory pressure, prone positioning, and ventilation with a high FiO_2. Therapies that permit lower airway pressures and FiO_2 might reduce the risk of barotrauma and oxidant injury to the lung. In initial clinical studies, inhaling 18 ppm NO for 40 min reduced the shunt fraction by 5% and increased the PaO_2/FiO_2 ratio by 30% in patients with ARDS (Rossaint *et al.,* 1993). In a dose-ranging study of ARDS patients breathing NO, the ED_{50} (the dose producing 50% of maximal effect) for increasing the partial pressure of oxygen in arterial blood (PaO_2) was markedly less [10 to 100 parts per billion (ppb)] than the ED_{50} producing pulmonary vasodilation (1–10 ppm, see Fig. 3) (Gerlach *et al.,* 1993).

In a Phase II multicenter trial, the effects of NO inhalation were studied in 177 patients with ARDS (PaO_2/FiO_2 <200 mmHg within the last 72 hours, bilateral chest infiltrates, pulmonary capillary wedge pressure < 18 mmHg, requirement for positive end-expiratory pressure (PEEP) > 8 cm H_2O, and FiO_2 requirement > 0.5) (Dellinger *et al.,* 1998). Sixty-five percent of the patients who received inhaled NO (pooled results of patients receiving either 1.25, 5, 20, 40, 40, or 80 ppm NO) had a significant improvement in PaO_2 (defined as a 20% increase of PaO_2 after 4 hours of therapy). Only 24% of the patients receiving placebo (nitrogen) responded similarly (see Fig. 4). The improved oxygenation induced by NO allowed physicians to decrease FiO_2 and PEEP and thereby decreased the oxygenation index [(FiO_2 × mean airway pressure × 100)/PaO_2] for the first 4 days of therapy. The MPAP remained modestly lower in the inhaled NO group, compared with placebo, for 2 days. Similar transient improvements of PaO_2/FiO_2 during inhaled NO therapy in patients with ARDS have been demonstrated in prospective studies conducted by Michael *et al.* (1998) and Troncy *et al.* (1998).

Some patients do not respond to NO inhalation with pulmonary vasodilation and improved oxygenation. In studies of critically ill patients with ARDS who were ventilated with low concentrations of NO gas (<40 ppm), approximately 35% of patients have minimal or no response to inhaled NO. Varying responses to NO inhalation are also observed among different patients and during the course of acute lung injury in individual patients (Bigatello *et al.,* 1994; Lundin *et al.,* 1996, 1997; Mira *et al.,* 1994; Rossaint *et al.,* 1995b; Wysocki *et al.,* 1994). The precise mechanism for the variable responsiveness to NO is unknown. Clinicians are thus unable to accurately predict the responsiveness of individual patients to inhaled NO. In general, the baseline level of PVR appears to predict the degree of pulmonary vasoconstriction reversible by NO inhalation. Those with the greatest degree of pulmonary hypertension respond maximally to NO inhalation (Bigatello *et al.,* 1994; Puybasset *et al.,* 1995; Young *et al.,* 1994). A favorable response to inhaled NO may be related to the degree of alveolar recruitment (Putensen *et al.,*

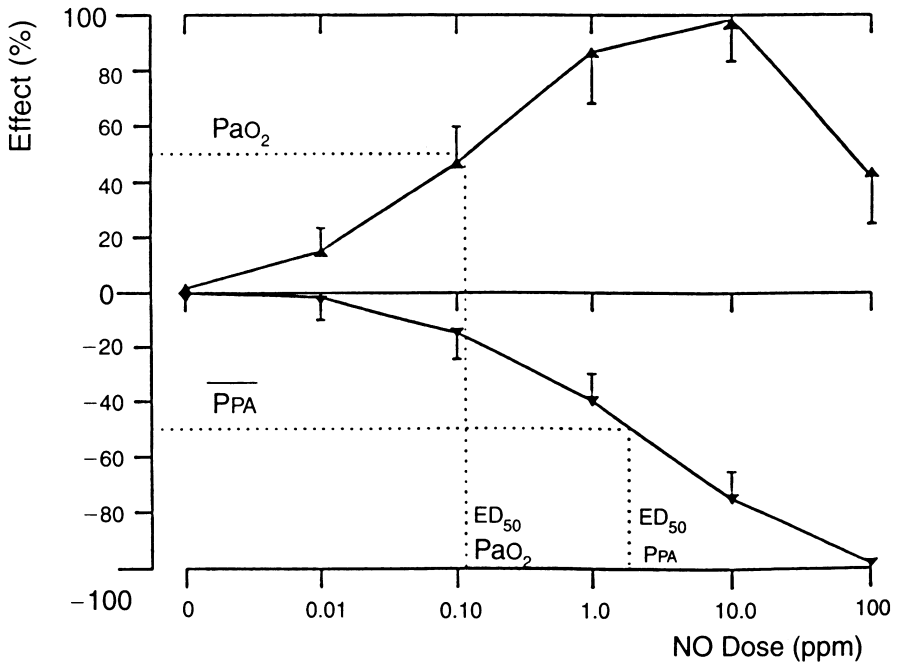


Figure 3 Dose–response to inhaled NO for PaO_2 (top) and mean pulmonary arterial pressure (bottom) in 12 patients with ARDS. The estimated ED_{50} (the dose producing 50% of maximal effect) for PaO_2 increase was 0.11 ppm and the estimated ED_{50} for mean pulmonary artery pressure (PPA) decrease was 1.2 ppm. Values are means ± SD. Reprinted with permission from Gerlach *et al.* (1993).

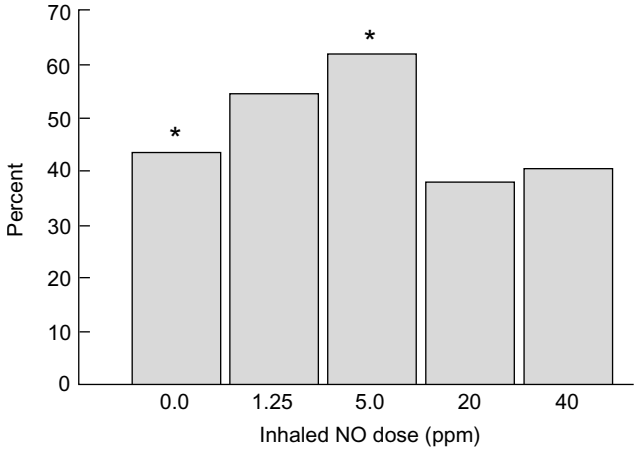


Figure 4 Percentage of ARDS patients who respond with a PaO_2 increase of 20% or more while receiving 0–40 ppm NO during a 4-hour inhalation period. Reprinted with permission from Dellinger *et al.* (1998).

1994; Puybasset *et al.,* 1995). Ventilatory techniques that increase alveolar recruitment, such as the use of high-frequency oscillation in neonates (Kinsella *et al.,* 1997), or prone positioning of ARDS patients (Papazian *et al.,* 1998a), may improve the response to inhaled NO. Enhancing pulmonary vasoconstriction while administering inhaled NO may accentuate the improvement in PaO_2 observed during inhaled NO therapy, presumably by improving $\dot{V}/\dot{Q}$ matching. The concomitant intravenous infusion of a vasoconstricting drug, almitrine, which increases the degree of hypoxic vasoconstriction in the lung, has been reported to enhance the beneficial effect of inhaled NO on PaO_2 (Payen *et al.,* 1993; Wysocki *et al.,* 1994). The concomitant infusion of norepinephrine during inhaled NO therapy appears to have a similar beneficial effect in septic patients with ARDS (Papazian *et al.,* 1998b).

Outcome

Early placebo-controlled trials of inhaled NO to treat ARDS have been disappointing, because they demonstrated only transient improvements in oxygenation (several days) and have failed to demonstrate an improved outcome. The Phase II U.S. multicenter study of the effects of inhaled NO on ARDS patients, discussed earlier, reported a mortality rate of 30% in both NO (all doses pooled) and placebo treated patients (Dellinger *et al.,* 1998). The patients were randomly assigned to receive 1.25, 5, 20, 40, or 80 ppm NO or nitrogen (N_2) placebo. Subgroups receiving the same dose of NO for the treatment period consisted of 8–34 patients. This severely limited the power of the study. The mortality rates in the subgroups were not statistically different: 32% (7/22 patients) in the 1.25 ppm group, 24% (8/24 patients) in the 5 ppm group, 31% (9/29) in the 20 ppm group, 30% (8/27 patients) in the 40 ppm group, and 38% in the 80 ppm group. A prospective randomized study reported by Troncy and associates (1998) similarly found no significant difference between ARDS patients receiving inhaled NO and controls with regard to 30-day mortality or days of mechanical

ventilation, but the small sample size (15 patients in each group) severely limited the power of the study to detect significant differences.

Are the beneficial effects of inhaled NO insufficient to alter outcome? Decreased pulmonary capillary pressure should decrease the extent of pulmonary edema, should improve lung compliance, and might improve resolution of lung injury. Improved oxygenation should permit a reduction of FiO_2 and airway pressure. If high airway pressures were being used, lower airway pressures could reduce barotrauma.

Although attractive, proving such hypotheses is difficult. The pulmonary artery pressure is usually only modestly elevated in ARDS. Even in the severe ARDS, the mean pulmonary artery pressure is usually about 30 mmHg (Zapol and Snider, 1977). This degree of pulmonary hypertension is usually well tolerated, and few patients with ARDS die of acute pulmonary hypertension.

Importantly, with the use of ventilatory strategies that are currently popular, in which airway pressures are limited to avoid pulmonary overdistention, slightly reducing airway pressure in patients with a moderate degree of lung injury may not improve outcome (Stewart *et al.,* 1998). Indeed, a worsened outcome might occur if NO-induced improvements in oxygenation are offset by prematurely reducing the level of positive end-expiratory pressure. Such reductions in PEEP can result in alveolar derecruitment and perhaps increased shear stress and barotrauma during mechanical ventilation (Amato *et al.,* 1998; Lachmann, 1992). Such premature derecruitment could explain the transitory nature of the improvement in PaO_2/FiO_2 noted in several prospective studies (Dellinger *et al.,* 1998; Michael *et al.,* 1998; Troncy *et al.,* 1998).

Improvements in oxygenation alone may not be sufficient to alter the clinical outcome of a population of ARDS patients. The survival rate of patients with ARDS appears to depend more on the occurrence of sepsis and multiple organ failure than on gas exchange. The majority of ARDS patients die from severe sepsis or multiple organ failure rather than refractory hypoxemia (Montgomery *et al.,* 1985; Suchyta *et al.,* 1992). It is unknown if patients with severe life-threatening hypoxemia would benefit from inhaled NO because such patients have been excluded from prospective studies.

The effect of inhaled NO varies among patients. Approximately one-third of patients fail to demonstrate a significant improvement in oxygenation or reductions of pulmonary artery pressure (Dellinger *et al.,* 1998; Manktelow *et al.,* 1997). Nevertheless, such patients must be included in studies that place subjects in the experimental or placebo group on an ''intention to treat'' basis. In addition, protocols may select a single fixed dose to administer. Such designs assume that the dose–response relationship of inhaled NO is similar among ARDS patients and over time. This may not be true (Gerlach *et al.,* 1999).

The beneficial effects of NO inhalation may be offset by its toxic effects. Doses as high as 80 ppm have been evaluated in both pediatric and adult studies. In experimental studies of ischemia–reperfusion injury during lung transplantation, low doses of inhaled NO were protective, but high doses were not (Murakami *et al.,* 1996). Before such studies, it was not well appreciated that the beneficial effects of inhaled NO could be lost at higher doses. The dose for NO inhalation may therefore have a narrow therapeutic range. Small doses, for example, 1–5 ppm, could be effective and improve survival; very low doses might be not effective, and larger doses could be toxic.

Lastly, the patients included in studies of ARDS rarely have a single disease of uniform severity. This might increase the heterogeneity of response or benefit to NO. The response to inhaled NO may be confounded by many factors including sepsis, excessive endogenous NO production, or a lack of endogenous production.

Performing such trials is difficult and expensive. The incidence of ARDS is relatively low, and the precipitating events are often multifactorial. Usually a large number of centers must participate to recruit sufficient numbers of patients. There may be significant differences in response rates and outcomes among different centers, as reported for PPHN patients by Kinsella *et al.* (1997). Different treatment strategies and the experience of individual institutions and caregivers may provide confounding variables. Study of well-defined subgroups of patients, such as those with marked pulmonary hypertension following lung injury or cardiac surgery, may be more successful.

Lung Transplantation

Inhaled NO may provide selective pulmonary vasodilation for both the native and transplanted lung and may reduce the necessity for cardiopulmonary bypass of the recipient during lung transplantation. Data from experimental animal studies suggest that reperfusion injury in lung transplantation may be reduced by the admininstration of inhaled NO (Bacha *et al.,* 1996; Murakami *et al.,* 1996). While patient studies evaluating the ability of inhaled NO to affect reperfusion injury have not been published yet, inhaled NO has been used to episodically treat the pulmonary artery hypertension that sometimes occurs immediately following lung transplantation (Adatia *et al.,* 1994). Inhaled NO also has been used to treat pulmonary dysfunction following transplantation. In a retrospective study by Date *et al.* (1996), 243 lung transplantations over 6 years were analyzed. Thirty-two patients had immediate severe graft dysfunction, as indicated by a PaO_2/FiO_2 ratio < 150 mmHg. Comparing patients in whom NO treatment was not available with later patients in whom NO treatment was begun after graft dysfunction was diagnosed, inhaled NO reduced MPAP and increased the PaO_2/FiO_2 ratio within the first hour of treatment. The requirement of ECMO was similar in both patient groups. Inhaled NO therapy appeared to reduce the rate of airway complications and hospital mortality (7% in NO group versus 24% in control group) in this patient pop-

ulation. Although this study suggests that inhaled NO may decrease pulmonary dysfunction following lung transplantation, as in all retrospective studies using historical controls, changes of treatment strategies and increased clinical experience over time must be considered.

Cardiac Surgery

Inhaled NO is widely used as a selective pulmonary vasodilator for cardiac surgical patients. Inhaled NO has been used to determine pulmonary vascular reactivity and the necessity for heart–lung versus lung transplantation alone (Adatia *et al.,* 1995). The successful intraoperative use of inhaled NO has been described in small case series (Fullerton *et al.,* 1996; Rich *et al.,* 1993). Postoperatively, inhaled NO has been used to treat RV dysfunction associated with pulmonary hypertension (Argenziano *et al.,* 1998; Wagner *et al.,* 1997).

Why is inhaled nitric oxide especially attractive for use in cardiac disease? Unlike intravenous pulmonary vasodilators, inhaled NO can reduce pulmonary vascular resistance while maintaining the coronary perfusion pressure. Although right ventricular (RV) dysfunction is relatively rare and often transient, its occurrence is catastrophic. Maintenance of aortic pressure and right coronary artery perfusion pressure is extremely critical in the presence of RV dysfunction (Hurford and Zapol, 1988).

The degree and reversibility of the preexisting pulmonary hypertension determine the various treatment options and outcome in children with congenital heart disease and PAH (Haworth, 1984). Roberts *et al.* (1993) demonstrated that inhaled NO (80 ppm for 10 min) decreased MPAP without causing systemic vasodilation in children between 3 months and 7 years of age with congenital cardiac lesions (e.g., atrioseptal defect, ventricular septal defect, atrioventricular canal). The ability of inhaled NO to decrease the PVR of children with congenital cardiac defects has been confirmed by others (Allman *et al.,* 1996; Berner *et al.,* 1996). In preoperative patients with severe right-to-left shunting, inhaled NO increases pulmonary blood flow, decreases extrapulmonary shunt flow, and improves oxygenation (Roze *et al.,* 1994). Chronic administration of inhaled NO therefore might be useful in the perioperative management of these patients.

After cardiopulmonary bypass, transient PAH is common and has been related to damage to the pulmonary vascular endothelium (Wessel *et al.,* 1994). Inhaled NO has been reported to ameliorate the postoperative PAH of congenital heart disease (Curran *et al.,* 1995; Journois *et al.,* 1994; Miller *et al.,* 1994) and decrease the need for postoperative ECMO (Goldman *et al.,* 1996a). Similar findings have been reported in adults following cardiac surgery for coronary artery or valvular disease (Fullerton *et al.,* 1996; Rich *et al.,* 1993).

Inhaled NO has been used to treat cardiac failure requiring a left ventricular assist device (LVAD) (Argenziano *et al.,* 1998; Wagner *et al.,* 1997). When an LVAD is used, systemic blood flow is directly dependent on pulmonary vascular resistance; a reduction of pulmonary resistance increases blood flow delivery to the LVAD and cardiac output. A similar physiological situation exists after a Fontan procedure, after which NO has also been reported to effectively increase cardiac output (Goldman *et al.,* 1996b).

In cardiac transplantation, small case series have documented that inhaled nitric oxide can reduce pulmonary vascular resistance and that sometimes this effect can be substantial (Kieler-Jensen *et al.,* 1994). In a study of patients following heart transplantation, inhalation of 20 ppm NO caused significant pulmonary vasodilation, but also decreased systemic vascular resistance (Auler *et al.,* 1996). The decreased systemic vascular resistance was most likely secondary to an improved cardiac output, since systemic arterial pressure and pulmonary capillary wedge pressure remained unchanged.

Chronic Lung Disease

Chronic Pulmonary Hypertension

PATHOPHYSIOLOGY

The pathophysiology of chronic PAH includes a partially reversible increase of MPAP in the early stages of the disease, leading to a nonreactive and irreversibly remodeled pulmonary vasculature after long-standing PAH (Meyrick and Reid, 1983). Vascular remodeling is characterized by muscularization of previously nonmuscular small resistance arteries, medial hypertrophy of proximal pulmonary arteries, and a reduced number of arteries within the lung. Endothelial-dependent relaxation may be impaired (Cremona *et al.,* 1991; Dinh Xuan *et al.,* 1990, 1991). Diagnosis and medical treatment of chronic PAH relies on vasodilator therapy. When medical treatment is not effective, lung transplantation remains as the last option to prolong life (Rich, 1998).

EVALUATION

The determination of pulmonary vascular responsiveness is essential for prognosis and long-term treatment. Drugs commonly used to assess pulmonary vasodilatory responses include intravenous prostacyclin (PGI_2), adenosine, and calcium-channel blockers (Kneussl *et al.,* 1996). The acute reduction of pulmonary vascular resistance in response to inhaled nitric oxide in such patients corresponds to the acute response to other vasodilators such as prostacyclin (Fig. 5) (Channick, 1999; Pepke-Zaba *et al.,* 1991). The pulmonary vascular response to inhaled NO is useful in the cardiac catheterization laboratory to suggest which patients may have pulmonary vascular recruitability and may respond to long-term vasodilator therapy. Because inhaled NO does not cause systemic hypotension during such therapeutic trials, its use could be safer, compared with intravenous or oral vasodilators (Sitbon *et al.,* 1995). Despite the investigational status of the drug, its use to test pulmonary vascular responsiveness

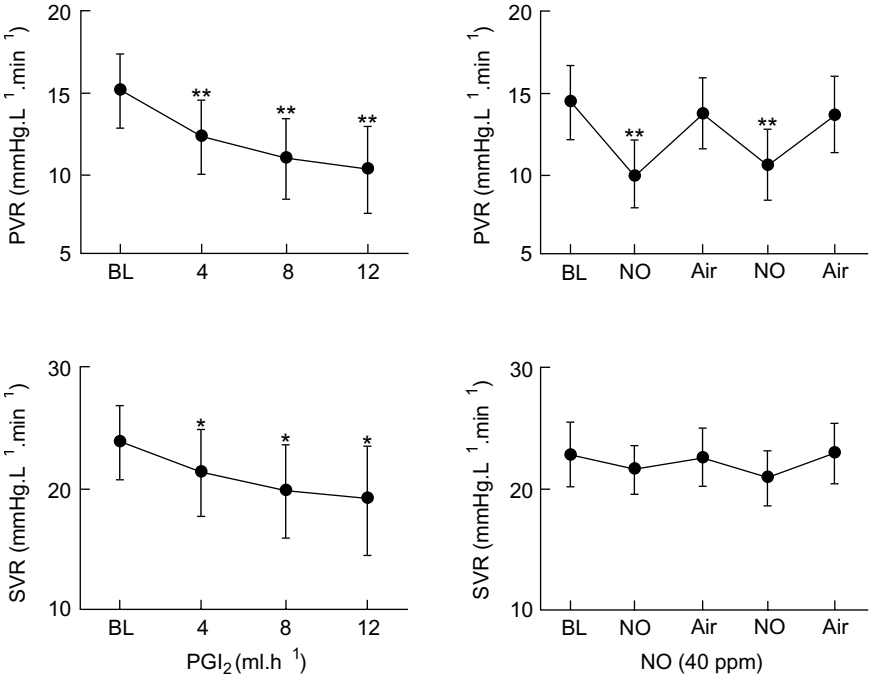


Figure 5 Pulmonary (PVR) and systemic (SVR) vascular resistance in eight patients with pulmonary hypertension receiving an intravenous infusion of prostacyclin (PGI_2) (0.5 mg in 250 ml) at rates of 4, 8, and 12 ml/hour, or inhalation of 40 ppm NO in air. Inhaled NO produced selective pulmonary vasodilation. Values are means ± SEM. * $p < 0.05$; ** $p < 0.01$. Reproduced with permission from Pepke-Zaba, J., Higenbottam, T. W., Dinh-Xuan, A. T., Stone, D., and Wallwork, J., Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension, Volume 338, pp. 1173–1174, © by The Lancet Ltd., 1991.

has been recommended (Fishman *et al.,* 1998), and a survey of long-term vasodilator treatment in patients with primary pulmonary hypertension reported that inhaled NO was used as the primary vasodilator to test pulmonary vascular responsiveness in 32% of the responding tertiary hospitals in the United States (Robbins *et al.,* 1998).

MANAGEMENT

In chronic PAH, a positive response to a short-term vasodilator trial usually results in continuing vasodilator therapy that may include a variety of systemic drugs (e.g., angiotensin converting enzyme inhibitors, calcium channel blockers, prostacyclin) and permanent anticoagulation. The efficacy of current vasodilator treatment is often limited by systemic hypotension.

On demand NO delivery devices to be used with nasal cannulas have been developed for chronic ambulatory treatment of patients intolerant to conventional vasodilators (Channick *et al.,* 1996). Channick (1999) has described chronic ambulatory administration inhaled NO for 13 weeks to 3 years in seven patients. There appears to be a durable, subjective response, pulmonary artery pressure decreases over time; and no averse effects have been observed. The mechanism of action remains uncertain. Long-term domiciliary NO inhalation as a bridge or alternative to lung-transplantation requires investigation in larger patient groups, after better delineation of the beneficial and toxic effects caused by long-term NO inhalation (Goldman *et al.,* 1995; Snell *et al.,* 1995).

Obstructive Airway Disease

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible airway obstruction, associated with irregular enlargement of alveoli and destruction of alveolar walls after chronic inflammation. Hypoxia produces pulmonary vasoconstriction, resulting in chronic PAH and right ventricular hypertrophy. Hypoxemia in COPD is primarily caused by a $\dot{V}/\dot{Q}$ mismatch, and not by intrapulmonary right-to-left shunting (as in ARDS). Hypoxic pulmonary vasoconstriction partially diverts blood flow from poorly ventilated regions and improves oxygenation. The administration of inhaled NO may oppose this effect. Inhaled nitric oxide may reach and have a prolonged residence time within partially obstructed, poorly ventilated lung regions. Release of hypoxic pulmonary vasoconstriction within these areas could worsen the matching of ventilation to perfusion (Hopkins *et al.,* 1997). Consequently, during air breathing, nitric oxide inhalation may worsen arterial oxygenation in patients with COPD (Barabrà *et al.,* 1996; Katayama *et al.,* 1997). When hypoxic pulmonary vasoconstriction is relieved by moderate concentrations of oxygen in such patients, however, the concomitant administration of inhaled NO increases PaO_2 and decreases MPAP to a greater extent than with O_2 therapy

alone (Yoshida *et al.,* 1997a). Inhaled nitric oxide has been demonstrated to prevent exercise-induced oxygen desaturation in patients with severe COPD (Roger *et al.,* 1997). This effect may be due to alterations of $\dot{V}/\dot{Q}$ distribution or the pattern of NO delivery. Patients with idiopathic pulmonary fibrosis who have both severe pulmonary artery hypertension and hypoxemia may benefit from inhaled NO, especially when it is combined with oxygen treatment (Yoshida *et al.,* 1997b).

Treatment of Bronchoconstriction

Inhaled NO has been reported to be a bronchodilator in many experimental animal models. Högman *et al.* (1993a) examined inhalation of 80 ppm NO in healthy volunteers, in patients with hyperreactive airways, bronchial asthma, and COPD. Inhaled NO caused mild bronchodilation in patients with asthma but not in COPD patients. In other studies, however, the bronchodilator action of NO has been reported to be much weaker than commonly used inhaled β_2-adrengeric agonists (Kacmarek *et al.,* 1996; Pfeffer *et al.,* 1996; Sanna *et al.,* 1994).

High-Altitude Pulmonary Edema

Severe (hypoxic) pulmonary vasoconstriction and hypertension characterize high-altitude pulmonary edema (HAPE). Scherrer *et al.* (1996) hypothesized that inhalation of NO would reduce MPAP and thus the severity of HAPE. Nitric oxide (40 ppm), inhaled at high altitude (4559 m), decreased MPAP in both subjects prone to HAPE and those with HAPE, but not in HAPE-resistant control subjects. In subjects with HAPE, perfusion scintigraphy demonstrated that inhaled NO redistributed pulmonary blood flow from edematous to nonedematous lung regions. This was associated with an improved PaO_2.

Effect on Blood Elements and Endothelial Cells

Sickle Cell Disease

Homozygous sickle cell anemia is a genetic disease characterized by severe hemolytic anemia, frequent vaso-occlusive events and a reduced life expectancy. A single amino acid substitution from valine to glutamic acid of the hemoglobin β-chain results in hemoglobin S (HbS) formation. On deoxygenation, a red blood cell containing HbS changes its shape from a biconcave disk into a crescent-shaped sickle cell due to intracellular hemoglobin polymerization. Sickle cells can occlude the microcirculation (Steinberg, 1996).

HbS has a markedly decreased O_2 affinity, reflected by an increased P_{50} (partial pressure of O_2 at half saturation of hemoglobin), compared with adult hemoglobin. In studies by Head *et al.* (1997), inhalation of 80 ppm NO for 45 min by patients with homozygous sickle cell disease shifted their blood O_2 dissociation curve 4.6 $\pm$ 2.0 mmHg to the left, significantly decreasing the P_{50} (see Fig. 6). Significant methemoglobinemia did not occur. In five of seven volunteers with sickle cell disease, the effect persisted for at least 60 min after discontinuing NO. In normal volunteers, the P_{50} was not affected by breathing NO. Precisely how inhaled NO alters sickle hemoglobin is unknown. One hypothesis is that the Cys-β93 residue of HbS is modified by NO, increasing HbS solubility and decreasing the tendency to polymerize on deoxygenation (Garel *et al.,* 1986; Schechter, 1997).

Other Effects

In experimental models, inhaled NO has been reported to display many actions with potential clinical importance, including inhibition of platelet adhesion and aggregation (Adrie *et al.,* 1996; Gries *et al.,* 1997; Högman *et al.,* 1993b, 1994; May *et al.,* 1991; Samama *et al.,* 1995), inhibition of leukocyte infiltration and inflammation (May *et al.,* 1991; Niu *et al.,* 1994; Provost *et al.,* 1994), inhibition of superoxide production and scavenging of oxygen free radicals (Clancy *et al.,* 1992; Hassoun *et al.,* 1995; Kanner *et al.,* 1991; Kavanagh *et al.,* 1994; Wink *et al.,* 1993), reduction of oxygen toxicity (McElroy *et al.,* 1997), and attenuation of vascular damage and smooth muscle proliferation (Cornwell *et al.,* 1994; Kouyoumdjian *et al.,* 1994; Roberts *et al.,* 1995). The clinical importance of these actions of NO remains to be fully explored.

Toxicity of Nitric Oxide Inhalation Therapy

Direct Toxicity

At high inspired levels, nitric oxide is directly toxic to tissues. An acute inhaled NO overdose (>500–1000 ppm) leads to rapid nitrogen dioxide (NO_2) formation, severe methemoglobinemia, pulmonary edema and alveolar hemorrhage, hypoxemia, and death within minutes to hours (Clutton-Brock, 1967; Greenbaum *et al.,* 1967). High levels of nitric oxide are also present in cigarette smoke (Norman and Keith, 1965). These levels, although much greater than the concentrations being considered for clinical use, are tolerated for short periods by millions of smokers. High levels of nitrogen dioxide production can be problematic with some delivery systems (Nishimura *et al.,* 1995), but very low inspired levels have been reported in randomized trials (Dellinger *et al.,* 1998). Commercial delivery systems and medical grade sources of NO gas have been developed, and inhaled NO doses of ≤20 ppm are commonly used (Kirmse *et al.,* 1998). Consequently, NO_2 levels have been kept at low levels.

Little is known about the long-term sequalae of NO inhalation in humans. In 12 newborns receiving NO inhalation treatment (<20 ppm) for up to 4 days, there were no signs of increased lipid peroxidation products, impaired surfactant activity, or changed cytokine profile (Hallman *et al.,* 1998).

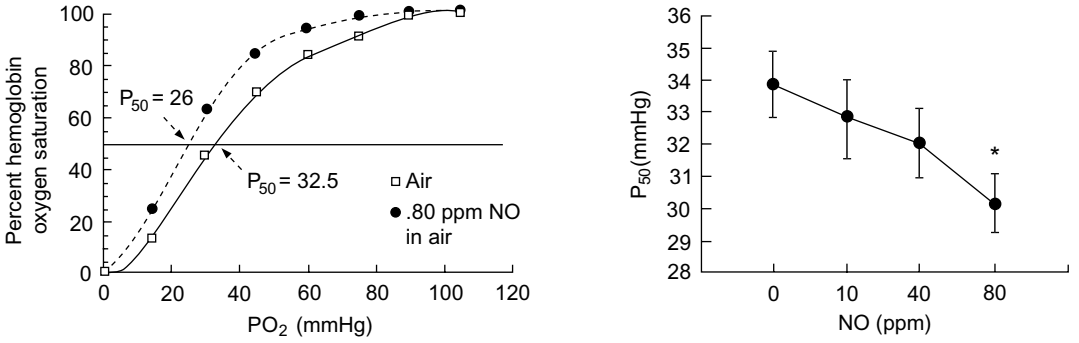


Figure 6 Effects of NO on oxygen affinity of red blood cells. Exposure to NO (80 ppm for 15 min) shifted to the left the oxygen dissociation curve of hemoglobin S erythrocytes (left). The effect of NO exposure on P50 of hemoglobin S erythrocytes was dose dependent (right). Values are means ± SE. Reprinted with permission from Head *et al.* (1997).

Nitrotyrosine residues were detected in the airway specimens of two infants requiring prolonged ventilation with NO in this study. The relative contribution of NO inhalation and endogenous NO formation to nitrotyrosine formation in the lung is unclear, however, because nitrotyrosine formation has been demonstrated in acutely injured lungs without the exogenous administration of NO (Haddad *et al.,* 1994; Kooy *et al.,* 1995). Studies of survivors of ARDS treated with inhaled NO reported no obvious differences in pulmonary function compared with ARDS patients not treated with NO (Luhr *et al.,* 1998). The doses of NO in current clinical use are less than that received with cigarette exposure and are nearly within the range encountered while breathing the air of many urban centers (Lee *et al.,* 1997).

Methemoglobinemia

Blood methemoglobin concentrations have been regularly monitored in clinical trials of inhaled NO in adults and neonates (Dellinger *et al.,* 1998; Kinsella *et al.,* 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997a,b; Roberts *et al.,* 1997). The incidence of methemoglobinemia has been low. Its occurrence is more common in neonates and with high inhaled doses, but it is usually well tolerated. There have been no reports of sequelae to methemoglobinemia in randomized studies. Methemoglobinemia is easily treated by reducing the dose of NO. Single reported cases of more severe methemoglobinemia during NO therapy have occurred in the setting of high doses (Hess *et al.,* 1997). Chemical therapies, such as methylene blue and ascorbic acid, are available, but they should not be necessary if methemoglobin levels are monitored.

Inhibition of Platelet Function

Inhaled NO inhibits platelet function. Increased bleeding times and decreased platelet aggregation have been reported in experimental animals and patients (George *et al.,* 1998; Högman *et al.,* 1993b, 1994; Samama *et al.,* 1995). In randomized studies of adults and term and nearly full-term infants, however, an increased incidence of clinical bleeding has not been substantiated (Dellinger *et al.,* 1998; Kinsella *et al.,* 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997a,b; Roberts *et al.,* 1997). Indeed, platelet inhibition could be therapeutic, rather than detrimental. Nevertheless, a cautious approach to the possibility of worsened bleeding during inhaled NO therapy remains prudent, especially in premature infants who have a high incidence of intracranial hemorrhages (Meurs *et al.,* 1997).

Adverse Hemodynamic Effects

Inhaled NO may also have adverse hemodynamic effects. Inhalation of NO may vasodilate the pulmonary circulation and increase blood flow entering the left ventricle. In patients with preexisting severe left ventricular dysfunction, an increased left ventricular end-diastolic pressure (Hayward *et al.,* 1996; Loh *et al.,* 1994; Semigran *et al.,* 1994) and pulmonary edema (Bocchi *et al.,* 1994) during NO breathing have been reported. This increase may be due to small increases in left ventricular volume associated with improved right ventricular function that, in turn, produced exaggerated increases in pulmonary capillary wedge pressure when the left ventricle is poorly compliant. Monitoring of left ventricular function may be indicated when inhaled NO is administered to patients with severe left ventricular dysfunction.

