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The Emerging Multifaceted Roles of Nitric Oxide

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Nitric oxide (NO) is a highly reactive free radical with a multitude of organ specific regulatory functions. Since 1985, NO has been the subject of numerous research efforts and as a result, has been found to play a major role in the cardiovascular, pulmonary, gastrointestinal, immune, and central nervous systems. In addition, deranged NO synthesis is the basis for a number of pathophysiologic states, such as atherosclerosis, pulmonary hypertension, pyloric stenosis, and the hypertension associated with renal failure. Traditional NO donors such as sodium nitroprusside and new pharmacologic NO adducts such as S-nitrosothiols may serve as exogenous sources of NO for the treatment of NO-deficient pathologic states. This review is an attempt to acquaint the surgical community with the fundamentals of NO biochemistry and physiology. Increased knowledge of its functions in normal homeostasis and pathologic states will enable physicians to better understand these disease processes and utilize new pharmacologic therapies.

Recently, the free radical, nitric oxide (NO) has received a tremendous amount of attention in the biological and medical literature. Between 1981 and 1986, ten papers related to NO biosynthesis were published. During the next 5 years, there were more than 500 such publications.[1] A computer search for the keyword, nitric oxide, generated more than 1500 publications in the world literature during the calendar year 1993. Studies have implicated NO as a mediator, messenger, or regulator of cell function in physiologic states that include vascular tone, platelet function, short- and long-term memory, hepatocyte respiratory function, septic shock, and penile erectile function. The enthusiastic response to nitric oxide biology will be tempered by the test of time. The observations of a noted pharmacologist are particularly germane, "Any new drug discovery will begin with a wave of enthusiasm associated with uncontrolled use, followed by a wave of damnation, ultimately followed by studies that reveal the true worth of the drug." [2] Nevertheless, NO has a number of well-established functions within the human cardiovascular, pulmonary, gastrointestinal, nervous, and immune systems (Table 1). This review gives a broad overview of nitric oxide biochemistry and physiology to the surgical community. In particular, examples of NO-dependent pathophysiologic states pertinent to surgical practice are emphasized.

BACKGROUND

The current explosion in nitric oxide research is based on three initially independent lines of investigation. Early interest in the link between nitrosamines and carcinogenesis resulted in the observation that mammalian cells metabolized L-arginine to produce a reactive nitrogen oxide compound.[3] Second, the cytotoxic and cytostatic actions of activated macrophages were found to require L-arginine as a substrate. In addition, it was noted

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Table 1. CELLULAR DISTRIBUTION OF CONSTITUTIVE AND INDUCIBLE ISOFORMS OF NITRIC OXIDE SYNTHASE

Cell of Origin	cNOS	iNOS
Platelets	+	
Lymphocytes		+
Leukocytes	+	+
Macrophages		+
Endothelium	+	+
Vascular smooth muscle cells		+
Endomyocardium		+
Myocardium		+
Hepatocytes		+
Kupffer cells		+
Pancreatic islet β-cells	+	
Neurons	+	
Glial cells	+	+
Nonadrenergic/noncholinergic neurons		
Gastrointestinal	+	
Pulmonary	+	
Pancreatic	+	
Renal macula densa cells	+	
Carcinoma cells		+
Pulmonary epithelial cells		+

that N^G-substituted analogs of L-arginine blocked cytotoxicity. Nitric oxide was found to mimic the cytotoxic effects of activated macrophages, whereas NO scavengers blocked macrophage-associated cytotoxicity.[1,4] Finally, the work of Furchgott and colleagues concerning agonist-mediated vasodilatation brought the term endothelium-derived relaxing factor, or EDRF, to popular use.[5] Endothelium-derived relaxing factor was the agent thought to be responsible for endothelium-dependent vasodilatation and inhibition of platelet aggregation induced by a variety of agents that activated soluble guanylyl cyclase. Ultimately, the actions of EDRF were found to result from endothelial release of NO, either alone or coupled to a thiol carrier molecule.[1] Based on these seemingly disparate lines of research, understanding of the biochemistry of NO synthesis has been advanced greatly during the last 10 years.

In the presence of molecular oxygen, a guanidine nitrogen of L-arginine undergoes a five-electron oxidation to yield the gaseous free radical, nitric oxide, and L-citrulline in a process catalyzed by the enzyme, nitric oxide synthase (NOS). Flavin mononucleotide, flavin adenine dinucleotide, heme, and tetrahydrobiopterin are essential cofactors for this reaction. Nitric oxide exists as a stable colorless paramagnetic gas with moderate solubility in water (2 mM at 1 atm at 20 C). In solution, NO undergoes rapid oxidation to nitrite and nitrate and has an estimated half-life of < 4 minutes. In biological systems, NO has been estimated to have a half-life of only 3 to 30 seconds, is inactivated by superoxide anion, and binds to heme-containing proteins with Fe-S active sites. The activity of NO is ablated by binding to oxyhemoglobin, accounting in part for its short biological half-life. The molecular targets of NO are varied and include heme proteins such as soluble guanylyl cyclase, Fe-S proteins such as the tricarboxylic acid cycle enzyme, *cis*-aconitase, thiol groups at the active sites of enzymes, as typified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the superoxide anion. The interaction of NO with soluble guanylyl cyclase mediates vascular smooth muscle relaxation. The interaction of NO with *cis*-aconitase and other iron/sulfur-centered proteins in the Krebs cycle and complex 1 and 2 of the electron transport chain is responsible for cytostasis after activated macrophage release of NO. A more prominent component of the cytotoxic activity of macrophage-derived NO lies in its inhibition of ribonucleotide reductase, the rate-limiting enzyme of DNA synthesis. Inhibition of hepatocyte GAPDH activity is another recently discovered regulatory function of NO. Additional biologically relevant molecular reactions of NO are highly probable and remain to be characterized.[1,6-8]

The interaction of NO with oxygen-derived free radicals with its associated oxidant properties are particularly controversial areas of investigation. Although NO has been implicated in the microbicidal activities of activated macrophages, the true mediator may be a secondary oxidant derived from NO. In the presence of oxygen, NO can rapidly form NO_2 and the two-electron oxidants, N_2O_3 and N_2O_4. However, the physiologic relevance of these reactions are questionable as the rate of NO_2 formation is determined by the third order rate equation $k[NO]^2[O_2]$.[9] Another potentially relevant reaction is that of NO with superoxide. The rate constant of the reaction of NO with superoxide is on the order of 10^9/M-sec.[10] As a result of its reactivity with superoxide, NO was originally thought to be a free radical scavenger and hence, a protective factor.[11] However, the product, peroxynitrite (ONOO—), is a relatively long-lived, potent oxidant.[12] Peroxynitrite formation occurs after immunologic activation of macrophages and has been implicated in the pathophysiology of stroke, atherosclerosis, and immune-complex mediated pulmonary edema.[13-16] At a physiologic pH, peroxynitrite decomposes to form the species, HOONO, which reacts as an activated complex in a manner reminiscent of the hydroxyl radical.[17] In addition, more NO_2 is formed during peroxynitrite decomposition, lipid peroxidation is initiated, sodium channels are inactivated, and reactivity with transition metals results in formation of a nitronium ion-like nitrating agent.[18-21] Again, the physiologic relevance of these *in vitro* observations has been questioned. Peroxynitrite undergoes rapid decomposition at physiologic

pH, with a half-life of less than 1 second.[9] Typically, *in vitro* studies use peroxynitrite concentrations of 100 to 250 μM, whereas the *in vivo* rate of OONO— formation has been estimated to be only 0.1 nmol/10^6 cells-minute.[13] In addition, recent evidence from Clancy and coworkers suggests that NO synthesis by activated neutrophils decreases superoxide formation, eliminating a necessary substrate for OONO— formation.[22,23] Thus, the role of NO in oxidative stress mediated injury remains an area of active inquiry.