Rebound hypoxemia and pulmonary hypertension may occur after the sudden discontinuation of NO (Bigatello *et al.,* 1994; Lavoie *et al.,* 1996; Rossaint *et al.,* 1993). It has been suggested that the downregulation of endogenous NO synthesis by NO inhalation is responsible for rebound PAH (Ma *et al.,* 1996; Rengasamy and Johns, 1993). Data obtained in rats with hypoxic pulmonary hypertension, however, suggest that inhibition of endogenous NO synthesis play a minor role in rebound PAH: no changes of lung endothelial nitric oxide synthase (NOS) protein levels, NOS activity, endothelium-dependent and -independent vasodilation were reported after 3 weeks inhaling 20 ppm NO. Lung guanylate cyclase activity was transiently decreased after 1 week of NO inhalation, but guanylate cyclase activity was normal after 3 weeks of NO inhalation (Frank *et al.,* 1998).

Rebound hypoxemia and pulmonary hypertension can be anticipated, and attenuated by increasing the FiO_2, and, perhaps, through the administration of phosphodiesterase inhibitors (Hess *et al.,* 1997). The administration of a type V phosphodiesterase inhibitor, dipyridamole, has been reported to prevent rebound PAH following discontinuation of inhaled NO (al-Alaiyan *et al.,* 1996; Ivy *et al.,* 1998; Ziegler *et al.,* 1998).

Summary

Inhaled NO offers a novel therapy for the treatment of pulmonary hypertensive diseases and the symptomatic relief of hypoxemia. The use of inhaled NO reduces the necessity for ECMO in newborns and infants with acute hypoxemic respiratory failure. Proper indications, contraindications, dosing criteria, and implications of the toxic actions of NO remain to be fully delineated. Randomized clinical studies of patients with carefully defined specific acute disease states characterized by pulmonary hypertension or hypoxemia (e.g., pulmonary embolism, severe PAH, postpneumonectomy pulmonary edema, acute rejection following lung transplantation) and of premature newborns with respiratory failure remain to be completed. If such trials are carefully designed and conducted, we may define additional groups of patients that may benefit from, or may be harmed by, inhaled NO. Chronic ambulatory inhaled NO therapy may someday prove valuable for patients with pulmonary hypertension. The use of inhaled NO continues to be a unique and fascinating approach to studying and treating diseases as diverse as acute rejection of the transplanted lung and sickle cell crisis.

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References

Abman, S. H., Kinsella, J. P., Schaffer, M. S., and Wilkening, R. B. (1993). Inhaled nitric oxide in the management of a premature newborn with severe respiratory distress and pulmonary hypertension. *Pediatrics* **92,** 606–609.

Abman, S. H., Griebel, J. L., Parker, D. K., Schmidt, J. M., Swanton, D., and Kinsella, J. P. (1994). Acute effects of inhaled nitric oxide in children with severe hypoxemic respiratory failure. *J. Pediatr.* **124,** 881–888.

Adatia, I., Lillehei, C., Arnold, J. H., Thompson, J. E., Palazzo, R., Fackler, J. C., and Wessel, D. L. (1994). Inhaled nitric oxide in the treatment of postoperative graft dysfunction after lung transplantation. *Ann. Thorac. Surg.* **57,** 1311–1318.

Adatia, I., Perry, S., Landzberg, M., Moore, P., Thompson, J. E., and Wessel, D. L. (1995). Inhaled nitric oxide and hemodynamic evaluation of patients with pulmonary hypertension before transplantation. *J. Am. Coll. Cardiol.* **25,** 1656–1664.

Adrie, C., Bloch, K. D., Moreno, P. R., Hurford, W. E., Guerrero, J. L., Holt, R., Zapol, W. M., Gold, H. K., and Semigran, M. J. (1996). Inhaled nitric oxide increases coronary artery patency after thrombolysis. *Circulation* **94,** 1919–1926.

al-Alaiyan, S., al-Omran, A., and Dyer, D. (1996). The use of phosphodiesterase inhibitor (dipyridamole) to wean from inhaled nitric oxide. *Intensive Care Med.* **22,** 1093–1095.

Alberts, W. M. (1994). Indoor air pollution: NO, NO_2, CO, and CO_2. *J. Allergy Clin. Immunol.* **94,** 524–526.

Allman, K., Young, J., Carapiet, D., Stevens, J., Ostman-Smith, I., and Archer, L. (1996). Effects of oxygen and nitric oxide in oxygen on pulmonary arterial pressures of children with congenital cardiac defects. *Pediatr. Cardiol.* **17,** 246–250.

Amato, M. B. P., Barbas, C. S. V., Medeiros, D. M., Magaldi, R. B., Schettino, G. P., Lorenzi-Filho, G., Kairalla, R. A., Deheinzelin, D., Munoz, C., Oliveira, R., Takagaki, T. Y., and Carvalho, C. R. R. (1998). Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome. *N. Engl. J. Med.* **338,** 347–354.

Anderson III, H., Steimle, C., Shapiro, M., Delius, R., Chapman, R., Hirschl, R., and Bartlett, R. (1993). Extracorporeal life support for adult cardiorespiratory failure. *Surgery* **114,** 161–173.

Argenziano, M., Choudhri, A. F., Moazami, N., Rose, E. A., Smith, C. R., Levin, H. R., Smerling, A. J., and Oz, M. C. (1998). Randomized, double-blind trial of inhaled nitric oxide in LVAD recipients with pulmonary hypertension. *Ann. Thorac. Surg.* **65,** 340–345.

Auler, J. O., Jr., Carmona, M., Bocchi, E., Bacal, F., Fiorelli, A., Stolf, N., and Jatene, A. (1996). Low doses of inhaled nitric oxide in heart transplant recipients. *J. Heart Lung Transplant.* **15,** 443–450.

Bacha, E. A., Herve, P., Murakami, S., Chapelier, A., Mazmanian, G.-M., Montpreville, V. d., Detruit, H., Libert, J.-M., and Darteville, P. (1996). Lasting beneficial effect of short-term inhaled nitric oxide on graft function after lung transplantation. Paris-Sud University Lung Transplantation Group. *J. Thorac. Cardiovasc. Surg.* **112,** 590–598.

Barabrà, J. A., Roger, N., Roca, J., Rovira, I., Higenbottam, T. W., and Rodriguez-Roisin, R. (1996). Worsening of pulmonary gas exchange with nitric oxide inhalation in chronic obstructive pulmonary disease. *Lancet* **347,** 436–440.

Benzing, A., and Geiger, K. (1994). Inhaled nitric oxide lowers pulmonary capillary pressure and changes longitudinal distribution of pulmonary vascular resistance in patients with acute lung injury. *Acta Anaesthesiol. Scand.* **38,** 640–645.

Benzing, A., Bräutigam, P., Geiger, K., Loop, T., Beyer, U., and Moser, E. (1995). Inhaled nitric oxide reduces pulmonary transvascular albumin flux in patients with acute lung injury. *Anesthesiology* **83,** 1153–1161.

Berner, M., Berghetti, M., Spahr-Schopfer, I., Oberhansli, I., and Friedli, B. (1996). Inhaled nitric oxide to test the vasodilator capacity of the pulmonary vascular bed in children with long-standing pulmonary hypertension and congenital heart disease. *Am. J. Cardiol.* **77,** 532–535.

Bigatello, L. M., Hurford, W. E., Kacmarek, R. M., Roberts, J. D., Jr., and Zapol, W. M. (1994). Prolonged inhalation of low concentrations of nitric oxide in patients with severe adult respiratory distress syndrome. Effects on pulmonary hemodynamics and oxygenation. *Anesthesiology* **80,** 761–770.

Bocchi, E. A., Bacal, F., Auler Junior, J. O., Carmone, M. J., Bellotti, G., and Pileggi, F. (1994). Inhaled nitric oxide leading to pulmonary edema in stable severe heart failure. *Am. J. Cardiol.* **74,** 70–72.

Borland, C. D., and Higenbottam, T. W. (1989). A simultaneous single breath measurement of pulmonary diffusing capacity with nitric oxide and carbon monoxide. *Eur. Respir. J.* **2,** 56–63.

Centers for Disease Control (1988). Recommendations for occupational safety and health standard. *MM WR* **37,** 21.

Channick, R. N. (1999). Chronic use of inhaled nitric oxide for pulmonary hypertension. *Respir. Care* **44,** 212–221.

Channick, R. N., Newhart, J. W., Johnson, F. W., Williams, P. J., Auger, W. R., Fedullo, P. F., and Moser, K. M. (1996). Pulsed delivery of inhaled nitric oxide to patients with primary pulmonary hypertension: An ambulatory delivery system and initial clinical tests. *Chest* **109,** 1545–1549.

Clancy, R. M., Leszcyznska-Piziak, J., and Abramson, S. B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.* **90,** 1116–1121.

Clark, R. H., Kueser, T. J., Walker, M. W., Southgate, W. M., Huckaby, J. L., Perez, J. A., Roy, B. J., Keszler, M., and Kinsella, J. P. (2000). Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn. Clinical Inhaled Nitric Oxide Research Group. *N. Engl. J. Med.* **342,** 469–474.

Clutton-Brock, J. (1967). Two cases of poisoning by contamination of nitrous oxide with higher oxides of nitrogen during anaesthesia. *Br. J. Anaesth.* **39,** 388–392.

Cornwell, T. L., Arnold, E., Boerth, N. J., and Lincoln, T. M. (1994). Inhibition of smooth muscle growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Annu. Rev. Immunol.* **267,** C1405–C1413.

Cremona, G., Dinh Xuan, A. T., and Higenbottam, T. W. (1991). Endothelium-derived relaxing factor and the pulmonary circulation. *Lung* **169,** 185–202.

Curran, R. D., Mavroudis, C., Backer, C. L., Sautel, S., Zaples, V. R., and Wessel, D. L. (1995). Inhaled nitric oxide for children with congenital heart disease and pulmonary hypertension. *Ann. Thorac. Surg.* **60,** 1765–1771.

Date, H., Triantafillou, A. N., Trulock, E. P., Pohl, M. S., Cooper, J. D., and Patterson, G. A. (1996). Inhaled nitric oxide reduces human lung allograft dysfunction. *J. Thorac. Cardiovasc. Surg.* **111,** 913–919.

Dellinger, R. P., Zimmerman, J. L., Taylor, R. W., Straube, R. C., Hauser, D. L., Criner, G. J., Davis, K., Hyers, T. M., Papadakos, P., and the Inhaled Nitric Oxide in ARDS Study Group (1998). Effects of inhaled nitric oxide in patients with acute respiratory distress syndrome: Results of a randomized phase II trial. *Crit. Care Med.* **26,** 15–23.

Dinh Xuan, A. T., Higenbottam, T. W., Clelland, C., Pepke-Zaba, J., Cremona, G., and Wallwork, J. (1990). Impairment of pulmonary endothelium-dependent relaxation in patients with Eisenmenger's syndrome. *Br. J. Pharmacol.* **99,** 9–10.

Dinh Xuan, A. T., Higenbottam, T. W., Clelland, C. A., Pepke-Zaba, J., Cremona, G., Butt, A. Y., Large, S. R., Wells, F. C., and Wallwork, J. (1991). Impairment of endothelium-dependent pulmonary artery relaxation in chronic obstructive lung disease. *N. Engl. J. Med.* **324,** 1539–1547.

ECMO registry of the Extracorporeal Life Support Organization (ELSO) (1996). International Summary. University of Michigan Medical Center, Ann Arbor, Michigan.

Fishman, A. P., McGoon, M. D., Chazova, I. E., Fedullo, P. F., Kneussel, M., Peacock, A. J., and Torbicki, A. (eds.) (1998). Diagnosis and assessment of pulmonary hypertension. *In* ''Primary Pulmonary Hypertension: Executive Summary from the World Symposium—Primary Pulmonary Hypertension 1998'' (S. Rich, ed.). Available via Internet (http://www.who.int/ncd/cvd/pph.html), World Health Organization, Geneva, Switzerland.

Frank, D. U., Horstman, D. J., Morris, G. N., Johns, R. A., and Rich, G. F. (1998). Regulation of the endogenous NO pathway by prolonged inhaled NO in rats. *J. Appl. Physiol.* **85,** 1070–1078.

Frostell, C., Fratacci, M. D., Wain, J. C., Jones, R., and Zapol, W. M. (1991). Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. *Circulation* **83,** 2038–2047.

Fullerton, D. A., Jones, S. D., Jaggers, J., Piedalue, F., Grover, F. L., and McIntyre, R. C., Jr. (1996). Effective control of pulmonary vascular resistance with inhaled nitric oxide after cardiac operation. *J. Thorac. Cardiovasc. Surg.* **111,** 753–762.

Garel, M., Domenget, C., Caburi-Martin, J., Prehu, C., Galacteros, F., and Beuzard, Y. (1986). Covalent binding of glutathione to hemoglobin. I. Inhibition of hemoglobin S polymerization. *J. Biol. Chem.* **261,** 14704–14709.

George, T. N., Johnson, K. J., Bates, J. N., and Segar, J. L. (1998). The effect of inhaled nitric oxide therapy on bleeding time and platelet aggregation in neonates. *J. Pediatr.* **132,** 731–734.

Gerlach, H., Rossaint, R., Pappert, D., and Falke, K. J. (1993). Time-course and dose–response of nitric oxide inhalation for systemic oxygenation and pulmonary hypertension in patients with adult respiratory distress syndrome. *Eur. J. Clin. Invest.* **23,** 499–502.

Gerlach, M., Keh, D., and Gerlach, H. (1999). Inhaled nitric oxide for the acute respiratory distress syndrome. *Respir. Care* **44,** 184–195.

Goldman, A. P., Rees, P. G., and Macrae, D. J. (1995). Is it time to consider domiciliary nitric oxide? *Lancet* **345,** 199–200.

Goldman, A. P., Delius, R. E., Deanfield, J. E., de Leval, M. R., Sigston, P. E., and Macrae, D. J. (1996a). Nitric oxide might reduce the need for extracorporeal support in children with critical postoperative pulmonary hypertension. *Ann. Thorac. Surg.* **62,** 750–755.

Goldman, A. P., Delius, R. E., Deanfield, J. E., Miller, O. I., de Leval, M. R., Sigston, P. E., and Macrae, D. J. (1996b). Pharmacological control of pulmonary blood flow with inhaled nitric oxide after the fenestrated Fontan operation. *Circulation* **94** (9 Suppl.), 44–48.

Greenbaum, R., Bay, J., Hargreaves, M. D., *et al.* (1967). Effects of higher oxides of nitrogen on the anesthetized dog. *Br. J. Anaesth.* **39,** 393–404.

Gries, A., Bottiger, B. W., Dorsam, J., Bauer, H., Weimann, J., Bode, C., Martin, E., and Motsch, J. (1997). Inhaled nitric oxide inhibits platelet aggregation after pulmonary embolism in pigs. *Anesthesiology* **86,** 387–393.

Haddad, I. Y., Pataki, G., Hu, P., Galliani, C., Beckman, J. S., and Matalon, S. (1994). Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J. Clin. Invest.* **94,** 2407–2413.

Hallman, M., Bry, K., Turbow, R., Waffarn, F., and Lappalainen, U. (1998). Pulmonary toxicity associated with nitric oxide in term infants with severe respiratory failure. *J. Pediatr.* **132,** 827–829.

Hassoun, P. M., Yu, F. S., Zulueta, J. J., White, A. C., and Lanzillo, J. J. (1995). Effect of nitric oxide and cell redox status on the regulation of endothelial cell xanthine dehydrogenase. *Am. J. Physiol.* **268,** L809–L817.

Haworth, S. (1984). Pulmonary vascular disease in different types of congenital heart disease. Implications for interpretation for lung biopsy findings in early childhood. *Br. Heart J.* **52,** 557–571.

Hayward, C. S., Rogers, P., Keogh, A. M., Kelly, R., and Spratt, P. M. (1996). Inhaled nitric oxide in cardiac failure: Vascular versus ventricular effects. *J. Cardiovasc. Pharmacol.* **27,** 80–85.

Head, C. A., Brugnara, C., Martinez-Ruiz, R., Kacmarek, R. M., Bridges, K. R., Kuter, D., Bloch, K. D., and Zapol, W. M. (1997). Low concentrations of nitric oxide increase oxygen affinity of sickle erythrocytes *in vitro* and *in vivo*. *J. Clin. Invest.* **100,** 1193–1198.

Hess, D., Bigatello, L., and Hurford, W. E. (1997). Toxicity and complications of inhaled nitric oxide. *Respir. Care Clin. North Am.* **3,** 487–503.

Hoffman, G. M., Ross, G. A., Day, S. E., Rice, T. B., and Nelin, L. D. (1997). Inhaled nitric oxide reduces the utilization of extracorporeal membrane oxygenation in persistent pulmonary hypertension of the newborn. *Crit. Care Med.* **25,** 352–359.

Högman, M., Frostell, C. G., Hedenstrom, H., and Hedenstierna, G. (1993a). Inhalation of nitric oxide modulates adult human bronchial tone. *Am. Rev. Respir. Dis.* **148,** 1474–1478.

Högman, M., Frostell, C., Arnberg, H., and Hedenstierna, G. (1993b). Bleeding time prolongation and NO inhalation. *Lancet* **341,** 1664–1665.

Högman, M., Frostell, C., Arnberg, H., Sandhagen, B., and Hedenstierna, G. (1994). Prolonged bleeding time during nitric oxide inhalation in the rabbit. *Acta Physiol. Scand.* **151,** 125–129.

Hopkins, S. R., Johnson, E. C., Richardson, R. S., Wagner, H., De Rosa, M., and Wagner, P. D. (1997). Effects of inhaled nitric oxide on gas exchange in lungs with shunt or poorly ventilated areas. *Am. J. Respir. Crit. Care Med.* **156,** 484–491.

Hurford, W. E., and Zapol, W. M. (1988). The right ventricle and critical illness: A review of anatomy, physiology, and clinical evaluation of its function. *Intensive Care Med.* **14,** 448–457.

Ivy, D. D., Kinsella, J. P., Ziegler, J. W., and Abman, S. H. (1998). Dipyridamole attenuates rebound pulmonary hypertension after inhaled nitric oxide withdrawal in postoperative congenital heart disease. *J. Thorac. Cardiovasc. Surg.* **115,** 875–882.

Journois, D., Pouard, P., Mauriat, P., Malhere, T., Vouhe, P., and Safran, D. (1994). Inhaled nitric oxide as a therapy for pulmonary hypertension after operations for congenital heart defects. *J. Thorac. Cardiovasc. Surg.* **107,** 1129–1135.

Kacmarek, R. M., Ripple, R., Cockrill, B. A., Bloch, K. J., Zapol, W. M., and Johnson, D. C. (1996). Inhaled nitric oxide: A bronchodilator in mild asthmatics with methacholine-induced bronchospasm. *Am. J. Respir. Crit. Care Med.* **153,** 128–135.

Kanner, J., Harel, S., and Granit, R. (1991). Nitric oxide as an antioxidant. *Arch. Biochem. Biophys.* **289,** 130–136.

Katayama, T., Higenbottam, T. W., Diaz de Atauri, M. J., Cremona, G., Akamine, S., Barabŕa, J. A., and Rodriguez-Roisin, R. (1997). Inhaled nitric oxide and arterial oxygen tension in patients with chronic obstructive pulmonary disease and severe pulmonary hypertension. *Thorax* **52,** 120–124.

Kavanagh, B. P., Mouchawar, A., Goldsmith, J., and Pearl, R. G. (1994). Effects of inhaled NO and inhibition of endogenous NO synthesis in oxidant-induced acute lung injury. *J. Appl. Physiol.* **76,** 1324–1329.

Kieler-Jensen, N., Ricksten, S. E., Stenqvist, O., Bergh, C. H., Lindelov, B., Wennmalm, A., Waagstein, F., and Lundin, S. (1994). Inhaled nitric oxide in the evaluation of heart transplant candidates with elevated pulmonary vascular resistance. *J. Heart Lung Transplant.* **13,** 366–375.

Kinsella, J. P., Neish, S. R., Shaffer, E., and Abman, S. H. (1992). Low-dose inhalational nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet* **340,** 819–820.

Kinsella, J. P., Ivy, D. D., and Abman, S. H. (1994). Inhaled nitric oxide improves gas exchange and lowers pulmonary vascular resistance in severe experimental hyaline membrane disease. *Pediatr. Res.* **36,** 402–408.

Kinsella, J. P., Truog, W. E., Walsh, W. F., Goldberg, R. N., Bancalari, E., Mayock, D. E., Redding, G. J., deLemos, R. A., Sardesai, S., McCurnin, D. C., Moreland, S. G., Cutter, G. R., and Abman, S. H. (1997). Randomized, multicenter trial of inhaled nitric oxide and high-frequency oscillatory ventilation in severe, persistent pulmonary hypertension of the newborn. *J. Pediatr.* **131,** 55–62.

Kirmse, M., Hess, D., Fujino, Y., Kacmarek, R. M., and Hurford, W. E. (1998). Delivery of inhaled nitric oxide using the Ohmeda INOvent delivery system. *Chest* **113,** 1650–1657.

Kneussl, M. P., Lang, I. M., and Brenot, F. P. (1996). Medical management of primary pulmonary hypertension. *Eur. Respir. J.* **9,** 2401–2409.

Kooy, N. W., Royall, J. A., Ye, Y. Z., Kely, D. R., and Beckman, J. S. (1995). Evidence for *in vivo* peroxynitrite production in human acute lung injury. *Am. J. Respir. Crit. Care Med.* **15,** 1250–1254.

Kouyoumdjian, C., Adnot, S., Levame, M., Eddahibi, S., Bousbaa, H., and Raffestin, B. (1994). Continuous inhalation of nitric oxide protects against development of pulmonary hypertension in chronically hypoxic rats. *J. Clin. Invest.* **94,** 578–584.

Lachmann, B. (1992). Open up the lung and keep the lung open. *Intensive Care Med.* **17,** 375–376.

Lavoie, A., Hall, J. B., Olson, D. M., and Wylam, M. E. (1996). Life-threatening effects of discontinuing inhaled nitric oxide in severe respiratory failure. *Am. J. Respir. Crit. Care Med.* **153,** 1985–1987.

Lee, K. H., Tan, P. S., Rico, P., Delgado, E., Kellum, J. A., and Pinsky, M. R. (1997). Low levels of nitric oxide as contaminant in hospital compressed air: Physiologic significance? *Crit. Care Med.* **25,** 1143–1146.

Loh, E., Stamler, J. S., Hare, J. M., Loscalzo, J., and Colucci, W. S. (1994). Cardiovascular effects of inhaled nitric oxide in patients with left ventricular dysfunction. *Circulation* **90,** 2780–2785.

Luhr, O., Aardal, S., Nathorst-Westfelt, U., Berggren, L., Johansson, L. A., Wahlin, L., and Frostell, C. (1998). Pulmonary function in adult survivors of severe acute lung injury with inhaled nitric oxide. *Acta Anaesthesiol. Scand.* **42,** 391–398.

Lundin, S., Westfelt, U. N., Stenqvist, O., Blomqvist, H., Lindh, A., Berggren, L., Arvidsson, S., Rudberg, U., and Frostell, C. G. (1996). Response to nitric oxide inhalation in early acute lung injury. *Intensive Care Med.* **22,** 728–734.

Lundin, S., Mang, H., Smithies, M., Stenqvist, O., and Frostell, C. (1997). Inhalation of nitric oxide in acute lung injury: Preliminary results of a European multicenter study. *Intensive Care Med.* **23,** S2.

Ma, X. L., Lopez, B. L., Christopher, T. A., Birenbaum, D. S., and Vinten-Johansen, J. (1996). Exogenous NO inhibits basal NO release from vascular endothelium *in vitro* and *in vivo*. *Am. J. Physiol.* **271,** H2045–H2051.

McElroy, M. C., Wiener-Kronish, J. P., Miyazaki, H., Sawa, T., Modelska, K., Dobbs, L. G., and Pittet, J. F. (1997). Nitric oxide attenuates lung endothelial injury caused by sublethal hyperoxia in rats. *Am. J. Physiol.* **272,** L631–L638.

Manktelow, C., Bigatello, L. M., Hess, D., and Hurford, W. E. (1997). Physiologic determinants of the response to inhaled nitric oxide in patients with the acute respiratory distress syndrome. *Anesthesiology* **87,** 297–307.

May, G. R., Crook, P., Moore, P. K., and Page, C. P. (1991). The role of nitric oxide as an endogenous regulator of platelet and neutrophil activation within the pulmonary circulation of the rabbit. *Br. J. Pharmacol.* **102,** 759–763.

Meurs, K. P., Rhine, W. D., Asselin, J. M., and Durand, D. J. (1997). Response of premature infants with severe respiratory failure to inhaled nitric oxide. Preemie NO collaborative group. *Pediatr. Pulmonol.* **24,** 319–323.

Meyrick, B., and Reid, L. (1983). Pulmonary hypertension. Anatomic and physiologic correlated. *Clin. Chest Med.* **4,** 199–217.

Michael, J. R., Barton, R. G., Saffle, J. R., Mone, M., Markewitz, B. A., Hillier, K., Elstad, M. R., Campbell, E. J., Troyer, B. E., Whatley, R. E., Liou, T. G., Samuelson, W. M., Carveth, H. J., Hinson, D. M., Morris, S. E., Davis, B. L., and Day, R. W. (1998). Inhaled nitric oxide versus conventional therapy. Effect on oxygenation in ARDS. *Am. J. Respir. Crit. Care Med.* **157,** 1372–1380.

Miller, O. I., Celermajer, D. S., Deanfield, J. E., and Macrae, D. J. (1994). Very-low-dose inhaled nitric oxide: A selective pulmonary vasodilator after operations for congenital heart disease. *J. Thorac. Cardiovasc. Surg.* **108,** 487–494.

Mira, J. P., Monchi, M., Brunet, F., Fierobe, L., Dhainaut, J. F., and Dinh-Xuan, A. T. (1994). Lack of efficacy of inhaled nitric oxide in ARDS. *Intensive Care Med.* **20,** 532.

Montgomery, A. B., Stager, M. A., Carrico, C. J., and Hudson, L. D. (1985). Causes of mortality in patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **132,** 485–489.

Murakami, S., Bacha, E. A., Herve, P., Detruit, H., Chapelier, A. R., Dartevelle, P. G., and Mazmanian, G. M. (1996). Prevention of reperfusion injury by inhaled nitric oxide in lungs harvested from non-heart-beating donors. *Ann. Thorac. Surg.* **62,** 1632–1638.

Neonatal Inhaled Nitric Oxide Study Group (1997a). Inhaled nitric oxide in full-term and nearly full-term infants with hypoxic respiratory failure. The Neonatal Inhaled Nitric Oxide Study Group. *N. Engl. J. Med.* **336,** 597–604.

Neonatal Inhaled Nitric Oxide Study Group (1997b). Inhaled nitric oxide and hypoxic respiratory failure in infants with congenital diaphragmatic hernia. The Neonatal Inhaled Nitric Oxide Study Group (NINOS). *Pediatrics* **99,** 838–845.

Nishimura, M., Hess, D., Kacmarek, R. M., Ritz, R., and Hurford, W. E. (1995). Nitrogen dioxide production during mechanical ventilation with nitric oxide in adults. Effects of ventilator internal volume, air versus nitrogen dilution, minute ventilation, and inspired oxygen fraction. *Anesthesiology* **82,** 1246–1254.

Niu, X. F., Smith, W., and Kubes, P. (1994). Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ. Res.* **74,** 1133–1140.

Nong, Z., Hoyaerst, M., Van Pelt, N., Collen, D., and Janssens, S. (1997). Nitric oxide inhalation inhibits platelet aggregation and platelet-mediated pulmonary thrombosis in rats. *Circ. Res.* **81,** 865–869.

Norman, V., and Keith, C. H. (1965). Nitrogen oxides in tobacco smoke. *Nature* **205,** 915–916.

Papazian, L., Bregeon, F., Gaillat, F., Thirion, X., Gainnier, M., Gregoire, R., Saux, P., Gouin, F., Jammes, Y., and Auffray, J. P. (1998a). Respective and combined effects of prone position and inhaled nitric oxide in

patients with acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* **157,** 580–585.

Papazian, L., Bregeon, F., Gaillat, F., Kaphan, E., Thirion, X., Saux, P., Badier, M., Gregoire, R., Gouin, F., Jammes, Y., and Auffray, J. P. (1998b). Does norepinephrine modify the effects of inhaled nitric oxide in septic patients with acute respiratory distress syndrome? *Anesthesiology* **89,** 1089–1098.

Payen, D. M., Gatecel, C., and Plaisance, P. (1993). Almitrine effect on nitric oxide inhalation in adult respiratory distress syndrome. *Lancet* **341,** 1164.

Peliowski, A., Finer, N. N., Etches, P. C., Tierney, A. J., and Ryan, C. A. (1995). Inhaled nitric oxide for premature infants after prolonged rupture of the membranes. *J. Pediatr.* **126,** 450–453.

Pepke-Zaba, J., Higenbottam, T. W., Dinh-Xuan, A. T., Stone, D., and Wallwork, J. (1991). Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. *Lancet* **338,** 1173–1174.

Pesenti, A., Kolobow, T., and Gattinoni, L. (1988). Extracorporeal respiratory support in the adult. *Trans. Am. Soc. Artif. Intern. Organs* **34,** 1006–1008.

Pfeffer, K. D., Ellison, G., Robertson, D., and Day, R. W. (1996). The effect of inhaled nitric oxide in pediatric asthma. *Am. J. Respir. Crit. Care Med.* **153,** 747–751.

Provost, P., Lam, J. Y. T., Lacoste, L., Merhi, Y., and Waters, D. (1994). Endothelium-derived nitric oxide attenuates neutrophil adhesion to endothelium under arterial flow conditions. *Arterioscler. Thromb.* **14,** 331–335.

Putensen, C., Rasanen, J., Lopez, F. A., and Downs, J. B. (1994). Continuous positive airway pressure modulates effect of inhaled nitric oxide on the ventilation–perfusion distributions in canine lung injury. *Chest* **106,** 1563–1569.

Puybasset, L., Stewart, T., Rouby, J. J., Cluzel, P., Mourgeon, E., Belin, M. F., Arthaud, M., Landault, C., and Viars, P. (1994). Inhaled nitric oxide reverses the increase in pulmonary vascular resistance induced by permissive hypercapnia in patients with acute respiratory distress syndrome. *Anesthesiology* **80,** 1254–1267.

Puybasset, L., Rouby, J. J., Mourgeon, E., Cluzel, P., Souhil, Z., Law-Koune, J. D., Stewart, T., Devilliers, C., Lu, Q., Roche, S., Kalfon, P., Vicaut, E., and Viars, P. (1995). Factors influencing cardiopulmonary effects of inhaled nitric oxide in acute respiratory failure. *Am. J. Respir. Crit. Care Med.* **152,** 318–328.

Rengasamy, A., and Johns, R. A. (1993). Regulation of nitric oxide synthase by nitric oxide. *Mol. Pharmacol.* **44,** 124–128.

Rich, G. F., Murphy, G. D., Jr., Roos, C. M., and Johns, R. A. (1993). Inhaled nitric oxide: Selective pulmonary vasodilation in cardiac surgical patients. *Anesthesiology* **78,** 1028–1035.

Rich, S. (ed.) (1998). ''Primary Pulmonary Hypertension: Executive Summary from the World Symposium—Primary Pulmonary Hypertension 1998.'' Available via Internet (http://www.who.int/ncd/cvd/pph.html), World Health Organization.

Robbins, I. M., Christman, B. W., Newman, J. H., Matlock, R., and Loyd, J. E. (1998). A survey of diagnostic practices and the use of epoprostenol in patients with primary pulmonary hypertension. *Chest* **114,** 1269–1275.

Roberts, J. D., Jr. (1993). Inhaled nitric oxide for treatment of pulmonary artery hypertension in the newborn and infant. *Crit. Care Med.* **21,** S374–S376.

Roberts, J. D., Polaner, D. M., Lang, P., and Zapol, W. M. (1992). Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet* **340,** 818–819.

Roberts, J. D., Jr., Lang, P., Bigatello, L. M., Vlahakes, G. J., and Zapol, W. M. (1993). Inhaled nitric oxide in congenital heart disease. *Circulation* **87,** 447–453.

Roberts, J. D., Jr., Roberts, C. T., Jones, R. C., Zapol, W. M., and Bloch, K. D. (1995). Continuous nitric oxide inhalation reduces pulmonary arterial structural changes, right ventricular hypertrophy, and growth retardation in the hypoxic newborn rat. *Circ. Res.* **76,** 215–222.

Roberts, J. D., Jr., Fineman, J. R., Morin III, F. C., Shaul, P. W., Rimar, S., Schreiber, M. D., Polin, R. A., Zwass, M. S., Zayek, M. M., Gross, I., Heyman, M. A., and Zapol, W. M. (1997). Inhaled nitric oxide and persistent pulmonary hypertension of the newborn. *N. Engl. J. Med.* **336,** 605–610.

Roger, N., Barabrà, J. A., Roca, J., Rovira, I., Gomez, F. P., and Rodriguez-Roisin, R. (1997). Nitric oxide inhalation during exercise in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **156,** 800–806.

Rosenberg, A. A., Kennaugh, J. M., Moreland, S. G., Fashaw, L. M., Hale, K. A., Torielli, F. M., Abman, S. H., and Kinsella, J. P. (1997). Longitudinal follow-up of a cohort of newborn infants treated with inhaled nitric oxide for persistent pulmonary hypertension. *J. Pediatr.* **131,** 70–75.

Rossaint, R., Falke, K. J., Lopez, F., Slama, K., Pison, U., and Zapol, W. M. (1993). Inhaled nitric oxide for the adult respiratory distress syndrome. *N. Engl. J. Med.* **328,** 399–405.

Rossaint, R., Slama, K., Steudel, W., Gerlach, H., Pappert, D., Veit, S., and Falke, K. (1995a). Effects of inhaled nitric oxide on right ventricular function in severe acute respiratory distress syndrome. *Intensive Care Med.* **21,** 197–203.