Nitric oxide is synthesized by cell-specific isoforms of the enzyme, nitric oxide synthase (NOS). Nitric oxide synthase has been found in a variety of tissue types, including endothelial cells, vascular smooth muscle cells, hepatocytes, Kupffer cells, platelets, pancreatic islet β cells, and certain central neurons. Broadly categorized, NOS exists as two subtypes—constitutive and inducible. The constitutive isoform (cNOS) is present in endothelium, neurons, and platelets as a monomeric isoform and is calcium- and calmodulin-dependent, with a molecular weight of approximately 133 kD. cNOS is expressed continually in the absence of inducing agents, yielding continuous basal synthesis of NO in picomolar concentrations. In contrast, the inducible isoform (iNOS) is expressed in macrophages, hepatocytes, and vascular smooth muscle after stimulation with endotoxin and cytokines such as interferon-gamma, interleukin-1 (IL-1) and tumor necrosis factor (TNF). As the result of a tightly bound calmodulin subunit, this NOS isoform is calcium-independent and exists in the active form as a tetramer with a monomeric molecular weight of 130 kD. After induction, iNOS is active for a period of 4 to 24 hours and synthesizes NO in nanomolar concentrations, more than 100-fold greater than that produced by cNOS. In addition, the transcriptionally regulated induction of iNOS is inhibited by glucocorticoids, transforming growth factor-β, IL-4, and IL-10. No physiologic agents have been found that inhibit the enzyme activity of either cNOS or iNOS. Although other tissue-specific isoforms of NOS have been described with dependence on calcium or calmodulin, most discussions of NO physiology continue to categorize NOS within the constitutive or inducible framework.[1,7,8]

The molecular and cell biology of the constitutive and inducible NOS isoforms recently has been elucidated. Using a culture system of rat aortic endothelial cells, Michel and colleagues have demonstrated that agonist-induced phosphorylation of cNOS is associated with translocation of the enzyme from the plasma membrane to the cytosol. Agents that increased cNOS activity resulted in increased cNOS phosphorylation. These authors propose that enzyme phosphorylation and translocation were associated with ultimate NOS deactivation.[24] Other investigators have examined the role of the cytoskeleton in NO production. In the rat vascular smooth muscle, administration of colchicine and nocodazole, two distinct microtubule depolymerizing agents, inhibited NOS induction. Examples for similar involvement of microtubules in gene expression have been cited—e.g., endothelial plasminogen activity and hepatocyte acute phase protein synthesis.[25] Consideration the molecular biology of NO has led to the cloning of the human endothelial cNOS gene, which contains 26 exons spanning 21 kilobases of genomic DNA on chromosome 7.[26] Characterization of the 5′ flanking region revealed putative AP-1, AP-2, NF-1, heavy metal, acute-phase shear stress, and sterol regulatory elements. The AP-1 and AP-2 elements are thought to participate in the transcriptional response to phorbol esters or cyclic adenosine monophosphate, respectively. NF-1 has been implicated in transforming growth factor-β responses. The presence of the acute phase shear stress regulatory element corresponds to the observation that hemodynamic states reflective of increased endothelial membrane shear stress are associated with increased NO production. Similarly, the human inducible NOS gene has been isolated and cloned.[27] This gene is 37 kilobases in length, with 26 exons and 25 introns. The transcription initiation site was mapped 30 base pairs downstream of a TATA sequence. Consensus sequences for an NF-KB site and three gamma-IRE sites were identified; these are thought to be involved in lipopolysaccharide- and IFN-induced iNOS gene expression. The human iNOS gene has been localized to chromosome 17. As increased functional analysis of the human iNOS and cNOS promoter regions occurs, the complexities of NOS gene regulation can be examined. Ultimately, disease states with aberrant expression of the NOS genes may be identified and characterized.