Rossaint, R., Gerlach, H., Schmidt-Ruhnke, H., Pappert, D., Lewandowski, K., Steudel, W., and Falke, K. (1995b). Efficacy of inhaled nitric oxide in patients with severe ARDS. *Chest* **107,** 1107–1115.

Roze, J. C., Storme, L., Zupan, V., Morville, P., Dinh-Xuan, A. T., and Mercier, J. C. (1994). Echocardiographic investigation of inhaled nitric oxide in newborn babies with severe hypoxaemia. *Lancet* **344,** 303–305.

Samama, C. M., Diaby, M., Fellahi, J. L., Mdhafar, A., Eyraud, D., Arock, M., Guillosson, J. J., Coriat, P., and Rouby, J. J. (1995). Inhibition of platelet aggregation by inhaled nitric oxide in patients with acute respiratory distress syndrome. *Anesthesiology* **83,** 56–65.

Sanna, A., Kurtansky, A., Veriter, C., and Stanescu, D. (1994). Bronchodilator effect of inhaled nitric oxide in healthy men. *Am. J. Respir. Crit. Care Med.* **150,** 1702–1704.

Schechter, A. (1997). NO therapy? *J. Clin. Invest.* **100,** 955–956.

Scherrer, U., Vollenweider, L., Delabays, A., Savic, M., Eichenberger, U., Kleger, G. R., Fikrle, A., Ballmer, P. E., Nicod, P., and Bartsch, P. (1996). Inhaled nitric oxide for high-altitude pulmonary edema. *N. Engl. J. Med.* **334,** 624–629.

Semigran, M. J., Cockrill, B. A., Kacmarek, R., Thompson, B. T., Zapol, W. M., Dec, G. W., and Fifer, M. A. (1994). Hemodynamic effects of inhaled nitric oxide in heart failure. *J. Am. Coll. Cardiol.* **24,** 982–988.

Sitbon, O., Brenot, F., Denjean, A., Bergeron, A., Parent, F., Azarian, R., Herve, P., Raffestin, B., and Simonneau, G. (1995). Inhaled nitric oxide as a screening vasodilator agent in primary pulmonary hypertension. A dose–response study and comparison with prostacyclin. *Am. J. Respir. Crit. Care Med.* **151,** 384–389.

Skimming, J. W., Bender, K. A., Hutchison, A. A., and Drummond, W. H. (1997). Nitric oxide inhalation in infants with respiratory distress syndrome. *J. Pediatr.* **130,** 225–230.

Snell, G. I., Salamonsen, R. F., Bergin, P., Esmore, D. S., Khan, S., and Williams, T. J. (1995). Inhaled nitric oxide used as a bridge to heart–lung transplantation in a patient with end-stage pulmonary hypertension. *Am. J. Respir. Crit. Care Med.* **151,** 1263–1266.

Steinberg, M. (1996). Review: Sickle cell disease: Present and future treatment. *Am. J. Med. Sci.* **312.** 166–174.

Stewart, T. E., Meade, M. O., Cook, D. J., Granton, J. T., Hodder, R. V., Lapinsky, S. E., Mazer, C. D., McLean, R. F., Rogovein, T. S., Schouten, B. D., Todd, T. R. J., and Slutsky, A. S. (1998). Evaluation of a ventilation strategy to prevent barotrauma in patients at high risk for acute respiratory distress syndrome. Pressure- and Volume-Limited Ventilation Strategy Group. *N. Engl. J. Med.* **338,** 355–361.

Suchyta, M. R., Clemmer, T. P., Elliott, C. G., Orme, J. F. J., and Weaver, L. K. (1992). The adult respiratory distress syndrome. A report of survival and modifying factors. *Chest* **101,** 1074–1079.

Troncy, E., Collet, J. P., Shapiro, S., Guimond, J. G., Blair, L., Ducruet, T., Francoeur, M., Charbonneau, M., and Blaise, G. (1998). Inhaled nitric

oxide in acute respiratory distress syndrome: A pilot randomized controlled study. *Am. J. Respir. Crit. Care Med.* **157,** 1483–1488.

von Nieding, G., Wagner, H., and Kockeler, H. (1975). Investigation of the acute effects of nitrogen monoxide on lung function in man. *Staub Reinhalt. Luft* **35,** 175–178.

Wagner, F., Dandel, M., Gunther, G., Loebe, M., Schulze-Neick, I., Laucke, U., Kuhly, R., Weng, Y., and Hetzer, R. (1997). Nitric oxide inhalation in the treatment of right ventricular dysfunction following left ventricular assist device implantation. *Circulation* **96,** II-291–II-296.

Wessel, D. L., Adatia, I., Thompson, J. E., and Hickey, P. R. (1994). Delivery and monitoring of inhaled nitric oxide in patients with pulmonary hypertension. *Crit. Care Med.* **22,** 930–938.

Wink, D. A., Hanbauer, I., Krishna, M. C., DeGraff, W., Gamson, J., and Mitchell, J. B. (1993). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9813–9817.

Wysocki, M., Delclaux, C., Roupie, E., Langeron, O., Liu, N., Herman, B., Lemaire, F., and Brochard, L. (1994). Additive effect on gas exchange of inhaled nitric oxide and intravenous almitrine bismesylate in the adult respiratory distress syndrome. *Intensive Care Med.* **20,** 254–259.

Yoshida, M., Taguchi, O., Gabazza, E. C., Kobayashi, T., Yamakami, T., Kobayashi, H., Maruyama, K., and Shima, T. (1997a). Combined inhalation of nitric oxide and oxygen in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **155,** 526–529.

Yoshida, M., Taguchi, O., Gabazza, E. C., Yasui, H., Kobayashi, T., Kobayashi, H., Maruyama, K., and Adachi, Y. (1997b). The effect of low-dose inhalation of nitric oxide in patients with pulmonary fibrosis. *Eur. Respir. J.* **10,** 2051–2054.

Young, J. D., Brampton, W. J., Knighton, J. D., and Finfer, S. R. (1994). Inhaled nitric oxide in acute respiratory failure in adults. *Br. J. Anaesth.* **73,** 499–502.

Zapol, W. M., and Snider, M. T. (1977). Pulmonary hypertension in severe acute respiratory failure. *N. Engl. J. Med.* **296,** 476–480.

Ziegler, J. W., Ivy, D. D., Wiggins, J. W., Kinsella, J. P., Clarke, W. R., and Abman, S. H. (1998). Effects of dipyridamole and inhaled nitric oxide in pediatric patients with pulmonary hypertension. *Am. J. Respir. Crit. Care Med.* **158,** 1388–1395.

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CHAPTER 57

Airborne Nitric Oxide in Health and Disease

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DIRECT MEASUREMENTS OF NITRIC OXIDE (NO) IN BIOLOGICAL TISSUES ARE DIFFICULT TO PERFORM BECAUSE THIS GAS REACTS RAPIDLY WITH HEMOGLOBIN OR OTHER PROTEINS. THEREFORE, ONE MUST OFTEN RELY ON INDIRECT METHODS IN ORDER TO DETECT NO SYNTHESIS *IN VIVO.* THESE INCLUDE ANALYSIS OF NITRATE/NITRITE OR CITRULLINE, IMMUNOHISTOCHEMICAL DETECTION OF NITRIC OXIDE SYNTHASE (NOS), AND FUNCTIONAL STUDIES USING NOS INHIBITORS. UNLIKE THE SITUATION IN MOST BIOLOGICAL TISSUES, WHERE NO IS RAPIDLY METABOLIZED, NO IN THE GAS PHASE IS FAIRLY STABLE, ESPECIALLY AT LOW CONCENTRATIONS (BODY *ET AL.,* 1995). THEREFORE, NO PRODUCED IN SUPERFICIAL STRUCTURES OF HOLLOW ORGANS WILL DIFFUSE TO THE LUMEN AND MAY THUS BE DETECTABLE IN GAS COLLECTED FROM SUCH ORGANS. IN THIS CHAPTER WE WILL DISCUSS MEASUREMENTS OF LUMINAL NO IN THE AIRWAYS, GASTROINTESTINAL, AND UROGENITAL TRACTS AND ITS PHYSIOLOGICAL RELEVANCE IN HEALTH AND DISEASE.

Airways

The presence of NO in the exhaled breath of animals and humans was first demonstrated by Gustafsson *et al.* in 1991. The expired NO was detected in the low parts per billion (ppb) range with a chemiluminescence technique as well as with mass spectrometry, and it was decreased on intravenous (i.v.) administration of a NOS inhibitor. The chemiluminescence technique uses an excess of ozone (O_3), which reacts with NO to produce nitrogen dioxide (NO_2*) with an electron in an excited energy level. NO_2* changes back to the ground state while emitting electromagnetic radiation ranging between 600 and 3000 nm in wavelength. A photomultiplier tube proportionally converts the intensity of luminescence into an electrical signal (Archer, 1993; Fontijin *et al.,* 1970). The chemiluminescence technique is highly sensitive, and NO can be detected down to parts per trillion levels. Early studies showed that in healthy humans, basically all NO found in exhaled air originates from the upper airways, with only a minor contribution from the lower respiratory tract and the lungs (Alving *et al.,* 1993; Lundberg *et al.,* 1994; Gerlach *et al.,* 1994). Conscious tracheostomized subjects exhaled only low levels when breathing through the tracheostomy, whereas much higher levels were seen during oral or nasal breathing (Lundberg *et al.,* 1994a). In all healthy subjects, NO values are higher during nasal than during oral breathing, indicating a large NO release in the nasal airways (Alving *et al.,* 1993; Lundberg *et al.,* 1994a).

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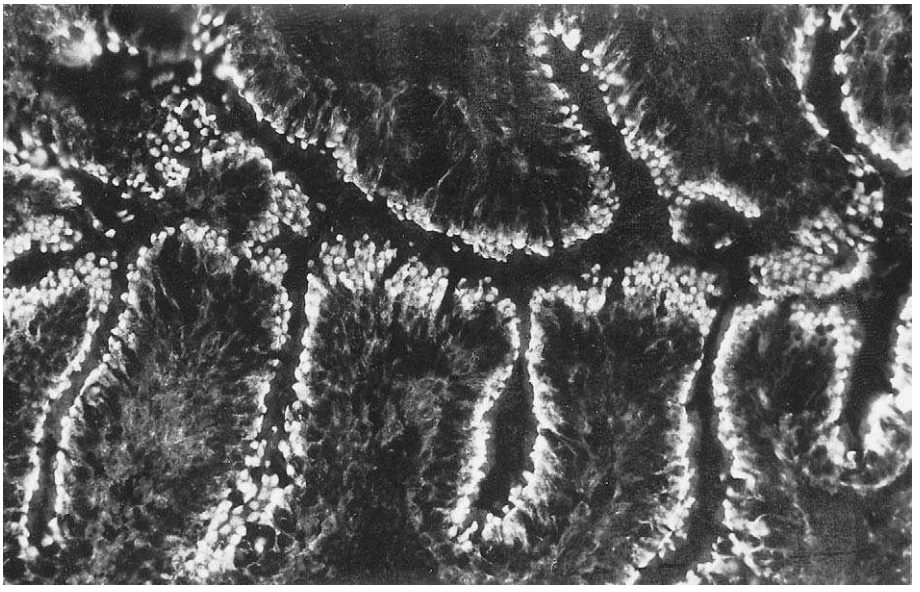


Figure 1 Immunohistochemical staining of inducible NOS (iNOS) in human paranasal sinus mucosa from a healthy individual showing intense signal apically in the epithelial cells. From Lundberg *et al.* (1995a) with permission.

NO in the Upper Airways

At present the precise origin of the NO in the upper airways and the relative contribution from different sources within the nasal airways are not known. There is a large production of NO in the paranasal sinuses in adult healthy humans (Lundberg *et al.,* 1995a). Air aspirated directly from the human sinuses contains NO in concentrations of 10–30 parts per million (ppm), which is similar to the highest permissible atmospheric pollution levels (Lundberg *et al.,* 1995a). NO release in the sinuses is markedly reduced by intrasinus instillation of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (Lundberg *et al.,* 1995a). Immunohistochemical (Fig. 1) and *in situ* hybridization studies show dense staining for NOS and its mRNA in sinus epithelium (Lundberg *et al.,* 1995a). Nasal NO levels are markedly reduced in patients who generally have mucus-filled paranasal sinuses and obstructed sinus ostia (Kartagener's syndrome) (Lundberg *et al.,* 1994a), cystic fibrosis (Lundberg *et al.,* 1996a), and acute sinusitis (Baraldi *et al.,* 1997), indicating a normal contribution from the paranasal sinuses to nasally measured NO (Fig. 2). Interestingly, nasal NO levels are high in humans and other primates (Schedin *et al.,* 1997) but very low in baboons (Lewandowski *et al.,* 1998), the only mammal lacking paranasal sinuses.

Both immunohistochemical and *in situ* hybridization studies indicate that the NOS found in healthy sinus epithelium (Lundberg *et al.,* 1995a) is identical to or very closely related to the inducible NOS (iNOS) that has been cloned from activated human hepatocytes (Geller *et al.,* 1993). Moreover, NOS activity in sinus mucosa is predominantly calcium independent, which is an attribute associated with iNOS (Lundberg *et al.,* 1996b). However, the regulation of the expression and the activity of sinus NOS seems to differ from what has previously been described for iNOS. Thus, sinus NOS is constantly expressed and seems to be resistant to steroids (Lundberg *et al.,* 1995a, 1996b), properties that are normally associated with the low-rate NO-producing endothelial and neuronal forms of NOS. Immunohistochemistry for NOS in the nasal mucosa has shown only weak staining for iNOS (Lundberg *et al.,* 1995a). Moreover, Ramis *et al.* (1996) found only calcium-dependent NOS activity in nasal mucosa from healthy subjects. However, human nasal epithelium from patients with nasal polyp disease expresses increased iNOS mRNA compared to normal epithelium (Watkins *et al.,* 1998). Nasal NO levels are only slightly reduced following topical administration of L-NAME, whereas the same inhibitor at lower concentrations caused a marked decrease in sinus NO output (Lund-

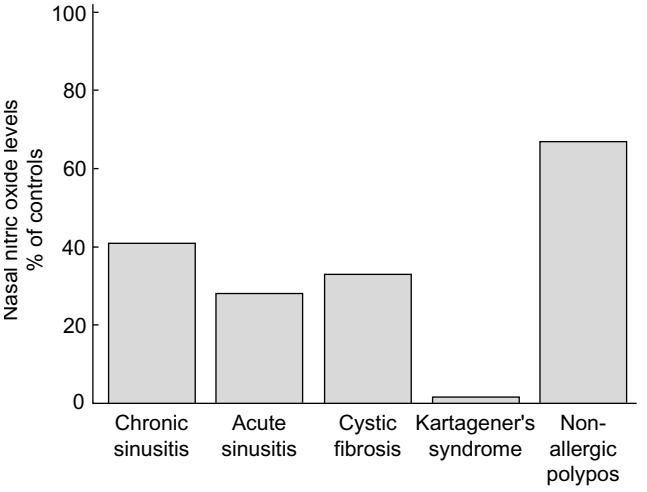


Figure 2 Nasal nitric oxide levels in various disorders involving the paranasal sinuses compared to healthy controls. Data from Lundberg *et al.* (1994a, 1996a), Lindberg *et al.* (1997a), and Arnal *et al.* (1999).

berg *et al.,* 1995a). Taken together these findings indicate that the nasal mucosa may not be a major source of NO in the upper airways.

NASAL NO MEASUREMENTS

Several techniques for collecting nasal air have been used. The most commonly presented way to measure nasal NO is to sample nasal air directly from one nostril (Lundberg *et al.,* 1996c; Kharitonov *et al.,* 1997a; ATS Taskforce, 1999). Using the intrinsic flow of the chemiluminescence analyzer or an external pump, air is aspirated from one nostril. The contralateral nostril is left open, and the subject either holds his breath or breathes through the mouth. Thereby, air is forced from one side of the nose to the other via the nasopharynx. To avoid contamination with lower airway NO, the subject can be asked to exhale orally against a resistance in order to close the soft palate (Kimberly *et al.,* 1996; Silkoff *et al.,* 1997). Another suggested method to measure nasal NO is to exhale through the nose after a full vital capacity inhalation. The exhalation should be made at a constant flow rate. Following this procedure the subject then exhales through the mouth at the same flow rate after a full vital capacity inhalation, and the NO values from oral exhalation is subtracted from the ones measured during nasal exhalation (Palm *et al.,* 2000). As NO is continuously released into the nasal airways, the concentration will be dependent on the flow rate by which the sample is aspirated (Lundberg *et al.,* 1996c). Thus, nasal NO concentrations are higher at lower flow rates. It may therefore be preferable to express nasal NO as the output per time unit (e.g., nl/min), which has been shown to be relatively independent of flow rate (Dubois *et al.,* 1998).

The optimal measurement technique for nasal NO is yet to be determined. A reliable and standardized method will allow for better comparisons of results between different laboratories. Indeed, consensus meetings have been arranged to standardize nasal NO measurements, and recommendations for suitable sampling techniques are available (Kharitonov *et al.,* 1997a; ATS Taskforce, 1999).

FACTORS INFLUENCING NASAL NO

Physiological Factors Nasal NO has been measured in healthy subjects at different ages ranging from zero to 70 years of age, and it has been found that NO is already present at birth (Lundberg *et al.,* 1995a; Schedin *et al.,* 1996). Schedin *et al.* (1996) also found significant nasal NO levels in newborns, including those delivered by cesarian section (Schedin *et al.,* 1996). There is no obvious decline in nasal levels on aging, although large population studies are yet to be performed. There is no evidence of sex differences in nasal NO; however, variations in relation to the menstrual cycle have not yet been studied. NO output in the nasal airways is acutely decreased by physical exercise (Lundberg *et al.,* 1996d; Imada *et al.,* 1996; Phillips *et al.,* 1996; Yasuda *et al.,* 1997). This decrease cannot be explained merely by dilution of nasal air due to changes in nasal cavity volume or increased ventilation. Instead, these changes have been attributed to a reduction in mucosal blood flow of the nasal airways with a concomitant decrease in substrate supply to the high-rate producing NOS (Lundberg *et al.,* 1996d).

Effects of Disease In children with Kartagener's syndrome, which is a triad consisting of sinusitis, bronchiectasis, and *situs inversus,* nasal NO levels are extremely low compared to healthy age-matched controls (Lundberg *et al.,* 1994a). Also in patients with cystic fibrosis nasal NO is very low (Lundberg *et al.,* 1996a; Balfour-Lynn *et al.,* 1996; Dotsch *et al.,* 1996) (see Fig. 2). Baraldi *et al.* (1997) measured nasal NO in a group of children with acute sinusitis and found low nasal NO. In these patients nasal NO increased when the patients improved following treatment with antibiotics. Also chronic sinusitis is associated with a more than 50% reduction in nasal NO (Lindberg *et al.,* 1997a). In general, the previously mentioned studies indicate that nasal NO is lower in subjects with sinus disorders. It has not been established if the low nasal values in patients with sinus disorders is a result of altered passage of NO gas from the sinuses to the nasal cavity or if the actual production of NO in the sinuses is decreased in these patients. However, in the case of Kartagener's syndrome it is likely that there is a primary defect in NO synthesis in the nasal airways since NO levels are extremely low despite open paranasal sinuses as assessed by a computed tomography scan (Arnal *et al.,* 1999).

Whether or not nasal NO is altered in patients with rhinitis of different etiology has not been fully established. Some groups have reported higher nasal NO in patients with allergic rhinitis (Martin *et al.,* 1996; Kharitonov *et al.,* 1997b; Arnal *et al.,* 1997). In one study nasal NO decreased following treatment with nasal topical steroids (Kharitonov *et al.,* 1997b). Kirsten *et al.* (1997) reported increased nasal NO in human subjects after nasal challenge with endotoxin-containing swine confinement dust. In contrast, no alterations in nasal NO were found in a group of children with perennial rhinitis (Lundberg *et al.,* 1996a). The reasons for the discrepancies between the studies are not clear. One might speculate that iNOS is upregulated in the nose during rhinitis (Furukawa *et al.,* 1996; Kawamoto *et al.,* 1998), as is the case in the lower airways in asthma (Hamid *et al.,* 1993). This would explain the higher levels of NO reported in some studies. On the other hand, the swelling of the nasal mucosa during rhinitis might also lead to partial blockage of the sinus ostia, which would result in less passage of sinus NO into the nasal cavity where NO is measured.

Ferguson and Eccles (1997) measured nasal NO in patients with a common cold but failed to see any differences in NO compared to healthy controls. In another study on experimental human influenza nasal NO levels decreased slightly during the symptomatic period (Murphy *et al.,* 1998).

Effects of Drugs Early reports on the effect of topical glucocorticoids showed no effect of these drugs on nasal NO in healthy individuals (Lundberg *et al.,* 1994a). In fact, not even systemic steroids in high doses seemed to alter normal nasal NO levels (Lundberg *et al.,* 1996b). Systemic

antibiotic treatment does not seem to affect nasal NO (Lundberg *et al.,* 1994a). Moreover, newborn babies delivered by cesarian section had high nasal NO levels as mentioned earlier (Schedin *et al.,* 1996). Therefore, nasal NO release seems to be independent of bacteria normally present in the nasopharynx.

Topical nasal decongestants such as oxymetazoline have been shown to decrease nasal NO (Holden *et al.,* 1999; Rinder *et al.,* 1996). The reason for this is not clear, but it has been suggested that the decrease in nasal/sinus blood flow induced by these drugs leads to a reduction of substrate supply to the high-producing iNOS in the upper airways (Rinder *et al.,* 1996). Indeed, substrate supply seems to be of importance for nasal/sinus NO release, because i.v. administration of L-arginine resulted in an increase in nasal NO levels (Lundberg *et al.,* 1996b). However, Rinder *et al.* (1996) looked at the effects of histamine and capsaicin on nasal NO but found no effects of these drugs.

The NO synthase inhibitor L-NAME has only minor effects on nasal NO when administered locally in the nose (Lundberg *et al.,* 1995a; Rinder *et al.,* 1996). In contrast, Albert *et al.* (1997) found a substantial decrease in nasal NO following i.v. administration of N^G-monomethyl-L-arginine (L-NMMA), another NOS inhibitor. Nasal NO has been reported to be lower in cigarette smokers (Gerlach *et al.,* 1994; Robbins *et al.,* 1997), but increases 1 week after smoking cessation (Robbins *et al.,* 1997).

Animal Models

Schedin *et al.* (1997) have measured upper airway NO in a large number of species and found high levels only in primates and in elephants. Lewandowski *et al.* (1998) measured nasal NO in baboons and found only low levels. Because of the large variation in nasal NO output between different species caution should be taken when comparing data from animal models with results from studies in humans.

Biological Effects of Nasal NO

Although the exact role of nasal NO is far from clear, it is reasonable to believe that this pluripotent gas is involved in a variety of physiological as well as pathophysiological events in the airways.

Host Defense Among the various biological properties of NO are its effects on the growth of various pathogens including bacteria, fungi, and virus (Xie and Nathan, 1994). NO produced by white blood cells is thought to be important in the killing of certain microorganisms by these cells. Furthermore, some bacteria are sensitive to authentic NO gas in concentrations as low as 100 ppb (Mancinelli and McKay, 1983). The fact that local NO concentrations in the nasal airways may be several hundred times higher than this supports the notion that NO is involved in local host defense in the upper airways. Local concentrations in the paranasal sinuses can reach 30,000 ppb, which may help to keep the sinuses sterile under normal conditions (Lundberg *et al.,* 1995a). The very low nasal NO in patients with Kartagener's syndrome or cystic fibrosis (Lundberg *et al.,* 1996a) may contribute to the increased susceptibility to airway infections in these patients. If this is correct, a stimulation of endogenous NO production could increase the resistance to airway infections in patients with low nasal NO as suggested earlier (Lundberg *et al.,* 1994a). It is not clear if NO itself acts directly on microorganisms or if it combines with other components to yield other reactive nitrogen intermediates that are toxic (Xie and Nathan, 1994).

Besides acting directly on microorganisms, NO may also contribute to local host defense by stimulating ciliary motility (Jain *et al.,* 1993). A study by Runer *et al.* (1998) in humans shows that application of an NO donor agent in the nasal mucosa indeed did cause an increase in ciliary beat frequency. Furthermore, the same group has shown that low levels of nasal NO correlate to impaired mucociliary function in the human upper airways (Lindberg *et al.,* 1997b). Interestingly, patients with primary ciliary dyskinesia have extremely low levels of nasal NO as mentioned earlier (Lundberg *et al.,* 1994a), and administration of L-arginine may increase both NO levels and improve ciliary function (Loukides *et al.,* 1998).

Nasal NO as an Airborne Messenger The effects of inhaled exogenous NO are currently being investigated in large clinical trials in treatment of patients with pulmonary hypertension and/or adult respiratory distress syndrome, and inhaled NO has presently found its clinical use in newborn children with persistent pulmonary hypertension (Davidson *et al.,* 1998). Several investigators have found clear effects on arterial oxygenation and pulmonary arterial pressure using concentrations of inhaled NO as low as 10–100 ppb (Puybasset *et al.,* 1994; Gerlach *et al.,* 1993). Interestingly, during normal breathing endogenous NO is inhaled at concentrations (~100 ppb) which are known to have vasodilating effects in the pulmonary circulation (Lundberg *et al.,* 1994a; Gerlach *et al.,* 1994). Nasal breathing reduces pulmonary vascular resistance and improves arterial oxygenation compared to oral breathing in subjects without lung pathology (Settergren *et al.,* 1998; Lundberg *et al.,* 1995b, 1996e). Addition of 100 ppb during oral breathing mimicked the effect of nasal breathing, whereas moistened air during oral breathing had no effect (Lundberg *et al.,* 1996e). Intubated patients are deprived of the natural inhalation of endogenous upper airway NO. Autosupplementation of nasal air to intubated patients treated with a ventilator also improves arterial oxygenation and reduces pulmonary vascular resistance (Lundberg *et al.,* 1995b) (Fig. 3). Moreover, Pinsky and co-workers have shown that hospital pressurized air may contain NO levels similar to those described earlier (6–500 ppb), which may consequently have effects on arterial oxygenation and pulmonary arterial pressure in mechanically ventilated patients (Lee *et al.,* 1997; Pinsky *et al.,* 1997). In another study, nostril widening by a nasal tape, improved arterial oxygenation in

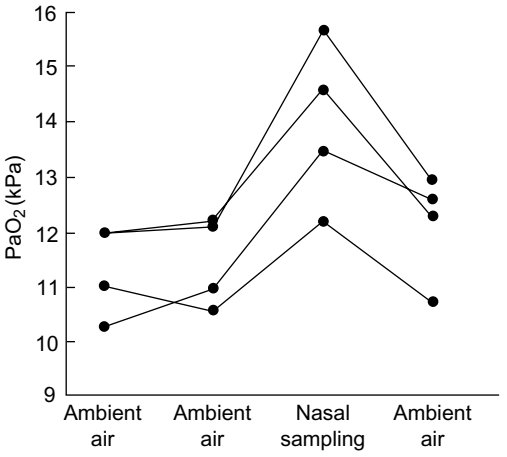


Figure 3 Arterial oxygen tension (PaO_2) in four intubated patients. Air was aspirated (2 liters/min) in 30-min periods from either ambient NO-free air (Ambient air) or from the nostril of the patient (Nasal sampling). The aspirated air was added to the inspiratory flow of the ventilator, and arterial oxygen tension was determined at the end of each sampling period. From Lundberg *et al.* (1995b) with permission.

spontaneously breathing patients, possibly by enhancing ventilation through the nasal airways thereby increasing the delivery of NO from the nasal airways to the lungs (Herulf *et al.,* 1999a).

These results show that NO derived from the upper airways is capable of improving oxygen uptake and reducing pulmonary vascular resistance. It is tempting to speculate that the marked production of NO in the nasal airways has the purpose of modulating lung function in humans. Because NO is produced proximally in the ariways, this vasodilating gas will only affect pulmonary vessels in contact with ventilated alveoli, thereby improving *V/Q* matching. This new physiological principle of NO as an airborne mediator may extend beyond vasodilation. Further studies will reveal if inhalation of endogenous NO will also have antimicrobial effects, upregulate ciliary motility, inhibit platelet aggregation, and downregulate inflammatory mediators in the lungs. If this is true the well-known complications associated with long-term intubation and ventilation, such as ciliary dysfunction and bacterial infections, may partly be explained by the lack of NO due to disruption of the natural low-dose flushing of the airways by self-inhalation of endogenous NO from the upper airways. In that case, a supplementation with physiological levels of NO to intubated patients would be beneficial.

Clinical Value of Nasal NO Measurements

Measurements of nasal NO are noninvasive and can easily be performed even in small children. The finding of low nasal NO in patients with chronic sinus disorders such as Kartagener's syndrome (Lundberg *et al.,* 1994a) and cystic fibrosis (Lundberg *et al.,* 1996a) are interesting. Nasal NO measurements may be used to facilitate early diagnosis of these two respiratory disorders.

The possible usefulness of nasal NO measurements in the diagnosis and therapeutic monitoring of allergic rhinitis clearly needs to be further evaluated. If nasal NO is a reliable marker of local inflammation, this simple test may be used to monitor patients with rhinitis of different etiology.

The clinical relevance of the fact that intubated patients are deprived of the autoinhalation of NO produced in the nasal airways is not clear. However, when considering the possible beneficial effects of nasal NO on pulmonary function, it is possible that a supplementation with NO at endogenous levels would be of benefit to these patients. One way of achieving this is to collect NO-containing air from the upper airways of the patient and to reintroduce this air to the inspiratory limb of the ventilator (Lundberg *et al.,* 1995b).

In light of the great impact NO has on various physiological as well as pathophysiological events, we now see drugs that effect endogenous NO synthesis emerging as candidates for clinical use. These drugs may either facilitate or decrease NO production, for example, by interfering with the activity or expression of the different forms of NOS. Measurement of nasal NO could be an interesting noninvasive means of monitoring the effects of such drugs in order to optimize the dosage. The nonselective NOS inhibitor L-NMMA is currently being investigated for use in patients with sepsis. Interestingly, this inhibitor, when administered intravenously, leads to a dose-dependent decrease in nasal NO (Albert *et al.,* 1997). Conversely, systemic administration of L-arginine increases nasal NO (Lundberg *et al.,* 1996b).

Lower Airways

Measurements of Exhaled NO

Orally exhaled NO can be measured either during single breath exhalations or during continuous breathing (Kharitonov *et al.,* 1997a). Rapid registration of exhaled NO levels during the course of a single exhalation allows fractioning of a breath into early and late phases. For single breath measurements, a subject takes a deep breath of NO-free air and exhales into a system of appropriate tubing (e.g., Teflon) from which an air fraction is continuously sampled, and NO is measured in the sample (Lundberg *et al.,* 1996c; Kharitonov *et al.,* 1997a; ATS Taskforce, 1999). The velocity of the exhalation will influence the concentration of NO in exhaled air and also the amount excreted from the respiratory tract (Lundberg *et al.,* 1996c; Silkoff *et al.,* 1997). Owing to the flow dependency of NO in exhaled air, it is vital to carefully monitor the exhalation flow rate when measuring NO. One further consideration regarding orally exhaled NO is the possible contamination of NO from the upper airways. It has been suggested that this unwanted addition of NO when one is studying the lower airways may be avoided by applying a positive mouth pressure during exhalation, thereby closing the soft palate (Kimberly *et al.,* 1996; Silkoff *et al.,* 1997). In urban areas, ambient NO levels may sometimes exceed 100 ppb, and in these situations a closed system is preferable, in which NO-free air is inhaled from a reservoir (Alving *et al.,* 1993). Also, the oral cavity is a source of NO which

may affect exhaled NO levels (Duncan *et al.,* 1995; Zetterquist *et al.,* 1999).

NO may also be measured during normal tidal breathing. This method is suitable for continuous measurements of NO over longer periods of time, for example, to study the effect of physical exercise on exhaled NO levels (Lundberg *et al.,* 1996c) and in the intensive care setting. This method has also been suggested when measuring in small children who may have problems in complying with single exhalation maneuvers. As with the single-breath measurements, one must also bear in mind the influence of flow rate on NO concentrations when using this method. As with nasal NO measurements, recommendations for suitable sampling techniques have been proposed (Kharitonov *et al.,* 1997a; ATS Taskforce, 1999).

NOS Isoforms in the Airways

It is likely that most of the NO found in exhaled air is endogenously produced by NOSs present in airway mucosal cells. In a number of studies, inhaled NOS inhibitors have markedly reduced airway excretion of NO (Gustafsson *et al.,* 1991; Lundberg *et al.,* 1995a; Yates *et al.,* 1995). However, a bacterial contribution to luminal NO cannot be entirely excluded, because some strains of bacteria are known to produce NO from nitrite (Ji and Hollocher, 1989). Furthermore, studies indicate that NO may be chemically formed in the oral cavity by reduction of salivary nitrite at the surface of the tongue (Duncan *et al.,* 1995; Zetterquist *et al.,* 1999).

All three known isoforms of NOS have been identified in human airway mucosa. Endothelial NOS (eNOS) is found in mucosal blood vessels and also in cultured human bronchial epithelium (Shaul *et al.,* 1994). Neuronal NOS (nNOS) has been identified in bronchial epithelial cells (Asano *et al.,* 1994) as well as in certain mucosal nerves. iNOS may be expressed in bronchial epithelial cells, for example, during inflammation (Hamid *et al.,* 1993) or *in vitro* after stimulation with certain cytokines (Asano *et al.,* 1994). However, there has been confusion in the literature as to whether this NOS isoform is also present in healthy bronchial epithelium. Hamid *et al.* (1993) and Asano *et al.* (1994) found no evidence of iNOS expression in healthy bronchial epithelium, whereas Guo *et al.* (1995) reported iNOS expression also in bronchial mucosa of healthy subjects. As discussed earlier, however, it seems quite clear that in certain areas of the upper airways iNOS is constitutively expressed (Lundberg *et al.,* 1995a).

Factors Influencing Exhaled NO

Today we know a little about the various physiological and pathological factors that influence levels of NO in exhaled air, although much still remains to be studied. In the following we will discuss some of these factors.

Physiological Factors No studies have shown that body posture has any influence on orally exhaled NO levels. In healthy men and children, intraindividual changes in orally exhaled NO over time seem to be minor, provided that the measurements are performed in the same way (Lundberg *et al.,* 1994a, 1996c). In menstruating women variations of exhaled NO in synchrony with the menstrual cycle have been reported (Kharitonov *et al.,* 1994). Thus, exhaled NO peaked at midcycle during ovulation, whereas it dropped during menstruations. Exhaled NO levels have been reported to remain fairly constant during the course of a normal pregnancy (Grunewald *et al.,* 1998; Morris *et al.,* 1995).

Several groups have investigated whether orally exhaled NO levels are affected by physical exercise (Trolin *et al.,* 1994; Iwamoto *et al.,* 1994; Persson *et al.,* 1993). The results are similar; absolute concentrations of exhaled NO decrease during exercise, but since the ventilation increases greatly, the calculated amounts of excreted NO are increased.

The amount of NO exhaled by cigarette smokers has been observed to be lower than normal (Gerlach *et al.,* 1994; Persson *et al.,* 1993). The reason for this is not clear, but it could be due to a downregulation of endogenous NO synthesis because of the high NO content in cigarette smoke (Norman and Keith, 1965), damage to NO-producing cells by toxic agents in smoke, or increased trapping of NO in the airway fluid line. The presence of NO in cigarette smoke also explains its vasodilatory effects on the pulmonary and bronchial circulation (Alving *et al.,* 1993). Because basal NO release from the lower airways is very low, as discussed earlier, it is likely that this downregulation occurs in the upper parts of the airways. Indeed, Gerlach *et al.* (1994) reported lower nasopharyngeal NO levels in smokers than in nonsmokers.

It has been shown that intake of nitrate, which is present, for example, in green leafy vegetables, increases NO in orally exhaled air significantly (Zetterquist *et al.,* 1999). This is probably due to increased production of NO in the oral cavity. Nitrate is actively secreted into the saliva (Spiegelhalder *et al.,* 1976), and reduction of salivary nitrate/nitrite by bacteria on the base of the tongue leads to NO generation. Zetterquist *et al.* (1999) could reduce this increment in exhaled NO after nitrate with an antibacterial mouthwash.

Asthma and Exhaled NO Inflammation in general has been reported to be associated with enhanced production of NO, and NO has been implicated in the pathogenesis of certain inflammatory diseases (Lundberg *et al.,* 1997a; Nussler and Billiar, 1993). In 1993, Alving *et al.* reported increased NO levels in orally exhaled air of asthmatics. These findings have since been confirmed by numerous research groups (for review, see Lundberg *et al.,* 1996c; Kharitonov *et al.,* 1997a; ATS Taskforce, 1999). Hamid *et al.* (1993) reported expression of iNOS in asthmatic bronchial epithelium but not in control bronchial tissue from healthy subjects. This may be one explanation for the elevation of the orally exhaled NO levels in asthmatic patients. However, other sources such as macrophages, mast cells, nerve cells, or other NO-generating cells in the inflamed mucosa cannot be excluded. Glucocorticoids are known to inhibit the expression of iNOS (Nathan and Xie, 1994). Therefore, one would expect that exhaled

NO levels would decrease in patients on treatment with such drugs. Indeed, treatment with inhaled steroids leads to a reduction in exhaled NO in asthmatics (Yates *et al.,* 1995). In asthmatic children a dose dependency has been observed; those treated with no or low to moderate doses of topical steroids showed increased exhaled NO, whereas those treated with the highest doses of steroids showed exhaled NO levels that did not differ from controls (Lundberg *et al.,* 1996a). Numerous studies have shown a dose-dependent reduction in exhaled NO with steroid treatment and correlation between other markers of airway inflammation such as eosinophilia in induced sputum and bronchoalveolar lavage fluid (Jatakanon *et al.,* 1998; Piacentini *et al.,* 1999). Also, newer anti-inflammatory agents used in asthma such as leuktriene inhibitors may reduce exhaled NO levels (Bisgaard *et al.,* 1999), whereas inhaled B agonists have only minor effects (Yates *et al.,* 1997; Fuglsang *et al.,* 1998) (Table I). Kharitonov *et al.* (1995a) reported further elevation of exhaled NO during the late-phase reaction of asthmatics. Measurements were made after antigen challenge, and only dual responders showed elevated exhaled NO levels. This indicates that the late-phase reaction increase in NO levels is due to an inflammation-driven increase in iNOS expression.

Altogether, many studies on exhaled NO in asthmatics have now been performed, and it is likely that the expression of an iNOS in asthmatic airways is responsible for the elevation in exhaled NO levels seen in these patients. Asthma therapy today is very much focused on treatment of the underlying inflammatory disease, and there is a great need for an objective marker of airway inflammation. It is tempting to speculate on the possible usefulness of NO in exhaled air as a marker of airway inflammation; these measurements are easy to perform, are noninvasive and objective, and rapidly provide information about a local process. However, more studies are needed to investigate the potential of this method in the diagnosis and monitoring of airway inflammation.

Other Diseases of the Respiratory Tract Asthma is not the only airway disease associated with alterations in exhaled NO. Alving *et al.* (1993a) reported transient elevations of orally exhaled NO in subjects with lower respiratory tract infection, and Kharitonov *et al.* (1995b) reported increased NO levels during the symptomatic period in subjects with upper airway infection. Increased NO levels have also been reported in patients with bronchiectasis (Kharitonov *et al.,* 1995c). In patients with chronic obstructive pulmonary disease, exhaled NO is increased during periods of exacerbation (Agusti *et al.,* 1999; Corradi *et al.,* 1999; Clini *et al.,* 1998). In lung transplant recipients exhaled NO levels have been reported to increase with signs of pulmonary graft dysfunction (Marczin *et al.,* 1997a; Silkoff *et al.,* 1998). Hence, both inflammation and infection in the airways may be associated with enhanced airway production of NO. This is not surprising because the known factors that induce expression of iNOS (e.g., certain proinflammatory cytokines and bacterial products) may be present in increased amounts in inflamed/infected tissues.

Table I Effects of Some Antiasthmatic Drugs on Exhaled NO in Asthmatics

Drug	Administration	Exhaled NO
Corticosteroids	Topical, intravenous	Decrease
β_2 adrenoceptor agonists	Topical	Minor increase
Leukotriene antagonists	Perorally	Decrease

Drugs Only little is known about the effects of drugs on NO excretion in the airways. However, some agents that are known to interfere with airway NO release will be discussed later.

Glucocorticoids have been mentioned earlier; these agents depress already increased exhaled NO levels in, for example, asthmatics. However, steroids do not influence basal exhaled NO levels in healthy individuals (Lundberg *et al.,* 1994a, 1996b). Yates *et al.* (1995) reported decreased orally exhaled NO levels in controls and in asthmatics following oral inhalation of an NOS inhibitor. In addition, the inhaled selective iNOS inhibitor aminoguanidine reduces exhaled NO in asthmatics (Yates *et al.,* 1996). L-Arginine, the substrate for enzymatic NO synthesis, has been shown to increase exhaled NO in a dose-dependent matter when taken orally (Kharitonov *et al.,* 1995d) or infused intravenously (Lundberg *et al.,* 1996b).

Persson *et al.* (1994a) reported decreased orally exhaled NO levels following ingestion of ethanol. Nitrovasodilators such as nitroglycerine act through the release of NO, and increased exhaled NO levels have been observed in experimental animals following i.v. administration of this drug (Persson *et al.,* 1994b). However, in humans data are conflicting as to whether NO-donating drugs may affect exhaled NO levels (Marczin *et al.,* 1997b; Dirnberger *et al.,* 1998).

Nitric Oxide and Airway Inflammation

NO produced by iNOS has also been implicated in the pathogenesis of inflammation (Nussler and Billiar, 1993), and NO synthesis is clearly enhanced locally at sites of inflammation (Lundberg *et al.,* 1997a), although the role of NO in the pathogenesis of inflammation is far from settled. Some studies indicate a harmful role of NO during inflammation, whereas others indicate the opposite. Possible proinflammatory actions of NO include activation of enzymes such as cyclooxygenase (Salvemini *et al.,* 1993) or metalloproteases (Murell *et al.,* 1995). Moreover, peroxynitrite, formed from the reaction of NO with superoxide, can exert toxic effects on tissues (Sato *et al.,* 1998; Beckman *et al.,* 1990). NO has also been implicated in the pathogenesis of pertussis, as NOS inhibitors dramatically attenuated the epithelial damaging effects of a cytotoxin that is released by *Bordetella pertussis* (Heiss *et al.,* 1994). The possible harmful effects of NO have been attributed to the large amounts

of this gas produced by iNOS during inflammation. However, the finding of a constantly expressed iNOS in the nasal airways (Lundberg *et al.,* 1995a) complicates this picture, because it clearly demonstrates that the sole expression of iNOS and the subsequent large production of NO is not associated with tissue damage. On the contrary, iNOS in the upper airways may serve important protective functions, as discussed earlier. It has also been suggested that upregulation of NO synthesis in asthma serves to counterbalance bronchoconstrictor stimuli (Gaston *et al.,* 1994).

Gastrointestinal Tract

Stomach

NONENZYMATIC INTRAGASTRIC NO PRODUCTION

Human saliva contains both nitrate and nitrite (Tannenbaum *et al.,* 1976). Dietary nitrate, derived mainly from green leafy vegetables, is absorbed in the gastrointestinal tract. For largely unknown reasons, up to 25% of circulating nitrate is actively taken up by the salivary glands and secreted in saliva (Spiegelhalder *et al.,* 1976). Bacteria in the oral cavity reduce parts of the salivary nitrate to nitrite (Bartsch *et al.,* 1988). Further reduction of nitrite to nitric oxide and other nitrogen oxides is a well-known chemical reaction that occurs in acidic solutions. It was shown that NO is formed in the stomach following reduction of salivary-derived nitrite (Lundberg *et al.,* 1994b; Benjamin *et al.,* 1994; McKnight *et al.,* 1997). Intragastric NO production from nitrite is dependent on a reducing/acidic environment, as stated earlier. Indeed, when acid production was blocked in the stomach with large doses of the proton-pump inhibitor omeprazole, NO production was almost completely abolished (Lundberg *et al.,* 1994b). Furthermore, *in vitro* studies using nitrite solutions at different pH showed a clear correlation between NO release and pH (Lundberg *et al.,* 1994b). The daily saliva production may exceed 1 liter, which ensures a continuous NO production in the acidic stomach. McKnight *et al.* (1997) reported mean values of about 14 ppm in the fasting stomach, and these values rose to about 90 ppm following ingestion of nitrate. In this and an earlier study (Lundberg *et al.,* 1994b), no attempts were made to calculate the actual production rate of NO in the stomach. Such estimations will be of help when further evaluating the possible biological role of NO in the stomach. Intragastric NO production is already initiated in the oral cavity by the reduction of nitrate to nitrite. Thus, commensal bacteria inhabiting the oral cavity produce the necessary substrate for the NO production that takes place in the stomach when nitrate-containing saliva is swallowed. This seems to be yet another example of symbiosis between organisms in nature.

The accumulation of nitrate in saliva seems to be part of a recycling of NO produced either nonenzymatically or by NOSs from L-arginine (Fig. 4). NO produced from L-arginine, for example, in endothelial cells, is converted to nitrate following reaction with hemoglobin in the blood (Moncada *et al.,* 1991). In fasting humans the L-arginine–NO pathway

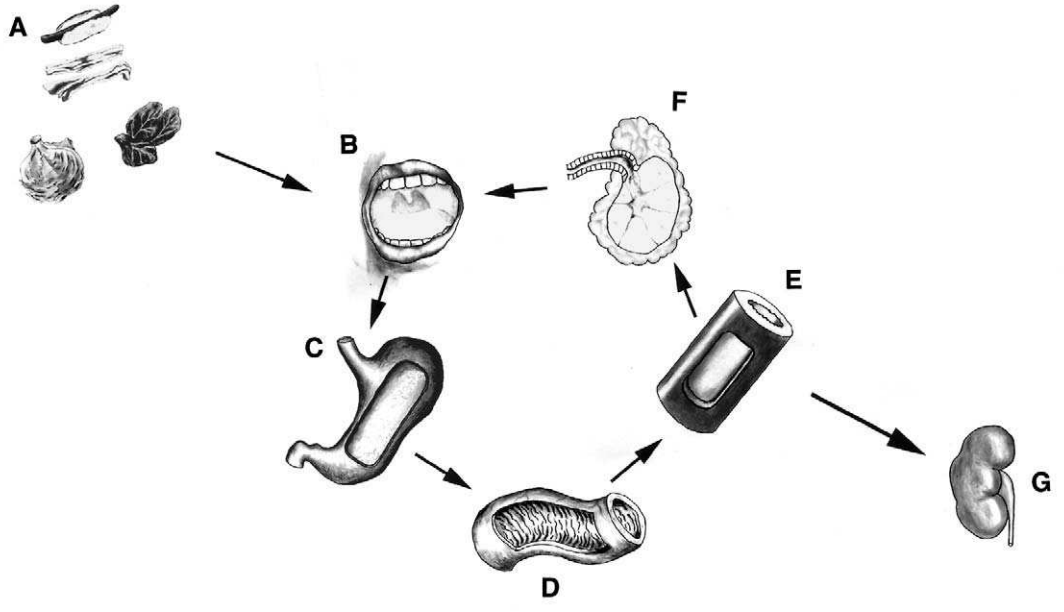


Figure 4 Intragastric generation of nitric oxide from nitrate and nitrite. (A) Dietary sources of nitrate and nitrite include green leafy vegetables and processed meat. (B) Nitrate is partly reduced to nitrite by bacteria in the oral cavity. (C) Nitric oxide and other nitrogen oxides are formed from nitrite in swallowed saliva in the acidic stomach. (D) Remaining nitrate is absorbed in the gastrointestinal tract. (E) Plasma nitrate originates from dietary sources as well as from oxidation of endogenously produced NO from the L-arginine/NOS pathway. (F) About 25% of plasma nitrate is actively taken up by the salivary glands and excreted in saliva. (G) A large portion of circulating nitrate is excreted in urine.

constitutes a major source of nitrate/nitrite levels in plasma (Rhodes *et al.,* 1995), and net nitrate synthesis ranges between 0.1 and 2 mmoles per day (Green *et al.,* 1981). In addition, similar amounts of nitrate are normally ingested (Forman *et al.,* 1985). Nitrate is taken up from the plasma by the salivary glands, and parts of it may eventually turn up as NO again in the stomach as described earlier. Because the substrate for intragastric NO production is derived not only from dietary sources, this production may occur even when intake of nitrate/nitrite is low.

Possible Role of NO in the Stomach

NO is a tiny lipophilic molecule that easily travels across biological membranes. At a concentration of 20–100 ppb, NO gas has been shown to cause vasodilation (Lundberg *et al.,* 1995b) and to inhibit the growth of certain bacteria (Mancinelli and McKay, 1985). Because intragastric NO levels are up to 4000 times higher (Lundberg *et al.,* 1994b; McKnight *et al.,* 1997), it is likely that locally produced NO has biological effects in the stomach. Indeed, it was shown that nitrite, when acidified, killed various gut pathogens much more effectively than did acid alone (Benjamin *et al.,* 1994). Interestingly, *Helicobacter pylori,* the bacterium involved in the pathogenesis of ulcer disease, is also sensitive to acidified nitrite (Dykhuizen *et al.,* 1998). It should be kept in mind that other nitrogen intermediates such as nitrogen dioxide (NO_2) and nitrous acid (HNO_2) are also produced from acidified nitrite, and these compounds may have bacteriostatic effects as well (Kaplan *et al.,* 1996; Kono *et al.,* 1994). Thus, NO may not account for all effects observed in the cited studies.

Intragastric NO may also be involved in regulation of mucosal blood flow and mucus formation. Indeed, it has been shown that instillation of an NO donor agent into the gastric lumen produces an increase in mucus formation that is inhibited by coadministration of oxyhemoglobin (an NO scavenger), suggesting that the effect was mediated by local NO release (Brown *et al.,* 1992). Furthermore, topical administration of nitrite to the gastric mucosa increases mucosal blood volume (Kitagawa *et al.,* 1990). An adequate blood flow is essential if the gastric mucosa is to withstand the challenge of both exogenous and endogenous aggressors, and local ischemia is an important mechanism underlying various forms of gastric damage (Whittle, 1993). As mentioned earlier, intragastric NO production is strongly pH-dependent, with more NO being produced at lower pH (Lundberg *et al.,* 1994b). Thus, NO formation in the stomach is enhanced in response to challenge with acid. In this way NO may help to counteract potential acid-induced damage by increasing superficial mucosal blood flow.

There has been concern that dietary nitrates/nitrites may have deleterious effects (Tannenbaum *et al.,* 1974; Bartsch *et al.,* 1988). This concern is not primarily related to the nitrate ion but to the fact that nitrate-reducing bacteria in the oral cavity reduce nitrate to nitrite. In the stomach, nitrogen oxides formed from nitrite may react with amines, resulting in the formation of harmful N-nitroso compounds (NOCs), a versatile class of carcinogens in animals (Tanneburm *et al.,* 1974; Bartsch *et al.,* 1988). However, results from epidemiological studies in humans are conflicting. In fact, a study by Forman *et al.* (1988) showed a negative correlation between salivary nitrate/nitrite concentrations and the risk of developing gastric cancer. The reason for this was not clear, but it was speculated that the high ascorbate content in nitrate-rich vegetables could have a protecting effect. Indeed, epidemiological studies have clearly shown that compounds known to inhibit NOC formation also have a protective effect against cancers, in particular gastric cancer (Bartsch *et al.,* 1988). Interestingly, a great variety of agents (including vitamin C) that inhibit NOC formation do so by reducing the nitrosating agent to NO, which is not a nitrosating agent (Bartsch *et al.,* 1988; Licht *et al.* 1988).

Oral Cavity

A similar mechanism of NO production from acidified nitrite has also been described in the oral cavity (Duncan *et al.,* 1995; Zetterquist *et al.,* 1999), even though less NO is being produced during the day, most likely owing to a higher pH in the mouth compared to the stomach. Nevertheless, it has also been suggested that NO may play a role in host defense in the mouth (Duncan *et al.,* 1995). NO production from nitrite in the oral cavity may interfere with measurements of exhaled NO (Zetterquist *et al.,* 1999). This is further discussed earlier in the airway section.

Intestines

Nitric oxide is present in the lumen of the small and large intestines in humans (Lundberg *et al.,* 1994c; Everts *et al.,* 1999). The levels in healthy individuals vary between 30 and 200 ppb, as measured with chemiluminescence during colonoscopy (Lundberg *et al.,* 1994c). The exact origin of the luminal NO in healthy subjects is not known. NO may be constitutively generated in the mucosa, submucosa, and muscle from neural, endothelial, and epithelial cells (Salzmann, 1995; Whittle, 1994). It is, however, likely that the luminal NO is derived from the nearby epithelium, in which both constitutive and inducible NOS have been found (Salzmann, 1995; Whittle, 1994). An additional source of nitrogen oxides from the gastrointestinal tract arises from the production of NO by luminal anaerobic microorganisms that use nitrate and nitrite for their respiration (Whittle, 1994). However, luminal NO, measured in the rectum in humans, is unaltered with or without the presence of fecal matter (Herulf *et al.,* 1998), indicating only a minor contribution of NO from bacterial reduction of nitrate/nitrite. NO may play a role in different physiological events in the gastrointestinal tract. It participates in regulation of perfusion to match the demand required for digestion, tissue oxygenation, and mucosal integrity. The release of NO from the nonadrenergic–noncholinergic (NANC) enteric nervous system plays a critical role in coordination of bowel motility (Whittle, 1994). It has also been suggested that NO has a role in fluid and electrolyte transport in the gut (Salzmann, 1995).

INFLAMMATION

In inflammatory bowel diseases (IBD) there is an increased production of NO. This was first described by Middleton *et al.* in 1993, who reported increased citrulline relative to other amino acids as indirect evidence of increased NO synthesis in rectal biopsy specimens from patients with active ulcerative colitis. Later the same year, Boughton-Smith *et al.* (1993) could demonstrate an eightfold increase in NOS activity in mucosal specimens from inflamed areas taken from six patients with ulcerative colitis. Singer *et al.* (1996) examined the cellular distribution of iNOS in normal bowel, ulcerative colitis, and Crohn's disease using immunoperoxidase staining with a monospecific human iNOS antibody. Intense focal iNOS labeling has been localized to inflamed colonic epithelium in ulcerative colitis and Crohn's disease but negative in controls (Singer *et al.,* 1996; Kimura *et al.,* 1998; Ikeda *et al.,* 1997). Also, in celiac disease increased iNOS activity has been described (Beckett *et al.,* 1998). However, in contrast to the localization of increased iNOS to the epithelial cells in ulcerative colitis, iNOS activity in celiac disease was primarily increased in the lamina propria (Beckett *et al.,* 1998). Forte *et al.* (1999) reported increased urinary output of the isotope ^{15}N in patients with acute infective gastroenteritis on administration of L-[^{15}N]arginine indicating an increased activity of the L-arginine–NO pathway in this disease. In 1994 Lundberg *et al.* reported results from analysis of aspirated luminal gas obtained during colonoscopy in patients with ulcerative colitis (Lundberg *et al.,* 1994c). The gas was obtained from different parts of the colon using a thin catheter and a syringe, and it was subsequently injected into a chemiluminescence analyzer. Six patients with active ulcerative colitis and 12 controls were examined; luminal NO concentrations were more than 100-fold higher in gas samples from patients than in gas from controls, and there was no overlap between the groups (Fig. 5). Also, in Crohn's disease of the colon, luminal NO levels are very high (Lundberg *et al.,* 1995c). The same group has developed a minimally invasive technique to measure luminal NO. In this procedure, a balloon-tipped catheter is inserted in the rectum of humans, and then the balloon is inflated with NO-free air, after which luminal NO is allowed to diffuse into the balloon. With this technique, patients with active ulcerative colitis and active Crohn's disease had values 10- to 100-fold higher than did healthy controls or patients with inactive disease (Herulf *et al.,* 1998) (Fig. 5). As mentioned earlier, the presence of intestinal fecal matter did not lead to higher values in that study. Also, in two other chronic inflammatory bowel diseases, collagenous and lymphocytic colitis, luminal NO levels are greatly increased (Lundberg *et al.,* 1997b) (Fig. 5). It was also shown that rectal NO levels are markedly increased in acute infective gastroenteritis (Herulf *et al.,* 1999b). Everts *et al.* (1999) reported elevated luminal NO in the small intestine in patients with untreated celiac disease. We measured rectal NO in patients with treated celiac disease after local challenge with gluten in the rectum (E. Weitzberg and J. O. N. Lundberg, 2000, unpublished observation). NO increased dramatically 24 hours after challenge in about 60% of the patients, whereas no response was seen in the rest of the patients. The reason for this discrepancy is presently not known.

The role of NO in inflammatory bowel diseases is far from settled. As discussed earlier, NO may have potential harmful as well as beneficial effects. There are conflicting data in the literature when using NOS inhibitors (Perner and Rask-Madsen, 1998). McCafferty *et al.* (1997) have shown that mice lacking the iNOS gene develop a much more severe inflammation compared to wild-type controls, indicating a protective role of iNOS in bowel inflammation.

Urinary Tract

NO MEASUREMENTS IN THE BLADDER

Unlike the airways and the intestines, the urinary bladder is normally not an air/gas-containing organ. To measure luminal NO in the bladder, a method has been used in which

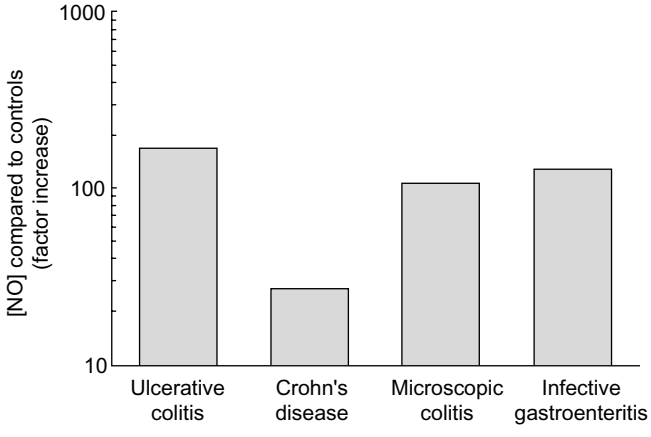


Figure 5 Luminal NO levels in patients with various intestinal inflammatory disorders compared to healthy controls. Gas was sampled from the colon or rectum. Data from Lundberg *et al.* (1994c, 1997b) and Herulf *et al.* (1998, 1999b).

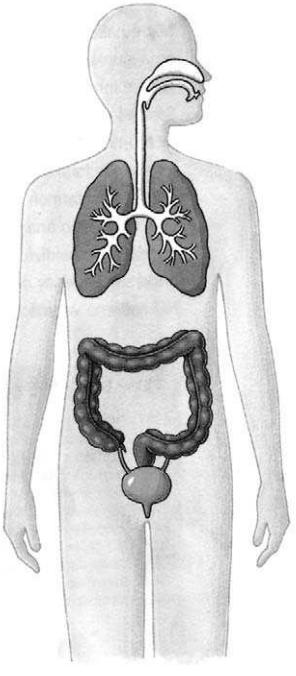
NO-free air was introduced into the bladder either during cystoscopy or through a urinary catheter (Lundberg *et al.,* 1996f). After a short incubation period, the air was removed and NO was measured with chemiluminescence technique as described earlier. This method has been further developed using the same technique as for rectal NO measurements. A silicon catheter with an inflatable balloon is introduced into the bladder, and the balloon is inflated with NO-free air, incubated for 5 to 10 min (allowing for NO to diffuse into the balloon), and then measured as described earlier. Using the previously described method, it has been shown that luminal NO in the urinary bladder is increased in patients with cystitis of different etiology (Ehren *et al.,* 1999a). The cellular source of this NO has not been pinpointed.

THE ROLE OF NO

The role of NO in cystitis is not clear. NO or subsequent reaction products may be cytotoxic to host cells when produced in excess as discussed earlier. On the other hand, increased NO production might enhance local host defense mechanisms in the bladder, keeping in mind the well-known bacteriostatic, antiviral, and antitumor properties of this gas. Thus, NOS expression in the urinary bladder mucosa may be induced in response to, for example, bacterial endotoxins. It will be of interest to study if the various pathogens that cause cystitis differ in their capacity to induce mucosal NO synthesis. Local instillation of Bacillus Calmette-Guérin (BCG) into the bladder is frequently used in the treatment of superficial recurrent bladder cancer. The mechanism of action is not fully understood, but it is thought to be related to a local inflammatory response involving upregulation of cytokines (Jackson *et al.,* 1995), many of which may promote iNOS expression. Interestingly, patients treated with BCG showed a marked increase in bladder NO production, suggesting that NO may contribute to the antitumor effects of this treatment (Lundberg *et al.,* 1996f). A study by Jansson *et al.* (1998) showed that NO donor agents caused inhibition of bladder tumor cell growth, suggesting an antitumor effect of NO in this model. The same may be true for irradiation therapy in cancer treatment. Thus, bladder concentrations of NO were also greatly increased in patients with irradiation cystitis (Lundberg *et al.,* 1996f).

CLINICAL USE OF BLADDER NO MEASUREMENTS

Lower urinary tract symptoms, for example, urgency, frequency, nocturia, discomfort, and pain, are common among patients referred to the urologist for evaluation. These symptoms may be due to inflammatory or noninflammatory functional disorders, and bladder biopsies are often required to distinguish between the two groups of patients. It is tempting to speculate on the possible usefulness of bladder NO measurements in the diagnosis and monitoring of cystitis. Measurements of NO in the bladder may be a simple way to detect inflammation in the lower urinary tract. A study by Ehren *et al.* (1999b) supports this notion. They looked at bladder NO levels in patients with urgency symptoms due to various noninflammatory disorders and compared the levels with healthy controls and patients with interstitial cystitis, an inflammatory disorder of the bladder with very similar symptoms. Interestingly, only the patients with interstitial cystitis had high NO levels (about 18-fold increase), whereas the other patient groups did not differ from controls. Thus,



Upper airways	
Inflammatory sinus disease	Decreased
Allergic rhinitis	Increased
Lower airways	
Asthma	Increased
Large intestine	
Inflammatory bowel disease	Greatly increased
Infective gastroenteritis	Greatly increased
Urinary bladder	
Cystitis	Greatly increased

Figure 6 Overview describing various locations where airborne NO has been measured in health and disease.

luminal NO seems to be a marker of ongoing inflammation in the bladder. It will be of interest to study whether NO excretion correlates to the severity of inflammation; however, this will require larger groups of patients.

Summary and Conclusion

NO produced in superficial layers of the mucosa may be detected in luminal structures such as the airways, the gastrointestinal tract, and the urinary tract by measuring NO content in gas collected from these hollow organs. This allows for direct on-line measurements of NO generation in humans. Measurements of airborne NO may be a helpful tool in further understanding the role of NO in health and disease.

A large production of NO normally takes place in the nasal airways and in the stomach of healthy individuals. The exact role of this NO is not known but may include host defense actions as well as regulation of blood flow.

Mucosal inflammation in diseases such as asthma, inflammatory bowel disease, and cystitis leads to greatly increased luminal NO levels in the affected organs (Fig. 6). Exhaled NO levels are elevated in asthma and substantial evidence now suggests NO to be a clinically useful noninvasive marker of airway inflammation. NO measurements may be a valuable tool in evaluating effects of anti-inflammatory treatment. Furthermore, we will likely witness new therapies emerging that are focused on pharmacological intervention of endogenous NO production in various diseases. In these situations NO measurements may prove to be helpful.

References

Agusti, A. G., Villaverde, J. M., Togores, B., and Bosch, M. (1999). Serial measurements of exhaled nitric oxide during exacerbations of chronic obstructive pulmonary disease. *Eur. Respir. J.* **14,** 523–528.

Albert, J., Schedin, U., Lindqvist, M., *et al.* (1997). Blockade of endogenous nitric oxide production results in moderate hypertension, reducing sympathetic activity and shortening bleeding time in healthy volunteers. *Acta Anaesthesiol. Scand.* **41,** 1104–1113.

Alving, K., Weitzberg, E., and Lundberg, J. M. (1993a). Increased amounts of nitric oxide in exhaled air of asthmatics. *Eur. Respir. J.* **6,** 1368–1370.

Alving, K., Fornhem, C., and Lundberg, J. M. (1993b). Pulmonary effects of endogenous and exogenous nitric oxide in the pig: Relation to cigarette smoke inhalation. *Br. J. Pharmacol.* **110,** 739–746.

Archer, S. (1993). Measurement of nitric oxide in biological models. *FASEB J.* **7,** 349–360.

Arnal, J., Didier, A., Rami, J., *et al.* (1997). Nasal nitric oxide is increased in allergic rhinitis. *Clin. Exp. Allergy* **27,** 347–350.

Arnal, J. F., Flores, P., Rami, J., *et al.* (1999). Nasal nitric oxide in paranasal sinus inflammatory disease. *Eur. Respir. J.* **13,** 307–312.

Asano, K., Chee, C. B. E., Gaston, B., Lilly, C. M., Gerhard, C., Drazen, J. M., and Stamler, J. S. (1994). Constitutive and inducible nitric oxide synthase gene expression regulation and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 10089–10093.

ATS Taskforce (1999). Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children. *Am. J. Respir. Crit. Care Med.* **160,** 2104–2117.

Balfour-Lynn, I., Laverty, A., and Dinwiddle, R. (1996). Reduced upper airway nitric oxide in cystic fibrosis. *Arch. Dis. Child.* **75,** 319–322.

Baraldi, E., Azzolin, N., Biban, P., *et al.* (1997). Effect of antibiotic therapy on nasal nitric oxide concentration in children with acute sinusitis. *Am. J. Respir. Crit. Care Med.* **155,** 1680–1683.

Bartsch, H., Ohshima, H., and Pignatelli, B. (1988). Inhibitors of endogenous nitrosation: Mechanisms and implications in human cancer prevention. *Mutat. Res.* **202,** 307–324.

Beckett, C. G., Dell'Olio, D., Ellis, H. J., Rosen-Bronson, S., and Ciclitira, P. J. (1998). The detection and localization of inducible nitric oxide synthase production in the small intestine of patients with coeliac disease. *Eur. J. Gastroenterol. Hepatol.* **10,** 641–647.

Beckman, J. S., Beckman, T. W., Chen, J., *et al.* (1990). Apparent hydroxyl radical production by peroxinitrite: Implications for endothelial cell injury from nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1620–1624.

Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., and Golden, M. (1994). Stomach NO synthesis. *Nature* **368,** 502.

Bisgaard, H., Loland, L., and Anhoj, J. (1999). NO in exhaled air of asthmatic children is reduced by the leuktriene receptor antagonist montelukast. *Am. J. Respir. Crit. Care Med.* **160,** 1227–1231.

Body, S. C., Hartigan, P. M., Shernan, S. K., Formanek, V., and Hurford, W. E. (1995). Nitric oxide: Delivery, measurement, and clinical application. *J. Cardiothoracic Vasc. Anesth.* **9,** 748–763.

Boughton-Smith, N. K., Evans, S. M., Hawkey, C. I., Cole, A. T., Balsitis, M., Whittle, B. J. R., and Moncada, S. (1993). Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet* **342,** 338–340.

Brown, J. F., Hanson, P. J., and Whittle, B. J. R. (1992). Nitric oxide donors increase mucus gel thickness in rat stomach. *Eur. J. Pharmacol.* **223,** 103–104.

Clini, E., Bianchi, L., Pagani, M., and Ambrosino, N. (1998). Endogenous nitric oxide in patients with stable COPD: Correlates with severity of disease. *Thorax* **53,** 881–883.