The mechanism of NO transport to its molecular targets remains unknown. It has been argued that the rate with which NO reacts with molecular oxygen, superoxide anion, heme, and nonheme iron makes diffusion an implausible mechanism for its *in vivo* paracrine effects. Nitric oxide is a reactive, free radical formed in a cellular milieu replete with molecules capable of stabilizing NO activity or promoting its degradation. Under physiologic conditions, nitrogen oxides readily combine with protein-bound thiol groups to form stable, biologically active S-nitroso-compounds. Recent work demonstrates that NO may circulate in mammalian plasma as an S-nitroso-adduct of albumin.[28,29] Therefore, a transport process using low-molecular-weight thiols as carrier molecules has been suggested.[28] These S-nitroso-compounds exhibit *in vitro* EDRF-like vasorelaxant and platelet inhibitory properties mediated by a cGMP-dependent process.[28] Molecules, such as S-nitroso-albumin and S-nitroso-L-cysteine, possess biologic properties similar to EDRF in *in vivo* experiments.[29] The physio-

logic half-life of S-nitrosothiols varies from 30 seconds for S-nitroso-L-cysteine to 12 hours for S-nitroso-albumin.[29] In *in vivo* and *in vitro* models, S-nitrosothiols are potent, physiologically relevant molecules that mimic NO activity and act as low-molecular-weight thiol donors.

Another candidate for *in vivo* NO transport is the dinitrosyl iron complex (DNIC). Critics deem nitrosothiol formation to be an unlikely candidate for EDRF because nitrosothiols are not thought to form in aqueous media under neutral pH and decompose rapidly unless present in an acidic milieu.[30] Vanin and colleagues have proposed that EDRF may be an Fe-nitrosyl compound with thiol-containing ligands $Fe(NO)_2(RS)_2$.[31] Such complexes are known to form in cells in a neutral aqueous environment. This paramagnetic species has unique electron spin resonance characteristics and has been termed a 2.03 complex based on its electron spin resonance-derived axial anisotropy (g_{ave}).[31] Dinitrosyl iron complex is formed in endothelial cells and cytokine-activated macrophages as the result of NO production.[32-33] Inhibition of NO synthesis ablates the characteristic electron spin resonance signal. The presence of low molecular weight thiol ligands, such as cysteine or glutathione, in DNIC facilitates intercellular and intracellular transport; however, the equilibrium distribution of DNIC between low molecular weight *versus* protein complexes greatly favors the latter.[31] The 2.03 complexes act as vasodilators in isolated blood vessel systems and inhibit platelet aggregation.[31] In a system of precontracted de-endothelialized rat aorta segments, DNIC vasodilator responses more closely resemble those of acetylcholine-mediated dilation than that of true NO. These findings suggest that DNIC with small thiol ligands may be the molecular species whose physiologic actions have been termed EDRF.[30] Still other investigators have proposed that EDRF is a nitroxyl species or hydroxylamine.[34,35] Although a number of prospective moieties may serve as carrier molecules for NO delivery, the true nature of EDRF continues to be controversial. In fact, Moncada and coworkers maintain that nitrosothiols, DNIC, nitroxyl, and hydroxylamine can be eliminated as candidates and contend that NO is EDRF.[36] These considerations notwithstanding, it is agreed that NO remains the final biochemical mediator for the actions of EDRF.

A variety of pharmacologic agents have been described that can promote or inhibit NO production. Promoters include the organic nitrates, such as nitroglycerin or sodium nitroprusside, and S-nitrosothiols. Although organic nitrates have been used clinically as vasodilators for decades, only recently has their mechanism of action as NO donors been appreciated. In addition, the concept that low molecular weight thiols may be the carrier molecules responsible for the paracrine effects of NO has led to the development of nitrosated thiols, such as S-nitroso-N-acetylcysteine and S-nitroso-albumin.[28] These compounds have been found to mimic the effects of NO in both *in vivo* and *in vitro* models.[28,29] Inhibitors of both cNOS and iNOS include the flavoprotein binders, calmodulin binders, heme binders, substrate analogs, and tetrahydrobiopterin-depleting agents. Because of their ease of use and ready availability, the substrate analogs have become the most commonly used inhibitors of NO synthesis. As structural analogs of the true substrate, L-arginine, these agents act as competitive substrate inhibitors of NOS activity. When added in the absence of L-arginine, these analogs are metabolized to end products that covalently bind NOS. This class of NOS inhibitors is accompanied by a variety of "L-N" abbreviations as typified by N^G-nitro-L-arginine (L-NNA), N^G-methyl-L-arginine (L-NMMA), and N^G-nitro-L-arginine methyl ester (L-NAME). The individual substrate analogs display variable affinities for either cNOS or iNOS, giving rise to the possibility of pharmacologically derived, isoform-specific NOS inhibitors. However, it also is important to realize that although these agents often are used to invoke NO's role in various physiologic phenomena, there are few data to address the claim that any of these are specific NO synthesis inhibitors.[1,7]