Corradi, M., Majori, M., Cacciani, G. C., Consigli, G. F., de'Munari, E., and Pesci, A. (1999). Increased exhaled nitric oxide in patients with stable chronic obstructive pulmonary disease. *Thorax* **54,** 572–575.

Davidson, D., Barefield, E., Kattwinkel, J., *et al.* (1998). Inhaled nitric oxide for the early treatment of persistent pulmonary hypertension of the term new-born: A randomized, double-masked, placebo-controlled, dose-response, multicenter study. The I-NO/PPHN Study Group. *Pediatrics* **101,** 325–334.

Dirmberger, E., Lucan, H., Eichler, H. G., Kastner, J., Pernerstorfer, T., and Jilma, B. (1998). Effects of nitroglycerin and sodium nitroprusside on endexpiratory concentrations of nitric oxide in healthy humans. *Life Sci.* **62,** 103–108.

Dotsch, J., Demirakca, S., Terbrack, H., *et al.* (1996). Airway nitric oxide in asthmatic children and patients with cystic fibrosis. *Eur. Respir. J.* **9,** 2537–2540.

Dubois, A., Douglas, J., Stitt, J., *et al.* (1998). Production and absorption of nitric oxide in the nose. *J. Appl. Physiol.* **84,** 1217–1224.

Duncan, C., Douglas, H., Johnston, P., Green, S., Brogan, R., Leifert, C., Smith, L., Golden, M., and Benjamin, N. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat. Med.* **1,** 546–551.

Dykhuizen, R. S., Fraser, A., McKenzie, H., Golden, M., Leifert, C., and Benjamin, N. (1998). *Helicobacter pylori* is killed by nitrite under acidic conditions. *Gut* **42,** 334–337.

Ehren, I., Hosseini, A., Herulf, M., Lundberg, J. O. N., and Wiklund, N. P. (1999a). Measurements of luminal nitric oxide in bladder inflammation using a silicon balloon catheter—a novel minimally invasive method. *Urology* **54,** 264–267.

Ehren, I., Hosseini, A., Lundberg, J. O. N., and Wiklund, N. P. (1999b). Nitric oxide: A useful gas in the detection of lower urinary tract inflammation. *J. Urol.* **162,** 327–329.

Everts, B., Stotzer, P., Olsson, M., Kilander, A., and Pettersson, A. (1999). Increased luminal nitric oxide concentrations in the small intestine of patients with coeliac disease. *Eur. J. Clin. Invest.* **29,** 692–696.

Ferguson, E., and Eccles, R. (1997). Changes in nasal nitric oxide concentration associated with symptoms of common cold and treatment with a topical nasal decongestant. *Acta Oto-Laryngol.* **117,** 614–617.

Fontijin, A., Sabadell, A., and Ronco, R. (1970). Homogenous chemilumi-

nescent measurement of nitric oxide with ozone: Implications for continuous selective monitoring of gaseous air pollutants. *Anal. Chem.* **42,** 575–579.

Forman, D., Al-Dabbagh, S., and Doll, R. (1985). Nitrates, nitrites and gastric cancer in Great Britain. *Nature* **313,** 620–625.

Forte, P., Dykhuizen, R. S., Milne, E., McKenzie, A., Smith, C. C., and Benjamin, N. (1999). Nitric oxide synthesis in patients with infective gastroenteritis. *Gut* **45,** 355–361.

Fuglsang, G., Vikre-Joergensen, J., Agertoft, L., and Pedersen, S. (1998). Effect of salmeterol treatment on nitric oxide level in exhaled air and dose–response to terbutaline in children with mild asthma. *Pediatr. Pulmonol.* **25,** 314–321.

Furukawa, K., Harrison, D. G., Saleh, D., *et al.* (1996). Expression of nitric oxide synthase in the human nasal mucosa. *Am. J. Respir. Crit. Care Med.* **153,** 847–850.

Gaston, B., Drazen, J. M., Loscalzo, J., and Stamler, J. S. (1994). The biology of nitrogen oxides in the airways. *Am. J. Respir. Crit. Care Med.* **149,** 538–551.

Geller, D. A., Lowenstein, C. J., Shapiro, R. A., *et al.* (1993). Molecular cloning and expression of inducible nitric oxide synthase from hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3491–3495.

Gerlach, H., Rossaint, R., Pappert, D., *et al.* (1993). Time-course and dose–response of nitric oxide inhalation for systemic oxygenation and pulmonary hypertension in patients with adult respiratory distress syndrome. *Eur. J. Clin. Invest.* **23,** 499–502.

Gerlach, H., Rossaint, R., Pappert, D., *et al.* (1994). Autoinhalation of nitric oxide after endogenous synthesis in nasopharynx. *Lancet* **343,** 518–519.

Green, L. C., Luzuriaga, K. R., Wagner, D. A., *et al.* (1981). Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7764–7768.

Grunewald, C., Carlström, K., Kumelin, G., Ringqvist, Å., and Lundberg, J. O. N. (1998). Exhaled oral and nasal nitric oxide during L-arginine-infusion in preeclampsia. *Gynecol. Obstet. Invest.* **46,** 232–237.

Guo, F. H., Raeve, H. R. D., Rice, T. W., Stuehr, D. J., Thunnissen, F. B., and Erzurum, S. C. (1995). Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7809–7813.

Gustafsson, L. E., Leone, A. M., Persson, M. G., Wiklund, N. P., and Moncada, S. (1991). Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. *Biochem. Biophys. Res. Commun.* **181,** 852–857.

Hamid, Q., Springall, D. R., Riveros-Moreno, V., *et al.* (1993). Induction of nitric oxide synthase in asthma. *Lancet* **342,** 1510–1513.

Heiss, L. N., Lancaster, J. R., Jr., Corbetti, J. A., and Goldman, W. E. (1994). Epithelial autotoxicity of nitric oxide: Role in respiratory cytopathology of pertussis. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 267–270.

Herulf, M., Ljung, T., Hellström, P. M., Weitzberg, E., and Lundberg, J. O. N. (1998). Increased luminal nitric oxide in inflammatory bowel disease as shown with a novel minimally invasive method. *Scand. J. Gastroenterol.* **33,** 164–169.

Herulf, M., Lundberg, J. O. N., and Weitzberg, E. (1999a). Nostril widening improves arterial oxygenation: A role for nasal nitric oxide? *Respir. Med.* **93,** 134–135.

Herulf, M., Lagergren, Å., Ljung, T., Svennungsson, B., Weitzberg, E., and Lundberg, J. O. N. (1999b). Increased rectal nitric oxide in infectious gastroenteritis. *J. Infect. Dis.* **180,** 542–545.

Holden, W. E., Wilkins, J. P., Harris, M., Milczuk, H. A., Giraud, G. D. (1999). Temperature conditioning of nasal air: Effects of vasoactive agents and involvement of nitric oxide. *J. Appl. Physiol.* **87,** 1260–1265.

Ikeda, I., Kasajima, T., Ishuyama, S., Shimojo, T., Takeo, Y., Nishikawa, T., Kameoka, S., Hiroe, M., and Mistunaga, A. (1997). Distribution of inducible nitric oxide synthase in ulcerative colitis. *Am. J. Gastroenterol.* **92,** 1339–1341.

Imada, M., Iwamoto, J., Nonaka, S., *et al.* (1996). Measurement of nitric oxide in human nasal airway. *Eur. Respir. J.* **9,** 556–559.

Iwamoto, J., Pendergast, D. R., Suzuki, H., and Krasney, J. A. (1994). Effect of graded exercise on nitric oxide in expired air in humans. *Respir. Physiol.* **97,** 333–345.

Jackson, A. M., Alexandroff, A. B., Kelly, R. W., Skibinska, A., Suvaranathan, K. E., Prescott, S., Chisholm, G. D., and James, K. (1995). Changes in urinary cytokines and soluable intercellular adhesion molecule-1 (ICAM-1) in bladder cancer patients after treatment with Bacillus Calmette-Guérin (BCG) immunotherapy. *Clin. Exp. Immunol.* **99,** 369–375.

Jain, B., Rubenstein, I., Robbins, R. A., *et al.* (1993). Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem. Biophys. Res. Commun.* **191,** 83–88.

Jansson, O., Morcos, E., Adolfsson, J., Lundberg, J. O. N., and Wiklund, N. P. (1998). The role of nitric oxide in antitumour treatment with BCG. *Br. J. Cancer* **78,** 588–592.

Jatakanon, A., Lim, S., Kharitonov, S. A., Chung, K. F., and Barnes, P. J. (1998). Correlation between exhaled nitric oxide and sputum eosinophils, and metacholine responsiveness in patients with mild asthma. *Thorax* **53,** 91–95.

Ji, X., and Hollocher, T. C. (1989). Nitrate reductase of *Escherichia coli* as a NO-producing nitrite reductase. *Biochem. Arch.* **5,** 61–66.

Kaplan, S., Lancaster, J., Basford, R., and Simmons, R. (1996). Effect of nitric oxide on staphylococcal killing and interactive effect with superoxide. *Infect. Immun.* **64,** 69–76.

Kawamoto, H., Takumida, M., Takeno, S., *et al.* (1998). Localization of nitric oxide synthase in human nasal mucosa with nasal allergy. *Acta Oto-Laryngol.* **539**(Suppl.), 65–70.

Kharitonov, S. A., Logan-Sinclair, R. B., Busset, C. M., and Shinebourne, E. A. (1994). Peak expiratory nitric oxide difference in men and women: Relation to menstrual cycle. *Br. Heart J.* **72,** 243–245.

Kharitonov, S., O'Connor, B. J., Evans, D. I., and Barnes, P. J. (1995a). Allergen-induced late asthmatic reactions are associated with elevation of exhaled nitric oxide. *Am. J. Respir. Crit. Care Med.* **151,** 1894–1899.

Kharitonov, S. A., Yates, D., and Barnes, P. J. (1995b). Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. *Eur. Respir. J.* **8,** 295–297.

Kharitonov, S. A., Wells, A. U., O'Connor, B. J., Cole, P. J., Hansell, D. M., Logan-Sinclair, R. B., and Barnes, P. J. (1995c). Elevated levels of nitric oxide in bronchiectasis. *Am. J. Respir. Crit. Care Med.* **151,** 1889–1893.

Kharitonov, S. A., Lubec, G., Hjelm, M., and Barnes, P. J. (1995d). L-Arginine increases exhaled nitric oxide in normal human subjects. *Clin. Sci.* **88,** 135–139.

Kharitonov, S., Alving, K., and Barnes, P. J. (1997a). Exhaled and nasal nitric oxide measurements: Recommendations. *Eur. Respir. J.* **10,** 1683–1693.

Kharitonov, S., Rajakulasingam, K., O'Connor, B., *et al.* (1997b). Nasal nitric oxide is increased in patients with asthma and allergic rhinitis and may be modulated by nasal glucocorticosteroids. *J. Allergy Clin. Immunol.* **99,** 58–64.

Kimberly, B., Nejadnik, B., Giraud, G., *et al.* (1996). Nasal contribution to exhaled nitric oxide at rest and during breathholding. *Am. J. Respir. Crit. Care Med.* **153,** 829–836.

Kimura, H., Hokari, R., Miura, S., Shigematsu, T., Hirokawa, M., Akiba, Y., Kurose, I., Higuchi, H., Fujimori, H., Tsuzuki, Y., Serizawa, H., and Ishii, H. (1998). Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis. *Gut* **42,** 180–187.

Kirsten, A., Jorres, R., Kirsten, D., *et al.* (1997). Effect of a nasal challenge with endotoxin-containing swine confinement dust on nasal nitric oxide production. *Eur. J. Med. Res.* **2,** 335–339.

Kitagawa, H., Takeda, F., and Kohei, H. (1990). Effect of endothelium derived relaxing factor on gastric lesion induced by HCI in rats. *J. Pharmacol. Exp. Ther.* **253,** 1133–1137.

Kono, Y., Shibata, H., Adachi, K., and Tanaka, K. (1994). Lactate-dependent killing of *Escherichia coli* by nitrite plus hydrogen peroxide: A possible role of nitrogen dioxide. *Arch. Biochem. Biophys.* **311,** 153–159.

Lee, K., Tan, P., Rico, P., *et al.* (1997). Low levels of nitric oxide as contaminant in hospital compressed air: Physiologic significance? *Crit. Care Med.* **25,** 1143–1146.

Lewandowski, K., Busch, T., Lohbrunner, H., *et al.* (1998). Low nitric oxide concentrations in exhaled gas and nasal airways of mammals without paranasal sinuses. *J. Appl. Physiol.* **85,** 405–410.

Licht, W., Tannenbaum, S. R., and Deen, W. M. (1988). Use of ascorbic acid to inhibit nitrosation:kinetic and mass transfer considerations for an *in vitro* system. *Carcinogenesis* **9,** 365–372.

Lindberg, S., Cervin, A., and Runer, T. (1997a). Nitric oxide [NO] production in the upper airways is decreased in chronic sinusitis. *Acta Oto-Laryngol.* **117,** 113–117.

Lindberg, S., Cervin, A., and Runer, T. (1997b). Low levels of nasal nitric oxide [NO] correlate to impaired mucociliary function in the upper airways. *Acta Oto-Laryngol.* **117,** 728–734.

Loukides, S., Kharitonov, S., Wodehouse, T., *et al.* (1998). Effect of arginine on mucociliary function in primary ciliary dyskinesia. *Lancet* **352,** 371–372.

Lundberg, J. O. N., Weitzberg, E., Nordvall, S. L., *et al.* (1994a). Primarily nasal origin of exhaled nitric oxide and absence in Kartagener's syndrome. *Eur. Respir. J.* **7,** 1501–1504.

Lundberg, J. O. N., Weitzberg, E., Lundberg, J. M., and Alving, K. (1994b). Intragastric nitric oxide production in humans: Measurements in expelled air. *Gut* **35,** 1543–1546.

Lundberg, J. O. N., Hellström, P. M., Lundberg, J. M., and Alving, K. (1994c). Greatly increased luminal nitric oxide in ulcerative colitis. *Lancet* **344,** 1673–1674.

Lundberg, J. O. N., Farkas-Szallasi, T., Weitzberg, E., *et al.* (1995a). High nitric oxide production in human paranasal sinuses. *Nat. Med.* **1,** 370–373.

Lundberg, J. O. N., Lundberg, J. M., Settergren, G., *et al.* (1995b). Nitric oxide, produced in the upper airways, may act in an aerocrine fashion to enhance pulmonary oxygen uptake in humans. *Acta Physiol. Scand.* **155,** 467–468.

Lundberg, J. O. N., Hellström, P. M., Lundberg, J. M., and Alving, K. (1995c). Nitric oxide in ulcerative colitis. *Lancet* **345,** 449.

Lundberg, J. O. N., Nordvall, S. L., Weitzberg, E., *et al.* (1996a). Exhaled NO in pediatric asthma and cystic fibrosis. *Arch. Dis. Childhood* **75,** 323–326.

Lundberg, J. O. N., Weitzberg, E., Rinder, J., *et al.* (1996b). Calcium-independent and steroid-resistant nitric oxide synthase activity in human paranasal sinus mucosa. *Eur. Respir. J.* **9,** 1344–1347.

Lundberg, J. O. N., Weitzberg, E., Lundberg, J. M., *et al.* (1996c). Nitric oxide in exhaled air. *Eur. Respir. J.* **9,** 2671–2680.

Lundberg, J. O. N., Rinder, J., Weitzberg, E., *et al.* (1996d). Heavy physical exercise acutely decreases nitric oxide levels in the nasal airways in man. *Acta Physiol. Scand.* **159,** 51–57.

Lundberg, J. O. N., Settergren, G., Gelinder, S., *et al.* (1996e). Inhalation of nasally derived nitric oxide modulates pulmonary function in humans. *Acta Physiol. Scand.* **158,** 343–347.

Lundberg, J. O. N., Adolphsson, J., Weitzberg, E., Jansson, O., Lundberg, J. M., Alving, K., and Wiklund, N. P. (1996f). Elevated luminal nitric oxide in the urinary bladder in infectious and noninfectious cystitis. *Urology* **48,** 700–702.

Lundberg, J. O. N., Lundberg, J. M., Alving, K., *et al.* (1997a). Nitric oxide and inflammation: The answer is blowing in the wind. *Nat. Med.* **3,** 30–31.

Lundberg, J. O. N., Olesen, M., Bohr, J., Wiklund, N. P., Hellström, P. M., and Tysk, C. (1997b). Increased nitric oxide production in patients with collagenous and lymphocytic colitis. *Eur. J. Clin. Invest.* **27,** 869–871.

McCafferty, D.-M., Mudgett, J. S., Swain, M. G., and Kubes, P. (1997). Inducible nitric oxide synthase plays a critical role in resolving intestinal inflammation. *Gastroenterology* **112,** 1022–1027.

McKnight, G. M., Smith, L. M., Drummond, R. S., Duncan, C. W., Golden, M., and Benjamin, N. (1997). Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* **40,** 211–214.

Mancinelli, R. L., and McKay, C. P. (1983). Effects of nitric oxide and nitrogen dioxide on bacterial growth. *Appl. Environ. Microbiol.* **46,** 198–202.

Marczin, N., Riedel, B., Royston, D., and Yacoub, M. (1997a). Intravenous nitrate vasodilators and exhaled nitric oxide. *Lancet* **349,** 1742.

Marczin, N., Riedel, B., Gal, J., Polak, J., and Yacoub, M. (1997b). Exhaled nitric oxide during lung transplantation. *Lancet* **350,** 1681–1682.

Martin, U., Bryden, K., Devoy, M., *et al.* (1996). Increased levels of exhaled nitric oxide during nasal and oral breathing in subjects with seasonal rhinitis. *J. Allergy Clin. Immunol.* **97,** 768–772.

Massaro, A. F., Mehta, S., Lilly, C. M., Kobzic, L., Reilly, J. J., and Drazen, J. M. (1996). Elevated nitric oxide concentrations in isolated lower airway gas of asthmatic subjects. *Am. J. Respir. Crit. Care Med.* **153,** 1510–1514.

Middleton, S. J., Shorthouse, M., and Hunter, J. D. (1993). Increased nitric oxide synthesis in ulcerative colitis. *Lancet* **341,** 465–466.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–141.

Morris, N. H., Carroll, S., Nicolaides, K. H., Steer, P. J., and Warren, J. B. (1995). Exhaled nitric oxide concentration and amniotic fluid nitrite concentrations during pregnancy. *Eur. J. Clin. Invest.* **25,** 138–141.

Murell, G. A. C., Jang, D., and Williams, R. J. (1995). Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochem. Biophys. Res. Commun.* **206,** 15–21.

Murphy, A., Plattmills, T., Lobo, M., *et al.* (1998). Respiratory nitric oxide levels in experimental human influenza. *Chest* **114,** 452–456.

Nathan, C., and Xie, Q.-W. (1994). Nitric oxide synthases: Roles, tolls, and controls. *Cell* **78,** 915–918.

Norman, V., and Keith, C. H. (1965). Nitrogen oxides in tobacco smoke. *Nature* **205,** 915–916.

Nussler, A. K., and Billiar, T. R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. *J. Leukocyte Biol.* **54,** 171–178.

Palm, J., Alving, K., Lidman, C., and Lundberg, J. O. N. (2000). Nasal nitric oxide is reduced in patients with HIV. *Acta Oto-Laryngol.* **120,** 420–423.

Perner, A., and Rask-Madsen, J. (1999). The potential role of nitric oxide in chronic inflammatory bowel disease. *Aliment. Pharmacol. Ther.* **13,** 135–144.

Persson, M. G., Wiklund, N. P., and Gustavsson, L. E. (1993). Endogenous nitric oxide in single exhalations and the change during exercise. *Am. Rev. Respir. Dis.* **148,** 1210–1214.

Persson, M. G., Cederqvist, B., Wiklund, C. U., and Gustafsson, L. E. (1994a). Ethanol causes decrements in airway excretion of endogenous nitric oxide in humans. *Eur. J. Pharmacol.* **270,** 273–278.

Persson, M. G., Agvald, P., and Gustafsson, L. E. (1994b). Detection of nitric oxide in exhaled air during administration of nitroglycerine *in vivo*. *Br. J. Pharmacol.* **111,** 825–828.

Phillips, C., Giraud, G., and Holden, W. (1996). Exhaled nitric oxide during exercise: Site of release and modulation by ventilation and flow. *J. Appl. Physiol.* **80,** 1865–1871.

Piacentini, G. L., Bodini, A., Costella, S., Vicentini, L., Mazzi, P., Sperandino, S., and Boner, A. L. (1999). Exhaled nitric oxide and sputum eosinophil markers of inflammation in asthmatic children. *Eur. Respir. J.* **13,** 1386–1390.

Pinsky, M., Genc, F., Lee, K., *et al.* (1997). Contamination of hospital compressed air with nitric oxide: Unwitting replacement therapy. *Chest* **111,** 1759–1763.

Puybasset, L., Rouby, J., Mourgeon, E., *et al.* (1994). Inhaled nitric oxide in acute respiratory failure: Dose–response curves. *Intensive Care Med.* **20,** 319–327.

Ramis, I., Lorente, J., Roselló-Catafau, J., *et al.* (1996). Differential activity of nitric oxide synthase in human nasal mucosa and polyps. *Eur. Respir. J.* **9,** 202–206.

Rhodes, P. M., Leone, A. M., Francis, P. L., Struthers, A. D., and Moncada, S. (1995). The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans. *Biochem. Biophys. Res. Commun.* **209,** 590–596.

Rinder, J., Lundberg, J. O. N., Änggård, A., *et al.* (1996). Effects of topical nasal decongestants, L-arginine and nitric oxide synthase inhibition on nasal cavity nitric oxide levels and nasal cavity volume in man. *Am. J. Rhinol.* **10,** 399–408.

Robbins, R. A., Millatmal, T., Lassi, K., Rennard, S., and Daughton, D.

(1997). Smoking cessation is associated with an increase in exhaled nitric oxide. *Chest* **112,** 313–318.

Runer, T., Cervin, A., Lindberg, S., *et al.* (1998). Nitric oxide is a regulator of muco ciliary activity in the upper respiratory tract. *Otolaryngol. Head Neck Surg.* **119,** 278–287.

Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7240–7244.

Salzmann, A. L. (1995). Nitric oxide in the gut. *New Horizons* **3,** 33–45.

Sato, M., Fukuyama, N., Sakai, M., *et al.* (1998). Increased nitric oxide in nasal lavage fluid and nitrotyrosine formation in nasal mucosa—Indices for severe perennial nasal allergy. *Clin. Exp. Allergy* **28,** 597–605.

Schedin, U., Gustafsson, L. E., Herin, P., *et al.* (1996). Endogenous nitric oxide in the upper airways of healthy new-born infants. *Pediatr. Res.* **40,** 148–151.

Schedin, U., Roken, B., Nyman, G., *et al.* (1997). Endogenous nitric oxide in the airways of different animal species. *Acta Anaesthesiol. Scand.* **41,** 1133–1141.

Settergren, G., Angelin, B., Liska, J., *et al.* (1998). Lower pulmonary vascular resistance during nasal breathing: Modulation by nitric oxide from the paranasal sinuses. *Acta Physiol. Scand.* **163,** 235–239.

Shaul, P. W., North, A. J., Wu, L. C., Wells, L. B., Brannon, T. S., Lau, K. S., Michel, T., Magraf, L. R., and Star, R. A. (1994). Endothelial nitric oxide synthase is expressed in human bronchial epithelium. *J. Clin. Invest.* **94,** 2231–2236.

Silkoff, P., McClean, P., Slutsky, A., *et al.* (1997). Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide. *Am. J. Respir. Crit. Care Med.* **155,** 260–267.

Silkoff, P. E., Caramori, M., Tremblay, L., McClean, P., Chaparro, C., Kesten, S., Hutcheon, M., Slutsky, A. S., Zamel, N., and Keshavjee, S. (1998). Exhaled nitric oxide in human lung transplantation. A noninvasive marker of acute rejection. *Am. J. Respir. Crit. Care Med.* **157,** 1822–1828.

Singer, I. I., Kawka, D. W., Scott, S., Weidner, J. R., Mumford, R. A., Riehl, T. E., and Stenson, W. F. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology* **111,** 871–885.

Spiegelhalder, B., Eisenbrand, G., and Preussman, R. (1976). Influence of dietary nitrate on nitrite content of human saliva: Possible relevance to *in vivo* formation of N-nitroso compounds. *Food Cosmet. Toxicol.* **14,** 545–554.

Tannenbaum, S. R., Sisnkey, A. J., Weisman, M., and Bishop, W. (1974). Nitrite in human saliva. Its possible relationship to nitrosamine formation. *J. Natl. Cancer Inst.* **53,** 79–84.

Tannenbaum, S. R., Weissman, M., and Fett, D. (1976). The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet. Toxicol.* **14,** 549–552.

Trolin, G., Anden, T., and Hedenstierna, G. (1994). Nitric oxide in expired air at rest and during exercise. *Acta Physiol. Scand.* **151,** 159–163.

Watkins, D., Lewis, R., Basclain, K., *et al.* (1998). Expression and localization of the inducible isoform of nitric oxide synthase in nasal polyp epithelium. *Clin. Exp. Allergy* **28,** 211–219.

Whittle, B. J. R. (1993). Neuronal and endothelium-derived mediators in the modulation of gastric microcirculation: Integrity in the balance. *Br. J. Pharmacol.* **110,** 3–17.

Whittle, B. J. R. (1994). Nitric oxide in gastrointestinal physiology and pathology. *In* ''Physiology of the Gastrointestinal Tract'' (L. R. Johnson, ed.), pp. 267–294. Raven, New York.

Xie, Q.-W., and Nathan, C. (1994). The high output nitric oxide pathway: Role and regulation. *J. Leukocyte Biol.* **56,** 576–582.

Yasuda, Y., Itoh, T., Miyamura, M., *et al.* (1997). Comparisons of exhaled nitric oxide and cardiorespiratory indices between nasal and oral breathing during submaximal exercise in humans. *Jpn. J. Physiol.* **47,** 465–470.

Yates, D. H., Kharitonov, S. A., Robbins, R. A., Thomas, P. S., and Barnes, P. J. (1995). Effect of a nitric oxide synthase inhibitor and a glucocorticosteroid on exhaled nitric oxide. *Am. J. Respir. Crit. Care Med.* **152,** 892–896.

Yates, D. H., Kharitonov, S. A., Thomas, P. S., and Barnes, P. J. (1996). Endogenous nitric oxide is decreased in asthmatic patients by an inhibitor of inducible nitric oxide synthase. *Am. J. Respir. Crit. Care Med.* **154,** 247–250.

Yates, D. H., Kharitonov, S. A., and Barnes, P. J. (1997). Effects of short and long-acting B_2 agonists on exhaled nitric oxide in asthmatic patients. *Eur. Respir. J.* **10,** 1483–1488.

Zetterquist, W., Pedroletti, C., Lundberg, J. O. N., and Alving, K. (1999). Salivary contribution to exhaled nitric oxide. *Eur. Respir. J.* **13,** 327–333.

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CHAPTER 58

Nitric Oxide and Persistent Pulmonary Hypertension in the Newborn

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THE TRANSITION OF THE PULMONARY CIRCULATION FROM A HIGH RESISTANCE–LOW FLOW CIRCULATION IN THE FETUS TO A LOW RESISTANCE–HIGH FLOW CIRCULATION IN THE NEWBORN IS A COMPLEX PROCESS THAT INVOLVES THE SIMULTANEOUS DOWNREGULATION OF VASOCONSTRICTOR MEDIATORS AND UPREGULATION OF VASODILATORY MEDIATORS SUCH AS NITRIC OXIDE (NO). IN THE VAST MAJORITY OF INFANTS, THIS PROCESS OCCURS SPONTANEOUSLY AND QUICKLY AND DOES NOT REQUIRE ANY INTERVENTION. OCCASIONALLY, HOWEVER, THE TRANSITION MAY BE DIFFICULT OR ABNORMAL, AND REQUIRE PROMPT AND EFFECTIVE RESUSCITATION TO ENSURE A SUCCESSFUL ADAPTATION TO THE EXTRAUTERINE ENVIRONMENT.

ONE DISEASE THAT LEADS TO COMPLICATIONS DURING TRANSITION IS PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN (PPHN) WHICH IS CHARACTERIZED BY FAILURE OF THE PULMONARY VASCULAR RESISTANCE (PVR) TO DECREASE AFTER BIRTH, RESULTING IN HYPOXIA AND SEVERE DISTRESS. UNDERSTANDING THE PHYSIOLOGY OF THE PULMONARY CIRCULATION DURING THE TRANSITION FROM FETUS TO NEWBORN HAS HELPED NEONATOLOGISTS DEVELOP NEW TECHNIQUES FOR THE MANAGEMENT OF THIS SERIOUS CONDITION ASSOCIATED WITH HIGH MORTALITY AND MORBIDITY. FOR EXAMPLE, BECAUSE INFANTS WITH PPHN EXHIBIT REDUCED NO SYNTHESIS, INHALED NO IS NOW WIDELY USED TO INCREASE PULMONARY BLOOD FLOW AND CORRECT HYPOXIA IN THESE NEONATES. OTHER METHODS TO INCREASE cGMP, SUCH AS INHIBITION OF cGMP SPECIFIC TYPE 5 PHOSPHODIESTERASE ISOFORM (PDE5), EITHER ALONE OR IN COMBINATION WITH NO, ARE BEING INVESTIGATED AS NOVEL THERAPEUTIC MODALITIES FOR TREATING THIS CONDITION. IT IS ANTICIPATED THAT THIS COMBINATION THERAPY WILL DECREASE THE CONCENTRATION OF NO REQUIRED FOR A THERAPEUTIC EFFECT; THUS ATTENUATING POTENTIALLY DELETERIOUS EFFECTS RESULTING FROM THE FREE RADICAL ACTIONS OF NO.

THIS CHAPTER WILL STRESS THE ROLE OF NO IN (1) THE NORMAL FETAL CIRCULATION, (2) THE TRANSITION OF THE CIRCULATION AT BIRTH, AND (3) THE ETIOLOGY AND TREATMENT OF PPHN IN NEWBORNS. IT IS IMPORTANT TO NOTE THAT ALTHOUGH THEY WILL BE DISCUSSED ONLY BRIEFLY, MULTIPLE FACTORS IN ADDITION TO NO CONTRIBUTE TO BOTH THE NORMAL TRANSITION AND THE PATHOPHYSIOLOGY OF PPHN.

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The Normal Fetal Circulation

In the adult, the lung is the organ of gas exchange and receives 50% of the combined ventricular output. In contrast, the placenta serves as the organ of gas exchange during fetal life, and as such receives 50% of the combined ventricular output. Because the lung is not required for gas exchange, pulmonary blood flow is low in the fetus (approximately 10% of the combined ventricular output by term) and PVR is high (Rudolph and Heymann, 1970). This unique physiological state allows adequate pulmonary blood flow for lung growth and development, yet permits venous blood to be shunted across the ductus arteriosus and the foramen ovale so that it can be directed to the placenta for gas exchange (Fig. 1A). Total systemic vascular resistance is low, mainly due to the low-resistance placental circulation.

Structure of Fetal Pulmonary Vessels

The morphologic development of the pulmonary vasculature affects the physiological changes that occur in the perinatal period. In the fetus, small pulmonary arteries have cuboidal endothelium and a thicker muscular coat relative to the external diameter than do similar arteries in the adult. This muscularity is believed to be at least partially responsible for the increased vasoreactivity and high PVR in the fetus, particularly near term. In fetal lungs, the medial smooth muscle coat is most prominent in the fifth and sixth generation arteries (Reid, 1986). In more peripheral vessels, there is only partial muscularization, and in precapillary arterioles the muscular layer disappears altogether.

During fetal growth in lambs, the number of small pulmonary arteries increases, both in absolute terms and per unit volume of the lung. In the last trimester in fetal lambs, the number of small blood vessels in the lungs increases 40-fold while the wet weight of the lungs increases fourfold. Thus, the number of small blood vessels per unit of lung increases 10-fold, a process which prepares the lungs to accept the 10-fold increase in blood per unit of lung that occurs at birth.

Mediators of High Pulmonary Vascular Tone

Despite the large increase in the cross-sectional area of the pulmonary circulation, PVR remains high in the near-term fetus. Mechanical factors such as absence of an air–liquid interface, compression of the small pulmonary artery (PA) by the fluid filled alveolar space, and lack of rhythmic distention contribute to the maintenance of high resting tone in the fetal lung. However, active vasoconstriction to the low oxygen tension of the normal fetus (17 to 20 mmHg) is a primary mechanism responsible for maintaining high pulmonary vascular resistance during fetal life.

Hypoxic pulmonary vasoconstriction develops over the period of gestation when the cross-sectional area of the vascular bed is increasing rapidly. Increasing or decreasing oxygen tension in the fetal lamb early in the third trimester (70% gestation) does not affect pulmonary vascular resistance (Fig. 2). In contrast, in the near-term fetal lamb, decreasing

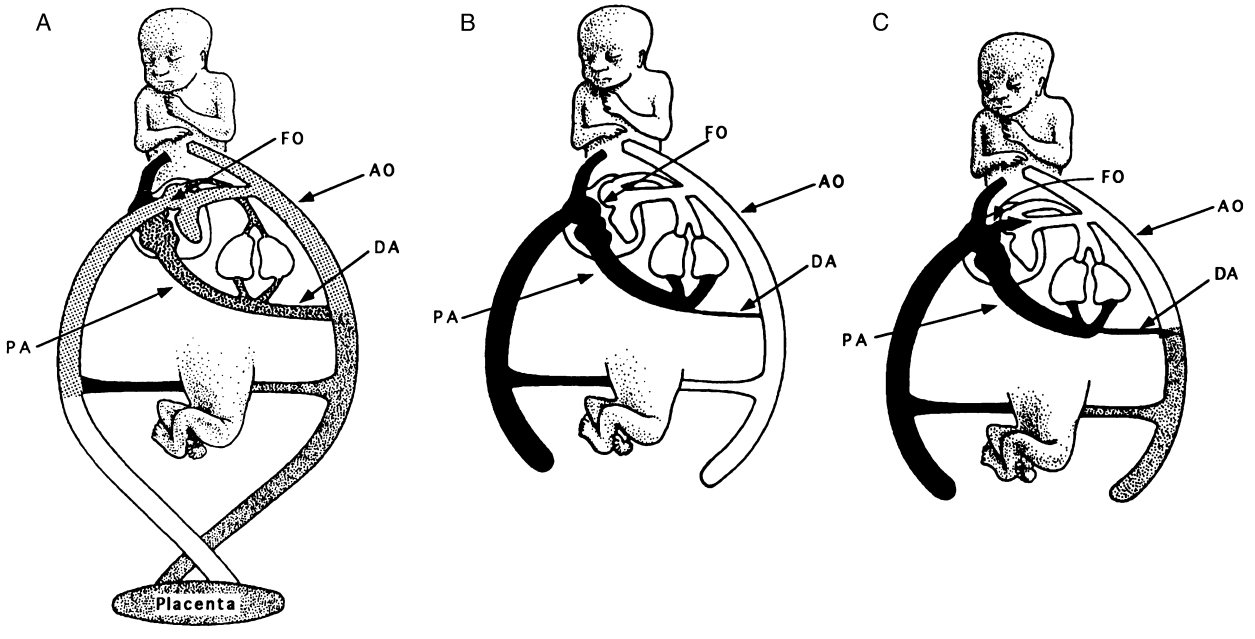


Figure 1 The circulations of the (A) normal fetus, (B) the normal newborn, and (C) the newborn with persistent pulmonary hypertension of the newborn. The whiter the vessels, the higher is the saturation of hemoglobin with oxygen. PA, pulmonary artery; FO, foramen ovale; DA, ductus arteriosus; AO, aorta; UA, umbilical artery; UV, umbilical vein; IV, inferior vena cava. From Morin III, F. C., and Stenmark, K. R. (1995). Persistent pulmonary hypertension of the newborn. *American Journal of Respiratory and Critical Care Medicine* **151,** 2010–2032. Official Journal of the American Thoracic Society © American Lung Association.

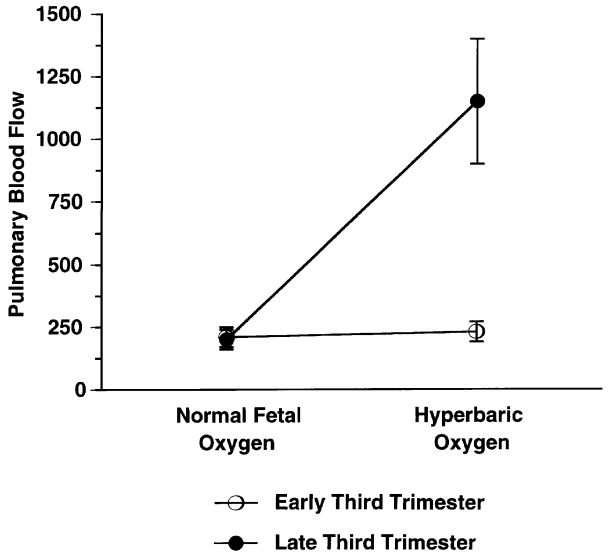


Figure 2 Effect of hyperbaric oxygen on pulmonary blood flow in early third trimester fetal lambs (open circles) and near-term fetal lambs (closed circles). From Morin and Egan (1992), with permission.

oxygen tension doubles PVR, and increasing oxygen tension markedly decreases PVR and increases pulmonary blood flow to normal newborn levels (Morin III and Egan, 1992). Inhibition (closure) of potassium channels in pulmonary vascular smooth muscle cells is a key event that links hypoxia to pulmonary vasoconstriction (Weir and Archer, 1995). Closure of potassium channels in response to hypoxia causes membrane depolarization and subsequent activation of voltage-gated calcium channels, leading to calcium influx and sustained contraction. Precisely which potassium channels are involved in maintaining vasoconstriction to hypoxia is controversial.

Role of Nitric Oxide

Distribution of NOS within the Fetal Lung

In the 1990s, nitric oxide became the agent most extensively studied in the perinatal pulmonary circulation. All three known nitric oxide synthase (NOS) isoforms, neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III), are developmentally regulated and differentially distributed in pulmonary vessels and airways of fetal lungs (Loesch and Burnstock, 1996; North *et al.,* 1994a; Sherman *et al.,* 1999; Xue *et al.,* 1996). Neuronal NOS and iNOS are found in the soluble fraction of cells, whereas eNOS is localized to the particulate fraction, primarily in the caveolar membrane fractions isolated from endothelial cell plasma membrane (Shaul *et al.,* 1996). Localization to this microdomain is likely to optimize eNOS activation and the extracellular release of NO.

In pulmonary vessels, eNOS is localized specifically to the endothelium, whereas iNOS and nNOS are found in both endothelial and smooth muscle cells (Sherman *et al.,* 1999; Tzao *et al.,* 1999). Immunostaining for endothelial cell eNOS in the ovine fetus decreases from large to small pulmonary arteries, whereas staining for nNOS, although abundant, is observed only in small pulmonary arteries. Both eNOS and nNOS are absent in the small pulmonary arteries of the adult sheep lung. In airways of fetal, newborn, and adult sheep, nNOS is found in the epithelium at all levels including the alveoli, whereas eNOS and iNOS are expressed primarily in proximal bronchiolar epithelia.

In addition to the NO produced by eNOS, iNOS, and nNOS in the pulmonary vessels and airways, high levels of NO can be produced by activation of iNOS in alveolar macrophages and cells recruited to the lung during inflammation. The adventitia of pulmonary vessels may provide a protective barrier by preventing the vasodilator effects of these extravascular sources of NO from influencing vessel tone. For example, NO produced locally within endothelial cells or applied to the luminal surface of pulmonary arteries is a potent vasodilator (Steinhorn *et al.,* 1994a). In contrast, application of NO to the adventitial surface has virtually no effect on pulmonary artery tone, whereas carbon monoxide and sodium nitroprusside are equipotent whether delivered to the endothelial or adventitial surface. These findings point to a highly localized regulation of pulmonary vascular tone by NO synthesized in the endothelium. The free radical nature of NO may be responsible for its localization by the adventitia, and this property may protect pulmonary vessels from high levels of NO produced by activation of iNOS in alveolar macrophages and inflammatory cells.

Ontogeny of the NO Pathway

Nitric oxide synthase, soluble guanylate cyclase (sGC), and phosphodiesterase (PDE) all serve to modulate cGMP levels and smooth muscle tone in the pulmonary vasculature. The expression and activity of all three enzymes change during fetal and postnatal development (Fig. 3 vs. Fig. 7). A variety of methods have been employed to assess the level of activity in the NO–cGMP pathway during development including: (1) vascular responses to NOS inhibitors and to endothelium-dependent and independent dilators, (2) measurement of NO levels, and (3) measurement of protein and message for eNOS, sGC, and PDE.

Nitric Oxide Synthase NOS activity can be demonstrated early in the third trimester of gestation in the fetal lamb. In these preterm fetal lambs, inhibition of NOS increases PVR, indicating that basal PVR is modulated by endogenous NO (Kinsella *et al.,* 1994). Basal NO production in the lung increases by twofold later in gestation, and the endothelial-dependent agonists acetylcholine and oxygen demonstrate progressive pulmonary vasodilator potency during this developmental period in the fetal lamb (Lewis *et al.,* 1976). The pulmonary vasodilation to acetylcholine is blocked by NOS inhibitors and restored by excess L-arginine (Abman *et al.,* 1990; Tiktinsky *et al.,* 1992). NOS inhibition blocks virtually the entire increase in fetal pulmonary blood flow caused by oxygenation of the late-gestation fetal lamb (Fig. 4) (Tiktinsky and Morin, 1993). Inhibition of NOS

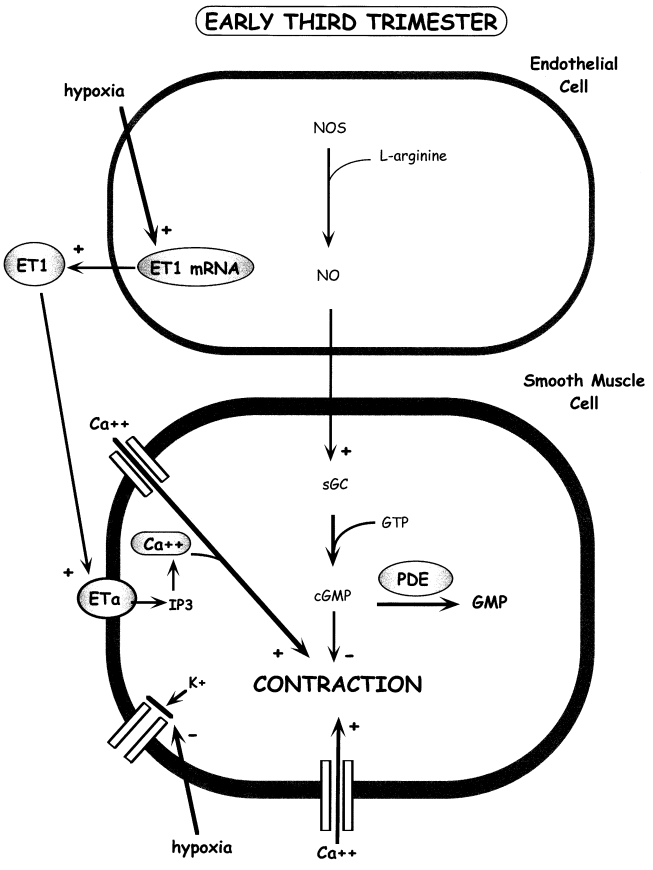


Figure 3 Diagram of the factors that favor high pulmonary vascular resistance during the early third trimester of fetal development. The size and density of labels and lines reflect relative concentrations and activity.

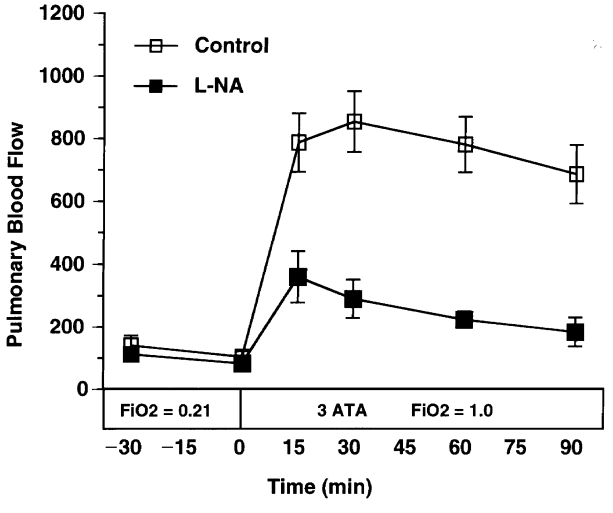


Figure 4 Effect of hyperbaric oxygenation on pulmonary blood flow in normal near-term fetal lambs (open squares). The dramatic increase in pulmonary blood flow is blocked by pretreatment with the NOS inhibitor N^G-nitro-L-arginine (L-NA). From Tiktinsky and Morin (1993), with permission.

immediately prior to delivery of the fetal lamb attenuates the increase in pulmonary blood flow and the decrease in PVR following birth (Abman *et al.,* 1990). Furthermore, chronic NOS inhibition in fetal lambs 10 days prior to birth similarly blunts the decrease in vascular tone and the increase in pulmonary blood flow normally seen at birth (Fineman *et al.,* 1994).

In contrast to the above findings, endothelial-independent agonists such as inhaled NO or the NO donor agent, sodium nitroprusside, produce greater changes in pulmonary blood flow than oxygen during early third trimester in the fetus (Abman and Accurso, 1991; Kinsella *et al.,* 1994). Infusion of N^G-nitro-L-arginine (L-NA) blocks the increase in pulmonary blood flow caused by oxygen but not inhaled NO. These findings indicate that the ability of the developing smooth muscle to respond to NO precedes the ability of the immature endothelium to increase and sustain the stimulated production and release of NO (Fig. 3).

Endothelial cell NOS (eNOS) protein and mRNA are detectable at 75% gestation and increase dramatically just prior to term in the fetal rat lung, thus optimizing the capacity for NO production at the time of birth (North *et al.,* 1994b). Immunostaining for eNOS is seen as early as 29% gestation in the fetal lamb lung, and peaks during mid-gestation (Halbower *et al.,* 1994). The mechanisms determining this pattern of expression are still not completely known, although estrogen appears to be quite important. Fetal circulating estrogen levels increase markedly during late gestation due to increasing placental production, surge just prior to birth, and subsequently fall. Fetal pulmonary arterial endothelial cells express estrogen receptors. Estrogen stimulates eNOS expression and activity in fetal pulmonary artery endothelial cells (Lantin-Hermoso *et al.,* 1997; MacRitchie *et al.,* 1997), and fetal plasma estrogen levels correlate well with the developmental changes in eNOS.

The vast majority of the previously mentioned studies on the regulation of pulmonary vascular resistance by NO have focused on the contribution of eNOS. More recently a role for iNOS and nNOS in modulating basal pulmonary vascular tone in the ovine fetus has been demonstrated (Rairigh *et al.,* 1998). In addition to the decrease in pulmonary vascular resistance at birth, the change from liquid breathing to air breathing also requires a marked decrease in lung liquid production. Nitric oxide decreases lung liquid production in fetal lambs (Cummings, 1997), and it is tempting to speculate that nNOS may be involved in this process due to its preferential distribution in alveoli, small airways, and small vessels.

Role of NOS in Hypoxic Vasoconstriction The capacity for hypoxic pulmonary vasoconstriction (HPV) is intrinsic to pulmonary vascular smooth muscle cells and is recognized as a primary mechanism for maintaining high pulmonary vascular tone in the fetus. Changes in nitric oxide synthase activity both directly and indirectly modulate hypoxic vasoconstriction. An acute decline in oxygen concentration depresses basal and acetylcholine-stimulated cGMP

production in pulmonary arteries from late gestation fetal lambs. This response to oxygen is absent in early third trimester (Shaul *et al.,* 1993), consistent with the developmental time course of fetal pulmonary vascular response to changes in oxygen tension described earlier. The activity and gene expression of nitric oxide synthase is reduced by hypoxia (McQuillan *et al.,* 1994; Shaul *et al.,* 1992). In addition, low oxygen tension (in part by NO-mediated mechanisms) induces the expression and secretion of the potent vasoconstrictor endothelin in cultured human endothelial cells (Kourembanas *et al.,* 1993).

Interaction between NO and Endothelin Endothelin-1 (ET-1) constricts pulmonary vascular smooth muscle by binding with high affinity to the ET_A receptors that are predominantly expressed in the smooth muscle cell layer. There appear to be two distinct subtypes of the ET_B receptor on pulmonary endothelial cells: the ET_{B1} vasodilator receptor and the ET_{B2} vasoconstrictor receptor (Perreault and Baribeau, 1995). All three receptors are present in the neonatal pulmonary circulation, and their expression increases with advancing gestation (Ivy *et al.,* 1998a). Nitric oxide appears to play a central role in endothelin-mediated vasodilation, as ET_{B1} receptor-mediated vasodilation is blocked by inhibition of nitric oxide synthase and hemoglobin (Zellers *et al.,* 1994).

The interaction between nitric oxide and endothelin is one likely mechanism responsible for the high PVR that occurs early in the third trimester of gestation (Fig. 3). ET-1 mRNA has been isolated from blood vessels within the perinatal lung (MacCumber *et al.,* 1989), and circulating immunoreactive ET-1 levels are very high in the fetal and transitional circulation (Kojima *et al.,* 1992) and decrease steadily in the postnatal period. Nitric oxide suppresses ET-1 synthesis (Kourembanas *et al.,* 1993), and cGMP inhibits ET-1-induced contraction and inositol phosphate production in fetal lamb pulmonary arteries (Millard *et al.,* 1998). Thus, decreased NO production by the endothelial cell during fetal life might contribute to high fetal PVR both by limiting vasodilation by stimulation of the endothelial receptor, and by allowing for increased production of ET-1 and inositol phosphate. Low oxygen tension, by decreasing nitric oxide production, induces the expression and secretion of ET-1 in cultured human endothelial cells (Kourembanas *et al.,* 1993). Nitric oxide also plays a role in the vascular response to endothelin. ET_A receptor blockade results in a dose-dependent increase in pulmonary blood flow, and selective ET_B agonists cause fetal pulmonary vasodilation (Ivy *et al.,* 1994, 1996). Both of these vasodilatory responses are blocked by inhibition of nitric oxide synthase.

Soluble Guanylate Cyclase Increases in NO lead to dilation of the pulmonary circulation by stimulating sGC and increasing cGMP concentrations in the cytosol of smooth muscle cells. The sGC enzyme in the pulmonary circulation is composed of $\alpha1$, $\alpha2$, and $\beta1$ subunits. The abundance of the $\alpha1$ subunit appears to correspond well with functional responses to NO in the lung (Tzao *et al.,* 1998). Two heterodimers ($\alpha1,\beta1$ and $\alpha2,\beta1$) with no functional difference have been found in the human placenta (Russwurm *et al.,* 1998). Levels of $\alpha1$ and $\beta1$ subunits of sGC in the lungs of late-gestation and neonatal Sprague-Dawley rats are high. Similar to the pattern of expression for eNOS, sGC levels are high late in gestation, and are greater than those observed in the adult lung (Bloch *et al.,* 1997). Pulmonary sGC enzyme activity correlates well with its expression, and is approximately sevenfold greater in newborn rats compared to adult rats.

The generally accepted mechanism for NO-induced vessel dilation involves the sGC-catalyzed production of cGMP with subsequent activation of protein kinase G (PKG), leading to extrusion of calcium from the cell cytosol and relaxation. However, activation of PKG by cGMP does not appear to be important in the NO-dependent modulation of the response to hypoxia in the normotensive rat, or in modulation of the increased vascular tone in the hypertensive rat lung (Fouty *et al.,* 1998). The mechanism responsible for this PKG-independent relaxation is not known.

Phosphodiesterase PDE activity in the smooth muscle of pulmonary vessels regulates vessel tone by rapidly converting cGMP to the inactive metabolite GMP. Although multiple phosphodiesterase isoforms have been identified, PDE5 appears to be the dominant isoform in the lung. Immunohistochemistry and *in situ* hybridization have demonstrated PDE5 protein mRNA in vascular smooth muscle of neonatal sheep and rat lungs (Hanson *et al.,* 1998a; Sanchez *et al.,* 1998). PDE5 activity is apparent early in gestation and progessively increases until term.

PULMONARY ARTERIES VERSUS PULMONARY VEINS

The vast majority of studies on NO–cGMP signaling in the lung have concentrated on the arterial side of the pulmonary circulation. However, isolated pulmonary veins are much more responsive to both endothelium-dependent and -independent nitric oxide than are pulmonary arteries (Gao *et al.,* 1995; Steinhorn *et al.,* 1993). Developmentally, large pulmonary veins from fetal and newborn lambs produce more NO than do veins from older lambs. In contrast, both basal and stimulated release of NO from large pulmonary arteries increases during the first few weeks of life.

Immunostaining for sGC and PDE5 also reveals interesting differences between arteries and veins. Staining for sGC is intensely positive in pulmonary veins of fetal lambs, is positive in small pulmonary arteries, and virtually absent in large pulmonary arteries (D'Angelis *et al.,* 1998) (Fig. 5). The distribution pattern for PDE5 is the exact opposite in fetal lambs, and is greater in pulmonary arteries than veins (D'Angelis, unpublished observation). This distribution of PDE5 is supported by activity assays showing greater PDE5 activity in ovine pulmonary arteries versus veins (Okogbule-Wonodi *et al.,* 1998). The differential distribution patterns of sGC and PDE5 in arteries versus veins may have important functional consequences for pulmonary blood flow at birth.

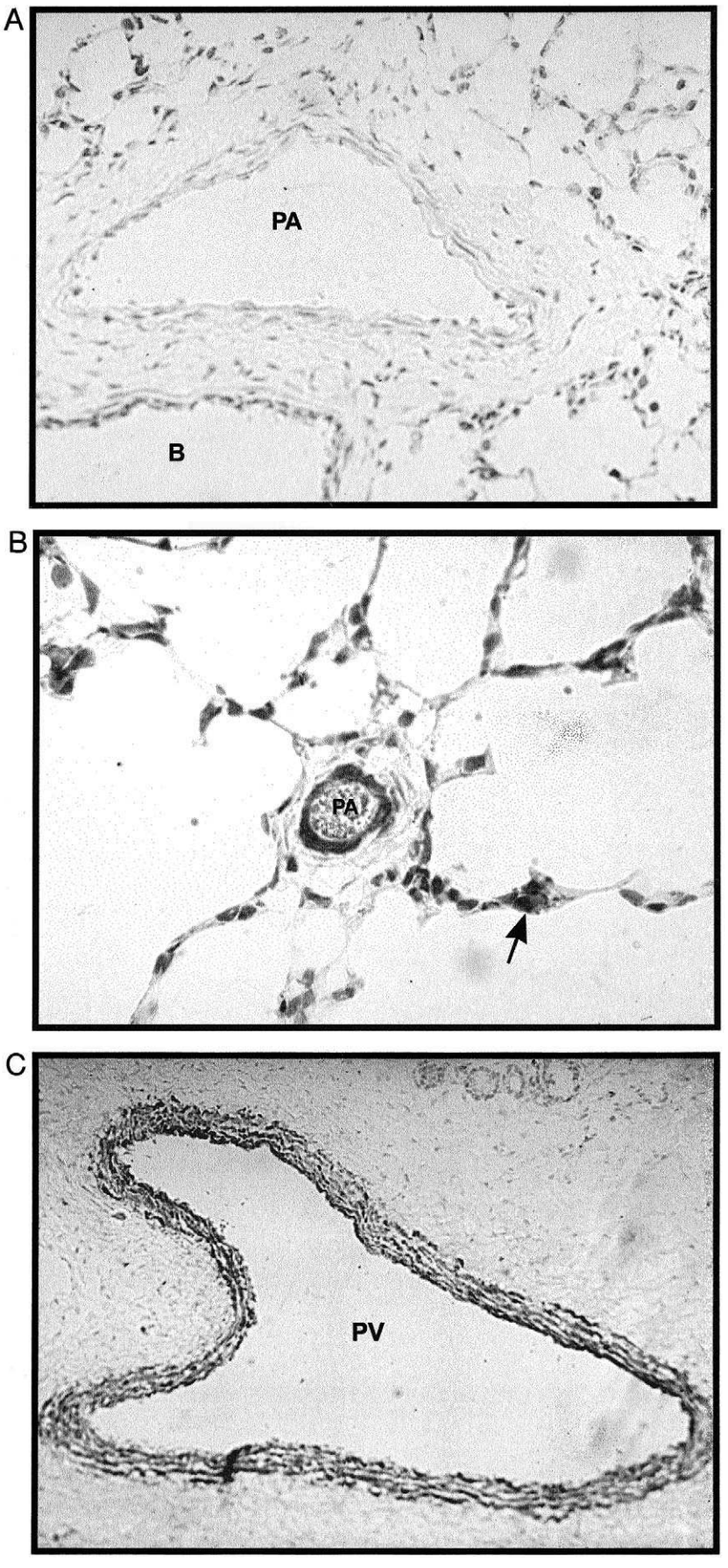


Figure 5 Differential distribution of soluble guanylate cyclase (sGC) in larger pulmonary arteries (A), small pulmonary arteries (B), and pulmonary veins (C) of near-term fetal lambs. Magnification: (A) 180×, (B) 800×, (C) 90×.

The low sGC/high PDE5 pattern in pulmonary arteries would favor a phasic dilator effect of NO, whereas the high sGC/low PDE5 pattern in pulmonary veins would favor a more sustained tonic dilator effect of NO. Rapidly changing levels of cGMP in pulmonary arteries may endow them with the ability to respond quickly to changes in oxygen levels and shear stress and readjust blood flow to different regions of the lung. More stable levels of cGMP in pulmonary veins might favor a low and uniform vascular resistance downstream of pulmonary capillaries.

Summary

The NO–cGMP pathway for vessel dilation is primed for maximal activity and functional effectiveness at birth, because levels of eNOS and sGC peak near term and PDE5 levels decrease. These conditions provide an optimal environment for the pulmonary vasodilatory response to NO during transition when the lung is both ventilated and exposed to a higher oxygen tension than exists *in utero.*

The Transition at Birth

As the fetal pulmonary circulation develops, there are major changes in the expression of humoral factors that influence PVR resistance and prime the pulmonary circulation for transition to the extrauterine environment. The development of the NO–cGMP signaling pathway plays a major role in this transition.

At birth, PVR drops immediately and permits an 8- to 10-fold rise in pulmonary blood flow (Fig. 6). Simultaneously, systemic vascular resistance increases, due to removal of the low vascular resistance bed of the placenta. As pulmonary pressure falls to less than systemic pressure, pulmonary blood flow increases and blood flow through the patent ductus arteriosus reverses direction. Functional closure of the ductus occurs over the first several hours of life; largely in response to the increased oxygen tension. Left atrial pressure also increases leading to closure of the foramen ovale. These events establish the normal postnatal circulatory pattern of separate pulmonary and systemic circulations by effectively eliminating the fetal right-to-left shunts (Fig. 1B). Within 24 hours after birth, PA pressure decreases to approximately 50% of mean systemic arterial pressure and adult values are normally reached within 2 to 6 weeks (Rudolph, 1985). It is critical that all of the previously mentioned events occur within the first several hours of life in order for the normal postnatal pattern of circulation to be established.

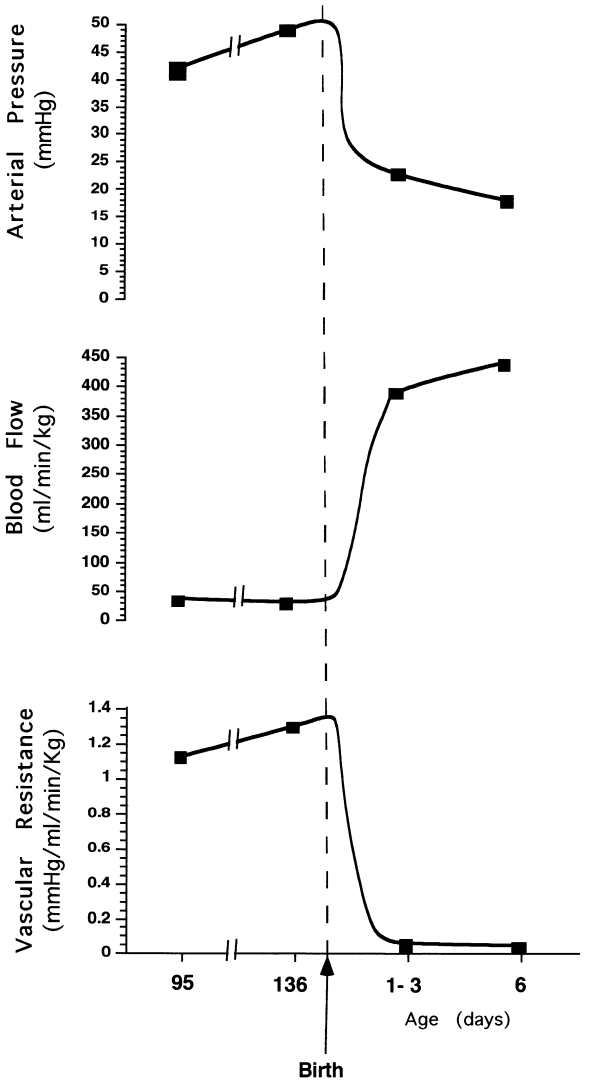


Figure 6 Changes in pulmonary hemodynamics during the last trimester of fetal life (to left of dashed line) and in the first days following birth (to right of dashed line).

Structural Adaptation to Extrauterine Life

Although there is no immediate postnatal reduction in the amount of vascular smooth muscle, structural adaptation rapidly occurs. Dilation and recruitment occur during the first 24 hours of life in the nonmuscular and partially muscular arteries of the precapillary bed. The external diameter of the nonmuscular arteries increases, and the prominent cuboidal endothelial cells seen in fetal life assume a flattened appearance. There is an increase in cell length and surface to volume ratio as the cells ''spread'' within the vessel wall to increase lumen diameter and lower resistance (Haworth *et al.,* 1987). The relative paucity of interstitial connective tissue at birth assists in this process by allowing for greater plasticity of the vessel. Dilation is followed by a reduction in muscularity, a process which begins in the first 24 hours of life and continues over subsequent weeks (Haworth, 1981). This overall reduction in muscularity further increases the lumen diameter, and functionally is associated with a reduction in the capacity of the PA to constrict with increasing age after birth. Through its antimitogenic effects, nitric oxide may be important in slowing replication of vascular smooth muscle cells and remodeling the pulmonary vasculature after birth.

Events that Initiate Transition

The stimuli that seem to be most important in decreasing PVR at birth are the rhythmic ventilation of the lungs with a gas and the increase in oxygen tension in the lungs. Each of these stimuli by itself will decrease PVR and increase pulmonary blood flow, but the largest effects are seen when the two events occur simultaneously (Teitel *et al.,* 1990). Studying the role of oxygenation independent of ventilation during transition is technically challenging. Chronically instrumented near-term fetal lambs have been studied while the ewe breathes oxygen in a hyperbaric chamber at three atmospheres. During hyperbaric oxygenation, fetal pulmonary vascular resistance decreases and pulmonary blood flow increases to levels comparable to after birth (Fig. 4).

Pulmonary endothelial cells play a central role in the pulmonary vascular transition through the production and release of numerous mediators that act on the subjacent smooth muscle cell layer. A complete discussion of their products is outside the scope of this chapter. However, the main endothelial products currently believed to be responsible for the pulmonary vasodilation at transition include arachidonic acid metabolites and nitric oxide. Prostacyclin (PGI_2) is the arachidonic acid metabolite most studied in the transition of the pulmonary circulation at birth. Prostacyclin may be important in pulmonary vasodilation following rhythmic distention of the lung, but does not appear to mediate the pulmonary vascular response to oxygenation in the fetus (Morin *et al.,* 1988). Despite a large body of research, the importance of prostacyclin in the transition at birth remains unclear.

Changes in NO–cGMP Signaling at Birth

Within 24 hours after birth, PA pressure decreases to approximately 50% of mean systemic arterial pressure (Fig. 6), and slowly declines to adult values within the next 2–6 weeks (Rudolph, 1985). The immediate increase in oxygen tension at birth alters the status of the smooth muscle and endothelial cells of the pulmonary vasculature (Fig. 7). One immediate effect of the increase in oxygen is reversal of the metabolic blockade of potassium channels in the smooth muscle cell layer leading to potassium efflux and a return of the membrane potential to the more polarized state typical in the adult pulmonary circulation. This closes voltage-dependent calcium channels leading to a decrease in cytosolic calcium, relaxation of pulmonary vessels, and a decrease in PVR.

After birth, eNOS abundance increases and peaks at 2–3 days. Subsequently, eNOS decreases in most vessels by 6 days and is nearly absent in the distal pulmonary arteries of adult animals (Halbower *et al.,* 1994; Hislop *et al.,* 1995). Despite the postnatal decrease of eNOS in small arteries, basal and stimulated production of NO from larger pulmonary arteries continues to increase after birth (Abman *et al.,* 1991; Steinhorn *et al.,* 1993).

The decrease in PVR is further augmented by a rapid increase in the oxygen-mediated availability of NO through a variety of mechanisms. Inhibition of nitric oxide synthase blocks the pulmonary vascular response of the near-term fetal lamb to hyperbaric oxygenation (Fig. 4). Oxygen directly increases basal and acetylcholine-stimulated cGMP production in the pulmonary vasculature (Shaul *et al.,* 1992). Acute changes in oxygen tension do not produce similar changes in mesenteric arteries, suggesting that this dramatic effect of oxygen is due to a direct and specific effect on NO production by fetal pulmonary arteries (Shaul and Wells, 1994). The acute oxygen modulation of pulmonary endothelial NO production does not appear to be a result of production of a local receptor agonist, or changes in availability of oxygen or L-arginine as substrates for NOS. Oxygen may, however, directly effect NOS by altering pulmonary endothelial cell calcium homeostasis.

Oxygen also causes a rapid increase in red blood cell adenosine triphosphate (ATP). ATP or its metabolite adenosine cause pulmonary vasodilation in the fetus, a response that is blocked by inhibition of NOS (Konduri *et al.,* 1992; Steinhorn *et al.,* 1994b). ATP may stimulate endothelial NO production either by binding to purinergic receptors or directly to NOS. Plasma ATP levels increase in the pulmonary arteries of fetal lambs during ventilation with oxygen, and the decrease in PVR that accompanies ventilation with oxygen is abolished by blockade of adenosine and ATP receptors (Konduri *et al.,* 1993). Thus, increased synthesis and release of ATP may cause pulmonary vasodilation in response to birth-related stimuli in the ovine fetus.

The peptide bradykinin stimulates endothelial NO production, and is a potent pulmonary vasodilator in the fetus. Ventilating fetal lambs with oxygen, or exposing the fetus to hyperbaric oxygen, increases the blood concentrations of bradykinin (Heymann *et al.,* 1969). However, blockade of bradykinin receptors does not block the pulmonary vasodilation to oxygen (Banerjee *et al.,* 1994), and it is unknown whether a direct interaction between bradykinin and NO plays a role in the development of the response to oxygen.

Closure of the ductus arteriosus at birth and the decrease in PVR lead to a large increase in pulmonary blood flow. This increase in pulmonary blood flow increases shear stress in the pulmonary vasculature and activates signaling cascades in endothelial cells which produce and/or potentiate pulmonary vasodilation via increased synthesis and release of NO. An increase in shear stress increases eNOS mRNA and protein expression in lung tissue (Black *et al.,* 1997). Therefore, whenever a stimulus initiates an increase in pulmonary blood flow during transition, it increases shear stress and creates a positive feedback loop in which increased NO synthesis increases pulmonary blood flow still further. At least a portion of the pulmonary vasodilation that results from shear stress may be attributed to NO-mediated activation of K^+-channels (Kv) in smooth muscle cells (Storme *et al.,* 1999).

The pattern of expression of soluble guanylate cyclase is similar to eNOS, with the highest levels of expression and activity noted in the first days following birth. Furthermore, within 1 hour following birth, PDE5 activity, protein, and mRNA dramatically decrease in newborn lamb and mouse

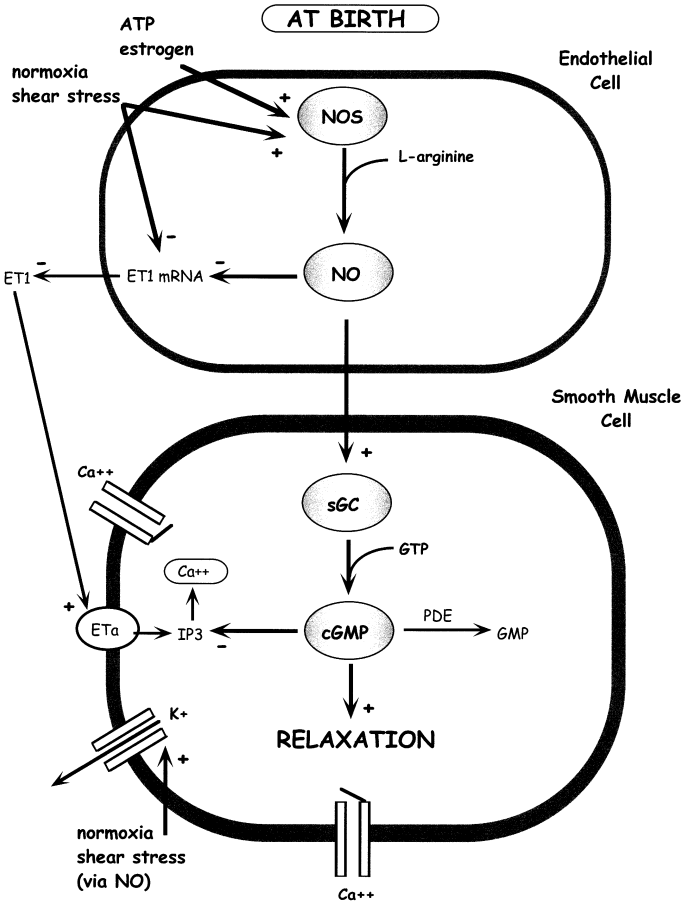


Figure 7 Diagram of the factors that favor low pulmonary vascular resistance at birth. The size and density of letters and lines reflect relative concentrations and activity.

lungs (Hanson *et al.,* 1998b). The low level of PDE5 activity following birth would be expected to enhance the effect of NO on pulmonary vascular smooth muscle by increasing cGMP levels in a manner that correlates well with the decrease in PVR early in transition.

Summary of Developmental Changes Leading to Transition

Early in fetal life PVR is high due to relatively few pulmonary capillaries, high vascular smooth muscle tone induced by hypoxia and endothelin, and a poorly developed NO–cGMP signaling pathway (Fig. 3). As lung development progresses toward term, the NO–cGMP pathway matures such that, at birth, it assumes a major role in the transition of the pulmonary circulation to a high flow/low pressure circuit (Fig. 7). Abnormalities in fetal lung development can interrupt maturation of the NO–cGMP pathway at several sites and interfere with this transition. The lack of a properly functioning NO–cGMP pathway for inhibition of pulmonary vascular tone at birth is a major contributing factor to the development of PPHN. The causes of PPHN, animal models for investigating this disease, and therapeutic regimens based on manipulation of the NO–cGMP pathway are discussed in the following sections.

Persistent Pulmonary Hypertension

Definition

The expected decrease in pulmonary vascular resistance and increase in pulmonary blood flow described earlier do not always occur normally during the transition to extrauterine life. Persistent pulmonary hypertension of the newborn (PPHN) is the result. This syndrome complicates more than 1 in 1000 live births and up to 10% of admissions to intensive care nurseries. There are no known genetic factors involved in its pathogenesis. Although it can occur in premature infants, PPHN is classically a disorder of the term infant.

PPHN is characterized by pulmonary hypertension causing right to left extrapulmonary shunting of blood and hypoxemia (Fig. 1C). It is a clinical syndrome rather than a specific disease, and is associated with a wide array of cardiac and respiratory disorders. PPHN may result when vasoconstriction of structurally normal pulmonary vessels

occurs in response to acute asphyxia, or alveolar hypoxia due to parenchymal disorders such as hyaline membrane disease or meconium aspiration syndrome. However PPHN can occur idiopathically in the absence of underlying parenchymal disease. It is believed that in these cases, the syndrome is the result of an abnormal remodeled vasculature that develops *in utero* in response to prolonged fetal stress, hypoxia, and pulmonary hypertension. PPHN causes substantial morbidity and mortality in otherwise normal term infants. Clinically, once recovery occurs, neurologic sequelae are a concern. Although there are theoretical concerns that an early insult may ''imprint'' the vasculature unfavorably, clinical outcome studies do not currently indicate that pulmonary hypertension recurs later in life.

Presently, over 1000 infants per year with PPHN require transport to specialized centers that provide extended heart lung bypass, known as extracorporeal membrane oxygenation or ECMO. Although ECMO is life-saving, it is expensive, invasive, and carries a substantial risk of morbidity. By design, cardiopulmonary bypass will decrease pulmonary blood flow and pressure and therefore may indirectly benefit the pulmonary vasculature. However it is not specific therapy designed to reverse PPHN. Therefore, understanding the role of mediators such as nitric oxide in the abnormal transition is important. This understanding should lead to development of safer, more effective therapies for PPHN and ultimately its prevention.

Human Studies

It is difficult to measure endogenous nitric oxide production in the newborn infant, and direct assay of NO production by pulmonary endothelial cells is not currently feasible. Therefore, the few studies in human infants have all have been based on more global measures of nitric oxide production. Urinary nitrites and nitrates are lower in infants with PPHN than in healthy term infants (Dollberg *et al.,* 1995). Plasma cGMP concentrations are also low, and increase rapidly in response to inhaled NO (Christou *et al.,* 1997). More recently, eNOS expression was found to be absent in umbilical vein endothelial cells cultured from four out of six infants who subsequently developed PPHN (Villaneuva *et al.,* 1998). Taken together, these studies provide indirect evidence for a deficiency of endogenous NO production in infants with PPHN. However, it is impossible to determine whether the absence of eNOS is the cause of PPHN, or whether PPHN leads to a loss of eNOS.

Effects of NO Synthase Inhibition or Disruption

Does a decrease in endogenous NOS activity produce clinical PPHN? Acute or chronic infusions of the nonspecific NO synthase inhibitor, L-NA, in fetal lambs produce physiological abnormalities consistent with PPHN following delivery (Abman *et al.,* 1990; Fineman *et al.,* 1994). Pulmonary arterial pressure and pulmonary vascular resistance are increased relative to control lambs, and hypoxemia results due to shunting of deoxygenated blood across the foramen ovale. These lambs are fully responsive to inhaled nitric oxide and nitric oxide donor agents, and following birth their pulmonary hypertension can be completely reversed with L-arginine. These findings suggest that inhibition or decreased activity of NOS could cause acute PPHN in some infants. Of particular interest is that persistent pulmonary hypertension develops in these lambs *without* associated pulmonary vascular remodeling. Therefore, reduction of endogenous NO production alone is probably not sufficient to produce the full physiological and anatomic picture of PPHN.

Mice with targeted disruption of endothelial or neuronal NOS offer an alternative experimental approach to infusions of pharmacological inhibitors, although measurements of pulmonary hemodynamics are technically challenging in adult animals, and currently not feasible in the newborn. Disruption of endothelial or neuronal NOS expression does not increase perinatal mortality. Adult eNOS −/− mice have at most a modest increase in pulmonary vascular resistance in a baseline unstressed state, but exhibit a striking exaggerated physiological response and increase in muscularization in peripheral arterioles in response to even mild hypoxia (Fagan *et al.,* 1999; Steudel *et al.,* 1998). Interestingly, even a 50% reduction of eNOS protein results in augmentation of the pulmonary vascular sensitivity to hypoxia.

Effect of PPHN on NO–cGMP Signaling

Most studies have addressed the converse of the previous question: Is PPHN associated with alterations in NOS expression and/or activity? Similar to the problem in evaluating the human studies, it must be kept in mind that it is difficult to differentiate whether alterations in specific enzymes are responsible for producing PPHN, or occur in response to it. Still, important insights into pathophysiology and therapeutic options can be gained from the study of animal models of PPHN.

Prenatal Development of Pulmonary Vascular Abnormalities

Newborns who die due to idiopathic persistent pulmonary hypertension of the newborn display an increase in pulmonary arterial medial smooth muscle and extension of muscle to normally nonmuscular pulmonary arteries (Haworth and Reid, 1976; Murphy *et al.,* 1981). The muscle cells are frequently surrounded by heavy elastic laminae, suggesting they formed several weeks before death. Further, these anatomic changes are observed in infants dying in the first 24 hours of life, which strongly suggests that an altered intrauterine environment may produce structural changes in the pulmonary circulation of the fetus.

To study the antenatal development of PPHN, intrauterine models of persistent pulmonary hypertension have been developed in the fetal lamb. The relatively large size of the fetal lamb makes it suitable for surgical intervention and physiological study as a fetus and immediate newborn. Fur-

thermore, the ewes tolerate uterine surgical intervention well, and are relatively resistant to premature labor.

Ductal Constriction or Ligation In the normal fetus, diversion of right ventricular output away from the lungs across the ductus arteriosus may be an important mechanism that protects against remodeling of pulmonary resistance arteries. PPHN is more common in postterm newborns, which may be due to intrauterine constriction of the fetal ductus arteriosus. A rare cause of PPHN in infants is prenatal constriction of the ductus arteriosus due to maternal ingestion of prostaglandin synthesis inhibitors. The most common model currently in use for the study of PPHN is the ductal ligation model.

Following surgical constriction of the ductus arteriosus, pulmonary blood flow acutely increases. However, within 2 hours pulmonary blood flow decreases back to baseline while pulmonary vascular resistance remains high. Fetal lambs born 7 to 14 days following ductal constriction or ligation have persistent pulmonary hypertension (Abman *et al.,* 1989; Morin, 1989), with all the physiological hallmarks of the human syndrome, including pulmonary arterial pressure equal to aortic pressure and hypoxemia unresponsive to ventilation with 100% oxygen. Structural alterations also occur, including extension of smooth muscle into the normally nonmuscular distal arteries and the formation of periadventitial fibrosis surrounding the intraacinar arteries (Wild *et al.,* 1989) (Fig. 8). These changes are identical to those observed in human infants.

Activity, message, and protein content of endothelial nitric oxide synthase are decreased by approximately 50% in lung extracts of ligated compared to control fetal lambs (Black *et al.,* 1998a; Shaul *et al.,* 1997; Villamor *et al.,* 1997). Preliminary studies indicate that neuronal NOS expression in the peripheral arterioles is also decreased (Tzao *et al.,* 1999). The pulmonary vasculature of ductal ligation lambs dilates in response to nitric oxide inhalation, but high concentrations are required to decrease pulmonary arterial pressure and pulmonary vascular resistance to near normal levels. This may be due in part to increased production of potent competing vasoconstrictors such as endothelin-1. However, this response pattern to exogenous NO can also be explained by other alterations in the nitric oxide–cGMP pathway as described later.

Relaxations to atrial natriuretic peptide and cGMP analogs are similar in control and hypertensive lambs, indicating that the remodeled pulmonary vessels relax normally when cGMP concentrations increase sufficiently. However, in addition to decreased endothelial NO synthase content and activity, soluble guanylyl cyclase content and activity are decreased. Pulmonary arteries isolated from PPHN lambs have diminished relaxations and cGMP accumulation in response to sodium nitroprusside and nitric oxide gas compared to controls (Steinhorn *et al.,* 1995). Protein contents for both the α and β subunits of soluble guanylate cyclase are decreased (Black *et al.,* 1998a; Tzao *et al.,* 1998), and immunostaining localizes the decrease in sGC expression to all levels of the arterial tree, with the most striking changes in the smallest resistance arterioles. Cyclic GMP specific phosphodiesterase activity appears to be elevated in PPHN lambs, by a mechanism that may involve posttranslational modification by phosphorylation. Increased phosphodiesterase activity would further depress the already decreased cGMP concentrations in response to endogenous and exogenous NO.

Abnormal Lung Growth Congenital diaphragmatic hernia (CDH) occurs when the diaphragmatic leaflets fail to fuse early in gestation, allowing the bowel to migrate into the chest cavity. Pulmonary hypoplasia occurs to a variable degree, probably in proportion to the volume and duration of intestinal herniation. In lung development, the vessels develop in parallel with the conducting airways. As a result, the pulmonary vascular bed in CDH is reduced in proportion to the degree of pulmonary hypoplasia, and abnormal muscularization of arterioles occurs as described earlier (Bohn *et al.,* 1987). Even with advanced support techniques such as ECMO, mortality has remained nearly 50%.

CDH can be induced in approximately 50% of rat fetuses after maternal ingestion of the herbicide nitrofen early in gestation. In this model, eNOS mRNA and protein abundance are decreased in the lung ipsilateral to the hernia compared to lungs from unaffected littermates. Congenital diaphragmatic hernia can also be surgically produced in the second trimester in the fetal lamb. Physiological and anatomic findings are similar to that seen in severely affected human infants. In this model, NO synthase content and functional activity are not altered in large pulmonary arteries (Karamanoukian *et al.,* 1995). However, NO synthase activity is abnormal in pulmonary veins, with the most striking abnormalities observed in veins isolated from the smaller lung ipsilateral to the hernia (Irish *et al.,* 1998). Relaxations to NO donor agents are normal in both pulmonary arteries and veins, indicating that at least prior to birth the vascular smooth muscle responds normally to NO. However, as described later, even when there is an initial dramatic improvement in oxygenation, infants with CDH are less likely to sustain a response to inhaled NO.

POSTNATAL DEVELOPMENT OF PULMONARY VASCULAR ABNORMALITIES

Hypoxic Vasoconstriction The development of acute vasoconstriction and chronic hypertension in response to hypoxia is a key feature that distinguishes the pulmonary circulation from the systemic circulation. In newborn and adult animals, acute hypoxia produces a prompt rise in pulmonary artery pressure and pulmonary vascular resistance. However, hypoxic vasoconstriction appears to be attenuated in the early newborn period, and increases strikingly with postnatal age (Fike and Hansen, 1987). This attenuation correlates well with the rapid alterations in NO–cGMP signaling that occur at birth and favor sustained increased cGMP concentrations and pulmonary vasodilation. The site of hypoxic vasoconstriction also varies with postnatal age. In contrast

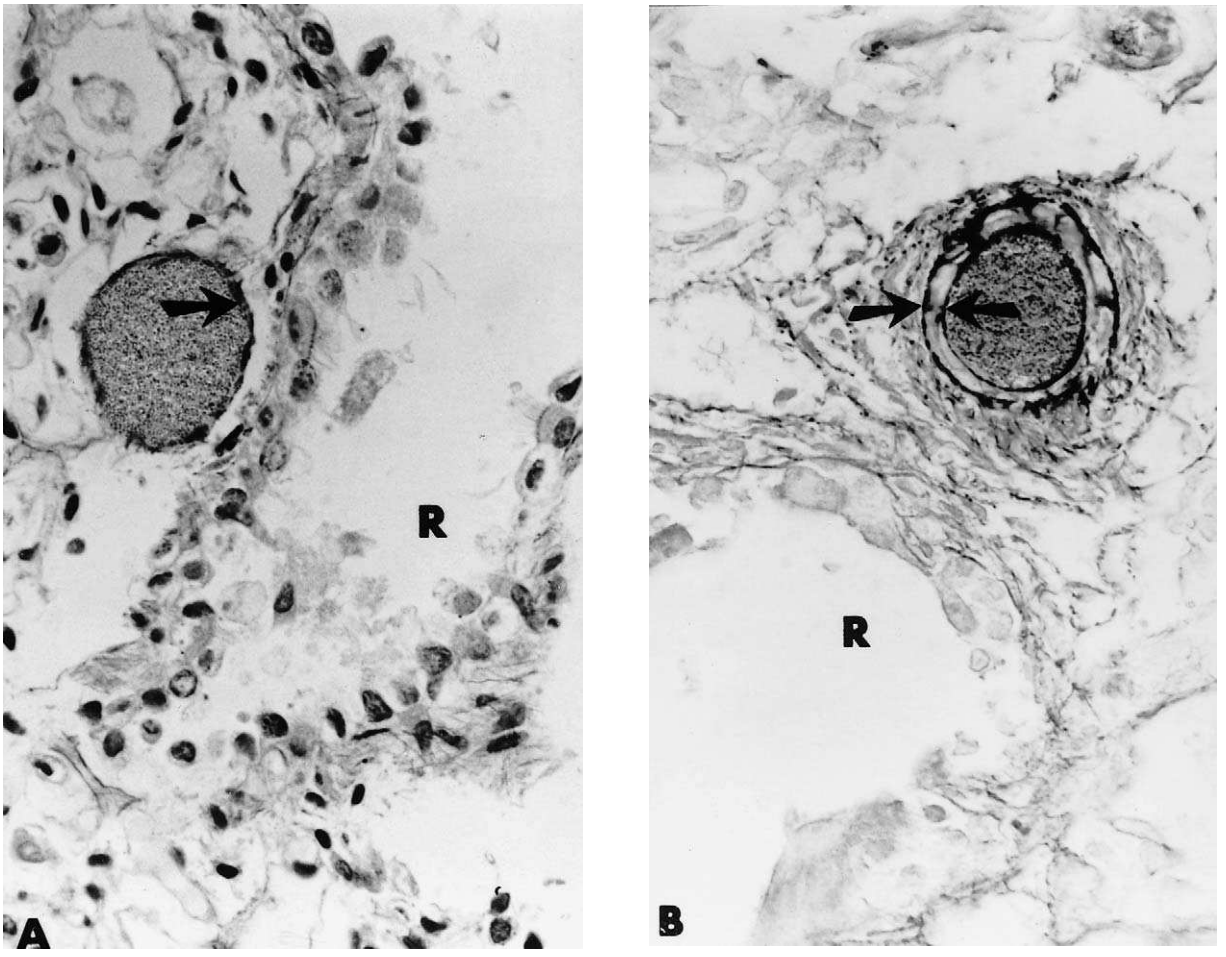


Figure 8 Example of pulmonary arterioles from normal near-term fetal lambs (A) and from lambs with PPHN following ductal ligation (B). In PPHN, the arterioles have a muscularized arterial wall, double elastic lamina (arrows), and adventitial proliferation. From Wild *et al.* (1989), with permission.

to adult lungs, in which the pulmonary arteries are the major site of hypoxic vasoconstriction, both pulmonary arterial and small diameter venous pressures increase following acute hypoxia in newborn lambs and piglets (Fike and Kaplowitz, 1992; Raj and Chen, 1986).

The role of NO synthase in modulating acute hypoxic pulmonary vasoconstriction remains controversial. Nitric oxide synthase inhibitors potentiate hypoxic vasoconstriction in newborn piglets and lambs (Gordon and Tod, 1993), suggesting that endogenous NO production may attenuate hypoxic vasoconstriction. However other studies demonstrate that decreased oxygen tension inhibits NO synthase expression and activity in fetal lambs (North *et al.,* 1996; Shaul *et al.,* 1992), and exhaled NO and NO metabolites fall during acute hypoxia in perfused lungs isolated from newborn piglets (Nelin *et al.,* 1996). Studies examining the newborn response to hypoxia following knockout of the NOS gene would be helpful, but have not been done because of the technical challenges.

Chronic Hypoxia Prolonged hypoxia following birth is an important cause of PPHN, and a widely used model for its study. Neonatal pulmonary vascular smooth muscle cells respond to hypoxia with a more vigorous proliferative response than adult cells, leading rapidly to structural changes. The vasculature of newborn piglets exposed to 72 hours of hypoxia from the moment of birth retains a fetal shape and spacial relationship, and the vascular smooth muscle cells retain a fetal phenotype (Haworth, 1988). In addition to retaining the fetal potential for smooth muscle cell proliferation, there is enhanced accumulation of extracellular matrix connective tissue components.

Important functional changes in NO-mediated relaxations accompany these structural changes. Endothelial nitric oxide synthase expression and activity are decreased in newborn animals exposed to chronic hypoxia (Fike *et al.,* 1998; Hislop *et al.,* 1997; Orton *et al.,* 1988). This is in contrast to the multiple studies indicating that in adult rats, prolonged hypoxia *enhances* expression of NOS (Lecras *et al.,* 1996; Shaul *et al.,* 1995), and that this upregulation occurs precisely at the onset of vascular remodeling (Xue and Johns, 1996). Further investigation will help delineate whether these opposite findings represent important development differences in NO synthase function, or whether chronic hy-

poxia produces alterations in the nitric oxide target enzyme pathways in the vascular smooth muscle cell which decrease sensitivity to endogenous and exogenous NO.

Alterations in smooth muscle response to NO may occur following chronic hypoxia. Relaxations and cGMP accumulation in response to both endogenous and exogenous NO are blunted following 7 days of hypoxia in adult rats, but responses to atrial natriuretic peptide (ANP) remain equivalent to controls (Crawley *et al.,* 1992). Rodman (1992), found that relaxations to endogenous NO, exogenous NO, and cGMP were all blunted in pulmonary arteries isolated from rats exposed to 35 days of hypoxia. Taken as a whole, these data indicate that in chronic hypoxia, defects in relaxation may develop over time, first at the level of the endothelium, followed by soluble guanylyl cyclase, followed by alterations in smooth muscle response to cGMP. Even briefer periods of hypoxia disrupt responses to exogenous nitric oxide in newborn piglets, indicating increased sensitivity to hypoxia during the early newborn period (Tulloh *et al.,* 1997).

Congenital Heart Disease Pulmonary hypertension commonly develops in infants with congenital heart lesions that are associated with increased pulmonary blood flow, such as truncus arteriosus or atrioventricular canal. If the heart lesion is not corrected, vascular changes of medial and intimal thickening occur, which ultimately lead to luminal obliteration. The obvious differences in pulmonary hypertension induced by a chronic increase in pulmonary blood flow from that induced by hypoxia has led to the development of specific animal models. As described earlier, increases in shear stress stimulate endothelial cells to produce several modulators of vascular tone, including NO. Large aortopulmonary shunts have been successfully placed in the late gestation ovine fetus, producing a model with the greatest similarity to children with congenital heart disease (Reddy *et al.,* 1995). At 1 month of age, the ratio of pulmonary to systemic blood flow is approximately 2 to 1; and while pulmonary vascular resistance is low after birth, it rises to near systemic values by 4 to 6 weeks of age. Morphological changes occur at this time, characterized by extension of muscle into small peripheral arteries, medial hypertrophy of small muscular arteries, and an increase in the total number of vessels (Reddy *et al.,* 1995).

High pulmonary blood flow from these shunts produces complex functional alterations in NO-mediated vasodilation. While endothelium-dependent vasodilation is decreased in 4 week old lambs, pulmonary vascular constriction to blockade of nitric oxide synthase is enhanced and plasma concentrations of cGMP are high (Reddy *et al.,* 1996). Expression of eNOS, the α and β subunits of soluble guanylyl cyclase, and Type 5 phosphodiesterase are all increased in lung parenchyma from shunted lambs, and *in situ* hybridization and immunohistochemistry localized the increase in eNOS to the endothelium of small and large pulmonary arteries (Black *et al.,* 1998b). These changes in NO–cGMP signaling are an interesting contrast to the changes observed in the ductal ligation model, indicating that pressure and flow may induce different abnormalities in endothelium–smooth muscle signaling.

Pulmonary hypertension can be dramatically exacerbated following cardiopulmonary bypass even in very young infants. Microemboli, neutrophil activation and sequestration, interruption of normal pulmonary blood flow, excessive thromboxane production, hypoxic vasoconstriction, and platelet adhesion all occur, and may disrupt endothelial function. If the endothelium is producing large amounts of NO prior to bypass, it is easy to envision that its disruption following bypass could shift the balance toward vasoconstrictors such as endothelin.

Clinical Importance of the NO–cGMP Pathway in PPHN

RESULTS OF CLINICAL TRIALS

To restore the normal transition in infants with PPHN, a vasodilator selective for the pulmonary circulation is needed. Nitric oxide is a gas, which allows it to be delivered directly to the lung. Further, the systemic circulation is protected because NO is rapidly inactivated by its combination with hemoglobin, forming nitrosohemoglobin and subsequently methemoglobin. Initial studies in animal models and human infants showed that NO inhaled at doses between 5 and 80 ppm improved systemic oxygenation in newborns with persistent pulmonary hypertension without decreasing systemic blood pressure. The animal studies further showed that inhalation of 80 ppm NO for 24 hours did not increase lung injury.

The clinical applications of inhaled NO have been studied in a wide range of populations and disease states, but to date the results are most compelling in the hypoxic newborn with PPHN. In two multicenter, randomized, placebo-controlled studies of term infants with PPHN (Table I), inhaled NO significantly improved systemic oxygenation and decreased the need for ECMO by approximately 30% (Neonatal Inhaled Nitric Oxide Study Group, 1997a; Roberts *et al.,* 1997). Although they report similar results (Fig. 9), the two studies used different concentrations of NO and enrolled quite different populations of infants. For example, although both studies enrolled infants with hypoxemia, documentation of pulmonary hypertension was only required for entry in the Roberts *et al.* (1997) trial. The similar outcomes in the two different patient populations may indicate that the clinical response to NO is not completely determined by the underlying disease state. A third large multicenter trial of NO inhalation studied patients earlier in the course of PPHN, and found a reduction in ECMO use similar to the two previous studies (Davidson *et al.,* 1998). However, *no* study has shown that NO reduces the incidence of death, neurologic sequelae, or chronic lung disease, findings that have been attributed to the availability and efficacy of ECMO. Although inhaled NO is an extraordinary advance in the therapeutic approach to PPHN, these studies clearly demonstrate that NO is not universally effective.

Table I Characteristics of Multicenter, Randomized, Placebo-Controlled Clinical Trials of Inhaled NO for PPHN

Authors	*N*	Entry criteria	Initial NO dose	Other therapies	Outcome measure
NINOS (1997a)	235	OI > 25 × 2, ≥34 weeks, ≤14 days old	20 ppm, may increase to 80 ppm	HFV permitted (55%), surfactant permitted (72%)	Death, ECMO
Roberts *et al.* (1997)	58	PPHN, ≥37 weeks, PaO_2 < 55	80 ppm	No HFV, surfactant permitted	Oxygenation (PaO_2 > 55)
Davidson *et al.* (1998)	155	PPHN, ≤72 hours old, >37 weeks, PaO_2 > 40 < 100	5, 20, or 80 ppm	No HFV, no surfactant	Death, ECMO, adverse sequelae

OI, oxygenation index; HFV, high frequency ventilation.

The correct clinical dose of inhaled NO is controversial. Animal studies, which allow for direct measurement of hemodynamics, indicate that pulmonary vascular resistance decreases in a dose-dependent fashion. However, in clinical studies, oxygenation and clinical efficacy were not different whether NO was inhaled at doses of 5, 20, or 80 ppm (Davidson *et al.,* 1998). Potential toxicities of NO are important in considering the ideal NO dose. NO is clinically delivered in combination with high concentrations of oxygen. This may favor the oxidation of NO to nitrogen dioxide, which even in very low concentrations can acutely injure the distal airways and alveoli, and disrupt the vascular endothelium. Furthermore, there may be increased production of superoxide due to inflammatory lung disease and high inspired oxygen concentrations. When NO comes into contact with superoxide, peroxynitrites are formed that may damage surfactant associated proteins, inhibit surfactant function, and cause cell damage. Another concern is that nitrosylhemoglobins formed when NO combines with hemoglobin are oxidized to methemoglobin. The balance of methemoglobin will depend on its rate of production, and the rate of elimination by methemoglobin reductase in the erythrocyte, an enzyme which has reduced activity in the newborn period. Significant increases in methemoglobin (> 7% of total hemoglobin) occur commonly during delivery of doses of 80 ppm to newborns. Finally, nitric oxide increases cGMP concentrations in platelets as well as in vascular smooth muscle, which can inhibit platelet aggregation and adhesion. NO inhalation increases bleeding time in healthy adult humans by 30%, and initial clinical trials indicate that premature infants treated with inhaled NO may have a very high incidence of intracranial hemorrhage.

CONGENITAL DIAPHRAGMATIC HERNIA: A UNIQUE SUBSET OF PATIENTS

Because of its unique pathophysiology, all of the previously mentioned clinical studies using inhaled NO specifically excluded infants with congenital diaphragmatic hernia. A multicenter trial enrolling only infants with CDH showed a significant *increase* in ECMO use following NO treatment, indicating that nitric oxide affects the hypoplastic lung adversely (Neonatal Inhaled Nitric Oxide Study Group, 1997b). The reason for this adverse effect is not clear. In some cases it may be due to poor lung recruitment due to surfactant deficiency. It is also likely that the hypoplastic pulmonary vasculature allows for sustained postnatal pul-

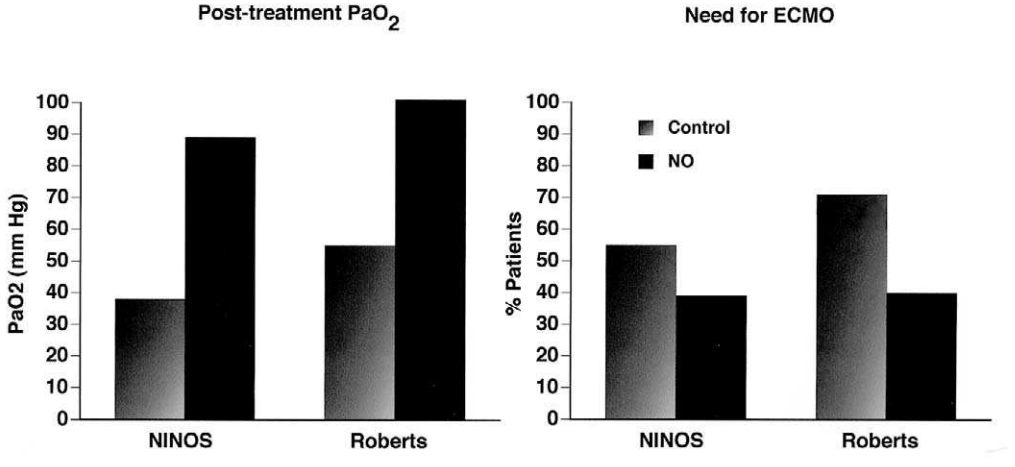


Figure 9 Comparison of results of two major clinical trials (NINOS, 1997a; Roberts *et al.,* 1997) of NO inhalation in infants with PPHN.

monary hypertension when pulmonary blood flow increases, subsequently leading to rapid vascular remodeling and decreased vascular smooth muscle responsiveness to NO. Infants with hypoplastic lungs who are initially refractory to inhaled NO do respond after a period of ECMO support (Karamanoukian *et al.,* 1994). This could result from improved lung recruitment due to restored surfactant synthesis, or possibly due to protection and recovery of the pulmonary vasculature during ECMO support.

CLINICAL PROBLEMS ENCOUNTERED USING NO

Inhaled NO is clearly not universally effective. In some cases, this may be due to lack of delivery to the target site. It is widely presumed that when NO is delivered as an inhaled gas, its small molecular weight allows it to simply diffuse through the pulmonary interstitium and vascular adventitia into the vascular smooth muscle cell. As discussed earlier, in reality, nitric oxide does not readily cross the adventitia of pulmonary vessels (Steinhorn *et al.,* 1994a). The clinical implication is that nitric oxide must be delivered to peripheral lung units to be effective, which can be difficult when parenchymal lung disease is present.

The clinical response to NO is more heterogenous than can be explained by lack of effective delivery to the lung periphery. Even a dramatic initial response to NO is often transient. To further complicate clinical use of NO, life-threatening rebound pulmonary hypertension may occur when NO is discontinued after only a few hours of inhalation (Miller *et al.,* 1995). Rebound pulmonary hypertension occurs even if the initial response to inhaled NO was modest, and may leave the patient in worse condition than prior to initiation of NO. It is attractive to theorize that decreased expression of endothelial NOS in response to exogenous NO is responsible for this response. NO donor agents acutely alter NOS activity, but do not alter eNOS expression in pulmonary artery endothelial cell cultures (Sheehy *et al.,* 1998). Chronic administration of NO downregulates soluble guanylyl cyclase activity in pulmonary vascular smooth muscle cells, indicating that the vascular smooth muscle cell may also alter its response to NO during prolonged exposures.

ENHANCEMENT OF NO EFFECT

Because NO must be delivered to peripheral lung units, clinical strategies designed to improve lung recruitment become critical components of successful therapy. Studies delivering inhaled nitric oxide in combination with high frequency oscillatory ventilation or exogenous surfactant indicate that these strategies may enhance the clinical efficacy of nitric oxide. Ventilation with oxygen-carrying perfluorochemicals is an exciting new experimental strategy that may improve lung recruitment in the face of severe surfactant deficiency or inactivation. Preliminary data indicate that the pharmacokinetics of inhaled nitric oxide are similar whether it is delivered to the perflurocarbon-filled or the conventionally gas-ventilated lung. The combination of these two therapies may therefore represent an additional way to deliver nitric oxide in the face of severe parenchymal lung disease.

Lung recruitment does not provide the whole answer to this clinical problem. It is important to note that the clinical efficacy of NO in the NINOS trial, which allowed use of lung recruitment strategies such as surfactant and high frequency ventilation, was not different than the Roberts *et al.* (1997) trial which restricted access to these therapies. After vascular injury, the adventitia, as well as the vascular smooth muscle cell, are sites of cellular proliferation in both animal models and human infants with PPHN. New reports indicate that the adventitia is metabolically active and produces superoxide. Furthermore, exogenous NO may induce superoxide formation in endothelial and other cells (Munzel *et al.,* 1995; Sheehy *et al.,* 1998). The relative intracellular activities of superoxide and superoxide dismutase (SOD) provide a potential pathway for modulating the effects of nitric oxide in the lung (Fig. 10). By reducing NO clearance by superoxide, SOD significantly enhances responses to nitric oxide *in vitro* (Cherry *et al.,* 1990). A newly developed recombinant human Cu,Zn-SOD is being tested in infants at high risk for lung injury. Experimental studies indicate that SOD in conjunction with nitric oxide may decrease oxidative lung injury and enhance the physiological effects of inhaled nitric oxide (Davis, 1998).

Similar to the animal models described earlier, some patients may not sustain a response to nitric oxide due to

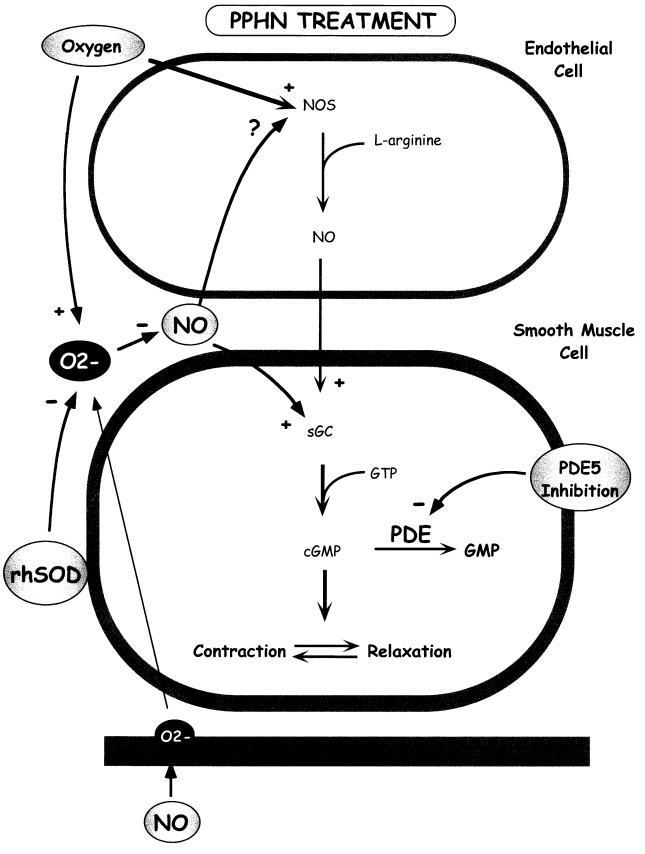


Figure 10 Diagram of potential points of the NO–cGMP signaling pathway that may allow for therapeutic intervention and enhancement of effect.

abnormalities of vascular development or function. This may be due to altered content or activity of soluble guanylyl cyclase or the cGMP phosphodiesterase isoenzyme. If so, inhibition of cGMP phosphodiesterase activity may provide an avenue for increasing efficacy of inhaled nitric oxide in human infants with PPHN by increasing cGMP concentrations (Fig. 9). Dipyridamole, which has been used for many years in humans, has significant inhibitory activity against cGMP phosphodiesterase. Dipyridamole augments the pulmonary vascular response to inhaled NO in adult patients with pulmonary hypertension following cardiopulmonary bypass (Fullerton *et al.,* 1997), and in some pediatric patients with pulmonary hypertension (Ziegler *et al.,* 1998). Dipyridamole may also prevent rebound pulmonary hypertension when inhaled NO is withdrawn (Ivy *et al.,* 1998b), and thereby allow for safer transport of infants who fail to respond to inhaled NO. There is little experience to date with its use in human infants. Dipyridamole selectively dilates the pulmonary circulation in the ovine fetus by augmenting the response to endogenous NO production (Ziegler *et al.,* 1995). However, dipyridamole inhibits other phosphodiesterase isoforms involved in cAMP metabolism, and its use must be approached with caution in the newborn period. In newborn lambs with PPHN, dipyridamole decreased pulmonary vascular resistance, but produced marked systemic hypotension at the same time (Dukarm *et al.,* 1998). Although the use of lower dipyridamole doses avoided systemic hypotension, they did not enhance the effect of inhaled NO.

Experimental pharmacological inhibitors such as zaprinast (M&B 22,948) and E4021 have more recently been developed for the Type 5 phosphodiesterase, and may be more potent and selective than dipyridamole. When zaprinast is administered in combination with a threshold dose of inhaled NO (6 ppm) in the ductal ligation lamb model, the drop in pulmonary vascular resistance and increase in oxygenation are quadrupled compared to nitric oxide alone (Thusu *et al.,* 1995). The combination of zaprinast and NO also significantly increases the duration of vasodilation to inhaled NO (Ichinose *et al.,* 1995).

If selective enough, phosphodiesterase inhibition may be effective even *without* the use of exogenous NO. For example, E4021, an experimental agent, is 100 times as potent as zaprinast for PDE5 (Saeki *et al.,* 1995), and has minimal to no inhibitory activity for other PDE isoenzymes. In newborn lambs with PPHN, increasing doses of E4021 result in selective pulmonary vasodilation without systemic hypotension. Using this strategy, a pulmonary vascular response equivalent to 50–100 ppm inhaled NO is possible by using E4021 alone.

Summary

Persistent pulmonary hypertension of the newborn is a syndrome that results from stresses on the pulmonary vasculature during critical developmental periods before or just after birth. Alterations in NO production and NO–cGMP signaling are clearly associated with PPHN, although it is not as clear whether they directly produce PPHN. Inhaled NO is now widely used to increase pulmonary blood flow and correct hypoxia in newborns with PPHN, but the clinical response is often absent or not sustained. Other methods to increase cGMP concentrations, such as inhibition of superoxide or cGMP-specific phosphodiesterase (PDE5), may enhance the response to inhaled NO and allow more infants to respond, to respond to lower concentrations of NO, and to sustain their response.

References

Abman, S. H., and Accurso, F. J. (1991). Sustained fetal pulmonary vasodilation with prolonged natriuretic factor and GMP infusions. *Am. J. Physiol.* **260,** H183–H192.

Abman, S. H., Shanley, P. F., and Accurso, F. J. (1989). Failure of postnatal adaptation of the pulmonary circulation after chronic intrauterine pulmonary hypertension in fetal lambs. *J. Clin. Invest.* **83,** 1849–1858.

Abman, S. H., Chatfield, B. A., Hall, S. L., and McMurtry, I. F. (1990). Role of endothelium-derived relaxing factor during transition of pulmonary circulation at birth. *Am. J. Physiol.* **259,** H1921–H1927.

Abman, S. H., Chatfield, B. A., Rodman, D. M., Hall, S. L., and McMurtry, I. F. (1991). Maturational changes in endothelium-derived relaxing factor activity of ovine pulmonary arteries *in vitro. Am. J. Physiol.* **260,** L280–L285.

Banerjee, A., Roman, C., and Heymann, M. A. (1994). Bradykinin receptor blockade does not affect oxygen mediated pulmonary vasodilation in fetal lambs. *Pediatr. Res.* **36,** 474–480.

Black, S. M., Johengen, M. J., Ma, Z. D., Bristow, J., and Soifer, S. J. (1997). Ventilation and oxygenation induce endothelial nitric oxide synthase gene expression in the lungs of fetal lambs. *J. Clin. Invest.* **100,** 1448–1458.

Black, S. M., Johengen, M. J., and Soifer, S. J. (1998a). Coordinated regulation of genes of the nitric oxide and endothelin pathways during the development of pulmonary hypertension in fetal lambs. *Pediatr. Res.* **44,** 821–830.

Black, S. M., Fineman, J. R., Steinhorn, R. H., Bristow, J., and Soifer, S. (1998b). Altered molecular expression of nitric oxide synthase in a lamb model of increased pulmonary blood flow. *Am. J. Physiol.* **275,** H1643–H1651.

Bloch, K. D., Filippov, G., Sanchez, L. S., Nakane, M., and delaMonte, S. M. (1997). Pulmonary soluble guanylate cyclase, a nitric oxide receptor, is increased during the perinatal period. *Am. J. Physiol.* **272,** L400–L406.

Bohn, D., Tamura, M., Perrin, D., Barker, G., and Rabinovitch, M. (1987). Ventilatory predictors of pulmonary hypoplasia in congenital diaphragmatic hernia, confirmed by morphologic assessment. *J. Pediatr.* **111,** 423–431.

Cherry, P. D., Omar, H. A., Farrell, K. A., Stuart, J. S., and Wolin, M. S. (1990). Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. *Am. J. Physiol.* **259,** H1056–H1062.

Christou, H., Adatia, I., VanMarter, L. J., Kane, J. W., Thompson, J. E., Stark, A. R., Wessel, D. L., and Kourembanas, S. (1997). Effect of inhaled nitric oxide on endothelin-1 and cyclic guanosine 5'-monophosphate plasma concentrations in newborn infants with persistent pulmonary hypertension. *J. Pediatr.* **130,** 603–611.

Crawley, D. E., Zhao, L., Giembycz, M. A., Liu, S., Barnes, P. J., Winter, R. J. D., and Evans, T. W. (1992). Chronic hypoxia impairs soluble guanylyl cyclase-mediated pulmonary arterial relaxation in the rat. *Am. J. Physiol.* **263,** L325–L332.

Cummings, J. J. (1997). Nitric oxide decreases lung liquid production in fetal lambs. *Am. J. Physiol.* **83,** 1538–1544.

D'Angelis, C., Nickerson, P. A., Steinhorn, R. H., and Morin III, F. C. (1998). Heterogeneous distribution of soluble guanylate cyclase in the pulmonary vasculature of the fetal lamb. *Anat. Rec.* **250,** 62–69.

Davidson, D., Barefield, E. S., Kattwinkel, J., Dudell, G., Damask, M., Straube, R., Rhines, J., and Chang, C. (1998). Inhaled nitric oxide for the early treatment of persistent pulmonary hypertension of the term newborn: A randomized, double-masked, placebo-controlled, dose-response, multicenter study. *Pediatrics* **101,** 325–334.

Davis, J. M. (1998). Superoxide dismutase: A role in the prevention of chronic lung disease. *Biol. Neonate* **74**(Suppl. 1), 29–34.

Dollberg, S., Warner, B. W., and Myatt, L. (1995). Urinary nitrite and nitrate concentrations in patients with idiopathic persistent pulmonary hypertension of the newborn and effect of extracorporeal membrane oxygenation. *Pediatr. Res.* **37,** 31–34.

Dukarm, R. C., Morin, F. C. I., Russell, J. A., and Steinhorn, R. H. (1998). Pulmonary and systemic effects of the phosphodiesterase inhibitor dipyridamole in newborn lambs with persistent pulmonary hypertension. *Pediatr. Res.* **44,** 831–837.

Fagan, K. A., Fouty, B. W., Tyler, R. C., Morris, K. G., Hepler, L. K., Sato, K., LeCras, T. D., Abman, S. H., Weinberger, H. D., Huang, P. L., McMurtry, I. F., and Rodman, D. M. (1999). The pulmonary circulation of homozygous or heterozygous eNos-null mice is hyperresponsive to mild hypoxia. *J. Clin. Invest.* **103,** 291–299.

Fike, C. D., and Hansen, T. N. (1987). Hypoxic vasoconstriction increases with postnatal age in lungs from newborn rabbits. *Circ. Res.* **60,** 297–303.

Fike, C. D., and Kaplowitz, M. R. (1992). Pulmonary venous pressure increases during alveolar hypoxia in isolated lungs of newborn pigs. *J. Appl. Physiol.* **73,** 552–556.

Fike, C. D., Kaplowitz, M. R., Thomas, C. J., and Nelin, L. D. (1998). Chronic hypoxia decreases nitric oxide production and endothelial nitric oxide synthase in newborn pig lungs. *Am. J. Physiol.* **274,** L517–L526.

Fineman, J. R., Wong, J., Morin III, F. C., Wild, L. M., and Soifer, S. J. (1994). Chronic nitric oxide inhibition *in utero* produces persistent pulmonary hypertension in newborn lambs. *J. Clin. Invest.* **93,** 2675–2683.

Fouty, B., Komalavilas, P., Muramatsu, M., Cohen, A., McMurtry, I. F., Lincoln, T. M., and Rodman, D. M. (1998). Protein kinase G is not essential to NO–cGMP modulation of basal tone in rat pulmonary circulation. *Am. J. Physiol.* **274,** H672–H678.

Fullerton, D. A., Jaggers, J., Piedalue, F., Grover, F. L., and McIntyre, R. C. (1997). Effective control of refractory pulmonary hypertension after cardiac operations. *J. Thorac. Cardiovasc. Surg.* **113,** 363–370.

Gao, Y., Zhou, H., and Raj, J. U. (1995). Heterogeneity in role of endothelium-derived NO in pulmonary arteries and veins of full-term fetal lambs. *Am. J. Physiol.* **268,** H1586–H1592.

Gordon, J. B., and Tod, M. L. (1993). Effects of *N*-nitro-L-arginine on total and segmental vascular resistances in developing lamb lungs. *J. Appl. Physiol.* **75,** 76–85.

Halbower, A. C., Tuder, R. M., Franklin, W. A., Pollock, J. S., Forstermann, U., and Abman, S. H. (1994). Maturation-related changes in endothelial nitric oxide synthase immunolocalization in developing ovine lung. *Am. J. Physiol.* **267,** L585–L591.

Hanson, K. A., Ziegler, J. W., Tybalkin, S. D., Miller, J. W., Abman, S. H., and Clarke, W. R. (1998a). Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity. *Am. J. Physiol.* **275,** L931–L941.

Hanson, K. A., Burns, F., Rybalkin, S. D., Miller, J. W., Beavo, J., and Clarke, W. R. (1998b). Developmental changes in lung cGMP phosphodiesterase-5 activity, protein, and message. *Am. J. Respir. Crit. Care Med.* **158,** 279–288.

Haworth, S. G. (1981). Normal structural and functional adaptation to extrauterine life. *J. Pediatr.* **98,** 915–918.

Haworth, S. G. (1988). Pulmonary vascular remodeling in neonatal pulmonary hypertension. *Chest* **93,** 133S–138S.

Haworth, S., and Reid, L. (1976). Persistent fetal circulation: Newly recognized structural features. *J. Pediatr.* **88,** 614–620.

Haworth, S., Hall, S. M., Chew, M., and Allen, K. (1987). Thinning of fetal pulmonary arterial wall and postnatal remodeling: Ultrastructural studies on the respiratory unit arteries of the pig. *Virchows Arch. A* **411,** 161–171.

Heymann, M. A., Rudolph, A. M., Nies, A. S., and Melmon, K. L. (1969). Bradykinin production associated with oxygenation of the fetal lamb. *Circ. Res.* **25,** 521–534.

Hislop, A. A., Springall, D. R., Buttery, L. K., Pollock, J. S., and Haworth, S. G. (1995). Abundance of endothelial nitric oxide synthase in newborn intrapulmonary arteries. *Arch. Dis. Child.* **73,** F17–F21.

Hislop, A. A., Springall, D. R., Oliveira, H., Pollock, J. S., Polak, J. M., and Haworth, S. G. (1997). Endothelial nitric oxide synthase in hypoxic newborn porcine pulmonary vessels. *Arch. Dis. Child.* **77,** F16–F22.

Ichinose, F., Adrie, C., Hurford, W. E., and Zapol, W. M. (1995). Prolonged pulmonary vasodilator action of inhaled nitric oxide by zaprinast in awake lambs. *J. Appl. Physiol.* **78,** 1288–1295.

Irish, M. S., Glick, P. L., Russell, J. A., Kapur, P., Bambini, D., Holm, B. A., and Steinhorn, R. H. (1998). Contractile properties of intralobar pulmonary arteries and veins in the fetal lamb model of congenital diaphragmatic hernia. *J. Pediatr. Surg.* **33,** 921–928.

Ivy, D. D., Kinsella, J. P., and Abman, S. H. (1994). Physiologic characterization of endothelin A and B receptor activity in the ovine fetal pulmonary circulation. *J. Clin. Invest.* **93,** 2141–2148.

Ivy, D. D., Ziegler, J. W., Dubus, M. F., Fox, J. J., Kinsella, J. P., and Abman, S. H. (1996). Chronic intrauterine pulmonary hypertension alters endothelin receptor activity in the ovine fetal lung. *Pediatr. Res.* **39,** 435–442.

Ivy, D. D., LeCras, T. D., Horan, M. P., and Abman, S. H. (1998a). Developmental regulation of endothelin gene expression in the ovine lung. *Pediatr. Res.* **43,** 287A.

Ivy, D. D., Kinsella, J. P., Ziegler, J. W., and Abman, S. H. (1998b). Dipyridamole attenuates rebound pulmonary hypertension after inhaled nitric oxide withdrawal in postoperative congenital heart disease. *J. Thorac. Cardiovasc. Surg.* **115,** 875–882.

Karamanoukian, H. L., Glick, P. L., Zayek, M., Steinhorn, R. H., Zwass, M. S., Fineman, J. R., and Morin III, F. C. (1994). Inhaled nitric oxide in congenital hypoplasia of the lungs due to diaphragmatic hernia or oligohydramnios. *Pediatrics* **94,** 715–718.

Karamanoukian, H., Glick, P., Wilcox, D., Rossman, J., and Azizkhan, R. (1995). Pathophysiology of congenital diaphragmatic hernia X: Localization of nitric oxide synthase in the intima of pulmonary artery trunks of lambs with surgically created congenital diaphragmatic hernia. *J. Pediatr. Surg.* **30,** 5–9.

Kinsella, J. P., Ivy, D. D., and Abman, S. H. (1994). Ontogeny of NO activity and response to inhaled NO in the developing ovine pulmonary circulation. *Am. J. Physiol.* **267,** H1955–H1961.

Kojima, T., Isozaki-Fikuda, Y., Takedatsu, M., Ono, A., Kurata, Y., and Kobayashi, Y. (1992). Plasma endothelin-1 like immunoreactivity levels in neonates. *Eur. J. Pediatr.* **151,** 913–915.

Konduri, G. G., Woodard, L. L., Mukhopadhyay, A., and Deshmukh, D. R. (1992). Adenosine is a pulmonary vasodilator in newborn lambs. *Am. Rev. Respir. Dis.* **146,** 670–676.

Konduri, G. G., Gervasio, C. T., and Theodorou, A. A. (1993). Role of adenosine triphosphate and adenosine in oxygen-induced pulmonary vasodilation in fetal lambs. *Pediatr. Res.* **33,** 533–539.

Kourembanas, S., McQuillan, L. P., Leung, G. K., and Faller, D. V. (1993). Nitric oxide regulates the expression of vasoconstrictors and growth factors by vascular endothelium under both normoxia and hypoxia. *J. Clin. Invest.* **92,** 99–104.

Lantin-Hermoso, R. L., Rosenfeld, C. R., Yuhanna, I. S., German, Z., Chen, Z., and Shaul, P. W. (1997). Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *Am. J. Physiol.* **273,** L119–L126.

Lecras, T. D., Xue, C., Rengasamy, A., and Johns, R. A. (1996). Chronic hypoxia upregulates endothelial and inducible NO synthase gene and protein expression in rat lung. *Am. J. Physiol.* **14,** L164–L170.

Lewis, A. B., Heymann, M. A., and Rudolph, A. M. (1976). Gestational changes in pulmonary vascular responses in fetal lambs *in utero*. *Circ. Res.* **39,** 536–541.

Loesch, A., and Burnstock, G. (1996). Ultrastructural localization of nitric oxide synthase and endothelin in rat pulmonary artery and vein during postnatal development and aging. *Cell Tissue Res.* **283,** 355–365.

MacCumber, M. W., Ross, C. A., Glaser, B. M., and Snyder, S. H. (1989). Endothelin: Visualization of mRNAs by *in situ* hybridization provides evidence for local action. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 7285–7289.

McQuillan, L. P., Leung, G. K., Marsden, P. A., Kostyk, S. K., and Kourembanas, S. (1994). Hypoxia inhibits expression of eNOS via transcriptional and post-transcriptional mechanisms. *Am. J. Physiol.* **267,** H1921–H1927.

MacRitchie, A. N., Jun, S. S., Chen, Z., German, Z., Yuhanna, I. S., Sherman, T. S., and Shaul, P. W. (1997). Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circ. Res.* **81,** 355–362.

Millard, S. L., Russell, J. A., Morin III, F. C., Adolf, M. A., Gugino, S. F., and Steinhorn, R. H. (1998). cGMP inhibition of endothelin-stimulated inositol phosphate production in the fetal lamb pulmonary artery. *Pulm. Pharm. Ther.* **11,** 201–204.

Miller, O. I., Tang, S. F., Keech, A., and Celermajer, D. S. (1995). Rebound pulmonary hypertension on withdrawal from inhaled nitric oxide. *Lancet* **346,** 51–52.

Morin III, F. C. (1989). Ligating the ductus arteriosus before birth causes persistent pulmonary hypertension in the newborn lamb. *Pediatr. Res.* **25,** 245–250.

Morin III, F. C., and Egan, E. A. (1992). Pulmonary hemodynamics in fetal lambs during development at normal and increased oxygen tension. *J. Appl. Physiol.* **73,** 213–218.

Morin III, F. C., Egan, E. A., and Norfleet, W. T. (1988). Indomethacin does not diminish the pulmonary vascular response of the fetus to increased oxygen tension. *Pediatr. Res.* **24,** 696–700.

Munzel, T., Sayegh, H., Freeman, B. A., Tarpey, M. M., and Harrison, D. G. (1995). Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J. Clin. Invest.* **95,** 187–194.

Murphy, J. D., Rabinovitch, M., Goldstein, J. D., and Reid, L. M. (1981). The structural basis of persistent pulmonary hypertension of the newborn infant. *J. Pediatr.* **98,** 962–967.

Nelin, L. D., Thomas, D. J., and Dawson, C. A. (1996). Effect of hypoxia on nitric oxide production in neonatal pig lung. *Am. J. Physiol.* **40,** H8–H14.

Neonatal Inhaled Nitric Oxide Study Group (NINOS) (1997a). Inhaled nitric oxide in full-term and nearly full-term infants with hypoxic respiratory failure. *N. Engl. J. Med.* **336,** 597–604.

Neonatal Inhaled Nitric Oxide Study Group (NINOS) (1997b). Inhaled nitric oxide and hypoxic respiratory failure in infants with congenital diaphragmatic hernia. *Pediatrics* **99,** 838–845.

North, A. J., Brannon, T. S., Michel, T., Wu, L. C., Wells, L. B., and Shaul, P. W. (1994a). Nitric oxide modulates nitric oxide synthase gene expression in fetal pulmonary artery endothelial cells. *Pediatr. Res.* **35,** 347A.

North, A. J., Star, R. A., Brannon, T. S., Ujiie, K., Wells, L. B., Lowenstein, C. J., Snyder, S. H., and Shaul, P. W. (1994b). Nitric oxide synthase type I and type III gene expression are developmentally regulated in rat lung. *Am. J. Physiol.* **266,** L635–L641.

North, A. J., Lau, K. S., Brannon, T. S., Wu, L. C., Wells, L. B., German, Z., and Shaul, P. W. (1996). Oxygen upregulates nitric oxide synthase gene expression in ovine fetal pulmonary artery endothelial cells. *Am. J. Physiol.* **270,** L643–L649.

Okogbule-Wonodi, A. C., Ibe, B. O., Yue, B. W., Hsu, S., and Raj, J. U. (1998). Phosphodiesterase activity in intrapulmonary arteries and veins of perinatal lambs. *Mol. Genet. Metab.* **65,** 229–237.

Orton, E. C., Reeves, J. T., and Stenmark, K. R. (1988). Pulmonary vasodilation with structurally altered pulmonary vessels and pulmonary hypertension. *J. Appl. Physiol.* **65,** 2459–2467.

Perreault, T., and Baribeau, J. (1995). Characterization of endothelin receptors in newborn piglet lung. *Am. J. Physiol.* **268,** L607–L614.

Rairigh, R. L., Cras, T. D. L., Ivy, D. D., Kinsella, J. P., Richter, G., Horan, M. P., Fan, I. D., and Abman, S. H. (1998). Role of inducible nitric oxide synthase in regulation of pulmonary vascular tone in the late gestation ovine fetus. *J. Clin. Invest.* **101,** 15–21.

Raj, J. U., and Chen, P. (1986). Micropuncture measurement of microvascular pressures in isolated lamb lungs during hypoxia. *Circ. Res.* **59,** 398–404.

Reddy, V. M., Meyrick, B., Wong, J., Khoor, A., Liddicoat, J. R., Hanley, F. L., and Fineman, J. R. (1995). *In utero* placement of aortopulmonary shunts. *Circulation* **92,** 606–613.

Reddy, V. M., Wong, J., Liddicoat, J. R., Johengen, M., Chang, R., and Fineman, J. R. (1996). Altered endothelium-dependent responses in lambs with pulmonary hypertension and increased pulmonary blood flow. *Am. J. Physiol.* **40,** H562–H570.

Reid, L. (1986). Structure and function in pulmonary hypertension. New perceptions. *Chest* **89,** 279–288.

Roberts, J. D., Fineman, J., Morin III, F. C., Shaul, P. W., Rimar, S., Schreiber, M. D., Polin, R. A., Thusu, K. G., Zayek, M., Zwass, M. S., Zellers, T. M., Wylam, M. E., Gross, I., Zapol, W. M., and Heymann, M. A. (1997). Inhaled nitric oxide gas improves oxygenation in PPHN. *N. Engl. J. Med.* **336,** 605–610.

Rodman, D. M. (1992). Chronic hypoxia selectively augments rat pulmonary artery Ca^{++} and K^{+} channel-mediated relaxation. *Am. J. Physiol.* **263,** L88–L94.

Rudolph, A. M. (1985). Distribution and regulation of blood flow in the fetal and neonatal lamb. *Circ. Res.* **57,** 811–821.

Rudolph, A. M., and Heymann, M. A. (1970). Circulatory changes during growth in the fetal lamb. *Circ. Res.* **26,** 289–299.

Russwurm, M., Behrends, S., Harteneck, C., and Koesling, D. (1998). Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. *Biochem. J.* **335,** 125–130.

Saeki, T., Adachi, H., Takase, Y., Yoshitake, S., Souda, S., and Saito, I. (1995). A selective type V phosphodiesterase inhibitor, E4021, dilates porcine large coronary artery. *J. Pharmacol. Exp. Ther.* **272,** 825–831.

Sanchez, L. S., Del La Monte, S. M., Filippov, G., Jones, R. C., Zapol, W. M., and Bloch, K. D. (1998). Cyclic-GMP-binding, cyclic-GMP-specific phosphodiesterase gene expression is regulated during rat pulmonary development. *Pediatr. Res.* **43,** 163–168.

Shaul, P. W., and Wells, L. B. (1994). Oxygen modulates nitric oxide production selectively in fetal pulmonary endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **11,** 432–438.

Shaul, P. W., Farrar, M. A., and Zellers, T. M. (1992). Oxygen modulates endothelium-derived relaxing factor production in fetal pulmonary arteries. *Am. J. Physiol.* **262,** H355–H364.

Shaul, P. W., Farrar, M. A., and Magness, R. R. (1993). Pulmonary endothelial nitric oxide production is developmentally regulated in the fetus and newborn. *Am. J. Physiol.* **265,** H1056–H1063.

Shaul, P. W., North, A. J., Brannon, T. S., Ujiie, K., Wells, L. B., Nisen, P. A., Lowenstein, C. J., Snyder, S. H., and Star, R. A. (1995). Prolonged *in vivo* hypoxia enhances endothelial and neuronal nitric oxide synthase gene expression in adult rat lung. *Am. J. Respir. Cell Mol. Biol.* **13,** 167–174.

Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G., and Michel, T. (1996). Acylation targets endothelial nitric oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271,** 6518–6522.

Shaul, P. W., Yuhanna, I. S., German, Z., Chen, Z., Steinhorn, R. H., and Morin III, F. C. (1997). Pulmonary endothelial NO synthase gene expression is decreased in fetal lambs with pulmonary hypertension. *Am. J. Physiol.* **272,** L1005–L1012.

Sheehy, A. M., Burson, M. A., and Black, S. M. (1998). Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: A role for superoxide. *Am. J. Physiol.* **274,** L833–L841.

Sherman, T. S., Chen, Z., Yuhanna, I. S., Lau, K. S., Margraf, L. R., and Shaul, P. W. (1999). Nitric oxide synthase isoform expression in the developing lung epithelium. *Am. J. Physiol.* **276,** L383–L390.

Steinhorn, R. H., Morin III, F. C., Gugino, S. F., Giese, E. C., and Russell, J. A. (1993). Developmental differences in endothelium-dependent responses in isolated ovine pulmonary arteries and veins. *Am. J. Physiol.* **264,** H2162–H2167.

Steinhorn, R. H., Morin III, F. C., and Russell, J. A. (1994a). The adventitia may be a barrier specific to nitric oxide in rabbit pulmonary artery. *J. Clin. Invest.* **94,** 1883–1888.

Steinhorn, R. H., Morin III, F. C., VanWylen, D. G. L., Gugino, S. F., Giese, E. C., and Russell, J. A. (1994b). Endothelium-dependent relaxations to adenosine in juvenile rabbit pulmonary arteries and veins. *Am. J. Physiol.* **266,** H2001–H2006.

Steinhorn, R. H., Russell, J. A., and Morin III, F. C. (1995). Disruption of cyclic GMP production in pulmonary arteries isolated from fetal lamb with pulmonary hypertension. *Am. J. Physiol.* **268,** H1483–H1489.

Steudel, W., Scherrer-Crosbie, M., Bloch, K. D., Weimann, J., Huang, P. L., Jones, R. C., Picard, M. H., and Zapol, W. M. (1998). Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase 3. *J. Clin. Invest.* **101,** 2468–2477.

Storme, L., Rairigh, R. L., Parker, T. A., Cornfield, D. N., Kinsella, J. P., and Abman, S. H. (1999). K^+ channel blockade inhibits shear stress-induced pulmonary vasodilation in the ovine fetus. *Am. J. Physiol.* **276,** L220–L228.

Teitel, D. F., Iwamoto, H. S., and Rudolph, A. M. (1990). Changes in the pulmonary circulation during birth-related events. *Pediatr. Res.* **27,** 372–378.

Thusu, K. G., Morin III, F. C., Russell, J. A., and Steinhorn, R. H. (1995). The cGMP phosphodiesterase inhibitor zaprinast enhances the effect of nitric oxide. *Am. J. Respir. Crit. Care Med.* **152,** 1605–1610.

Tiktinsky, M. H., and Morin III, F. C. (1993). Increasing oxygen tension dilates fetal pulmonary circulation via endothelium-derived relaxing factor. *Am. J. Physiol.* **265,** H376–H380.

Tiktinsky, M. H., Cummings, J. J., and Morin III, F. C. (1992). Acetylcholine increases pulmonary blood flow in intact fetuses via endothelium-dependent vasodilation. *Am. J. Physiol.* **262,** H406–H410.

Tulloh, R. M. R., Hislop, A. A., Boels, P. J., Deutsch, B. J., and Haworth, S. G. (1997). Chronic hypoxia inhibits postnatal maturation of porcine intrapulmonary artery relaxation. *Am. J. Physiol.* **272,** H2436–H2445.

Tzao, C., Russell, J. A., Nickerson, P. A., Gugino, S. F., and Steinhorn, R. H. (1998). Content of specific subunits of soluble guanylyl cyclase in pulmonary vessels from lambs with pulmonary hypertension. *Pediatr. Res.* **43,** 301A.

Tzao, C., Nickerson, P. A., Russell, J. A., and Steinhorn, R. H. (1999). Distribution of nitic oxide synthase (NOS) I and III and the α 1 subunit of soluble guanylate cyclase (sGC) in the pulmonary vasculature of the ovine fetus. *Am. J. Respir. Crit. Care Med.* **159,** A898.

Villamor, E., LeCras, T. D., Horan, M. P., Halbower, A. C., Tuder, R. M., and Abman, S. H. (1997). Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus. *Am. J. Physiol.* **272,** L1013–L1020.

Villaneuva, M. E., Zaher, F. M., Svinarich, D. M., and Konduri, G. G. (1998). Decreased gene expression of endothelial nitric oxide synthase in newborns with persistent pulmonary hypertension. *Pediatr. Res.* **44,** 338–343.

Weir, E. K., and Archer, S. L. (1995). The mechanism of acute hypoxic pulmonary vasoconstriction: The tale of two channels. *FASEB J.* **9,** 183–189.

Wild, L. M., Nickerson, P. A., and Morin III, F. C. (1989). Ligating the ductus arteriosus before birth remodels the pulmonary vasculature of the lamb. *Pediatr. Res.* **25,** 251–257.

Xue, C., and Johns, R. A. (1996). Upregulation of nitric oxide synthase correlates temporally with onset of pulmonary vascular remodeling in the hypoxic rat. *Hypertension* **28,** 743–753.

Xue, C., Reynolds, P. R., and Johns, R. A. (1996). Developmental expression of NOS isoforms in fetal rat lung: Implications for transitional circulation and pulmonary angiogenesis. *Am. J. Physiol.* **270,** L88–L100.

Zellers, T. M., McCormick, J., and Wu, Y. (1994). Interaction among ET-1, endothelium-derived nitric oxide and prostacyclin in pulmonary arteries and veins. *Am. J. Physiol.* **267,** H139–H147.

Ziegler, J. W., Ivy, D. D., Fox, J. J., Kinsella, J. P., Clarke, W. R., and Abman, S. H. (1995). Dipyridamole, a cGMP phosphodiesterase inhibitor, causes pulmonary vasodilation in the ovine fetus. *Am. J. Physiol.* **269,** H473–H479.

Ziegler, J. W., Ivy, D. D., Wiggins, J. W., Kinsella, J. P., Clarke, W. R., and Abman, S. H. (1998). Effects of dipyridamole and inhaled nitric oxide in pediatric patients with pulmonary hypertension. *Am. J. Respir. Crit. Care Med.* **158,** 1388–1395.

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