CARDIOVASCULAR SYSTEM

The identification of NO as the final chemical mediator of the actions of EDRF has given rise to an avalanche of scientific inquiry into its role in cardiovascular homeostasis. It currently is accepted that NO is the major physiological regulator of basal blood vessel tone. Nitric oxide is released continually by the arterial circulation. Vasodilatory agents such as acetylcholine and bradykinin act on endothelial cell-surface receptors to trigger NO release and stimulate soluble guanylyl cyclase, resulting in protein kinase-dependent relaxation of vascular smooth muscle. In the absence of endothelium, these stimuli lose their vasodilatory properties.[4] Nitric oxide also is released in response to pulsatile flow and shear stress from nonadrenergic, noncholinergic nerves. Within vascular networks, the basal release of NO may function to limit the work of perfusion and maintain flow distribution in the setting of variable flow rates by decreasing sympathetic outflow, and mediating L-glutamate-induced decreases in pulse and heart rate.[37,38] Renal arteriolar regulation suggests a role for NO in renin release and sodium and water homeostasis, making NO a key determinant of intravascular volume and vascular tone.[39] Furthermore, NO also modulates platelet adhesion and aggregation, leukocyte adhesion, endothelin generation, plasminogen activator enzymatic function,

and vascular smooth muscle proliferation.[1] Certainly, evidence to date suggests that basal endothelial production of NO by the cNOS isoform is critical to vascular homeostasis.

Altered endothelial production of NO is involved in a number of states of vascular dysfunction. In primates fed atherogenic diets, the NO-mediated vasodilator response to acetylcholine is reduced greatly in atherosclerotic vessels, whereas response to vasoconstrictors is enhanced. When atherosclerotic lesions have regressed after diet modification, vasodilator responses are restored.[40] In hypertensive animals, a similar derangement in vascular response is noted, which also is reversed by institution of antihypertensive therapy.[41] Furthermore, agonist-mediated vasodilatation is impaired in the coronary circulation of smokers, and children with familial hypercholesterolemia.[7] The results from these studies and others suggest that endothelial dysfunction in these pathologic states arise from either impaired release of NO from the endothelium or an alteration in the signal response characteristics of the vascular smooth muscle.

Derangement in endothelial NO synthesis may predispose to development of atherosclerosis. Evidence suggests that NO may play an artheroprotective role by inhibiting oxidation of lipoproteins and preventing oxidative membrane injury by free radicals. Low-density lipoprotein appears to inhibit NO-dependent vasorelaxation by a direct interaction between low-density lipoprotein and NO. As a result, the superoxide scavenging effect of NO is ablated, and increased amounts of superoxide remain available for oxidation of low-density lipopolyprotein, which then initiates the atherosclerotic process in endothelial and vascular smooth muscle cells. Moreover, chronic endothelial exposure to oxidized low-density lipopolyprotein causes irreversible inhibition of NO-dependent vasorelaxation.[42,43] Hayashi et al. have found that the atherosclerosis-resistant state associated with estradiol synthesis in premenopausal women is linked to enhanced basal endothelial NO release. Evidence from rabbit models has demonstrated that NO release from aortic rings of female rabbits is greater than that of male rabbits. This effect is ablated by oophorectomy.[44] Thus, atherosclerosis may occur as the result of lipoprotein-induced NO inactivation. Nitric oxide mediates endothelial interactions with circulating pro-atherosclerotic moieties, such as low-density lipolyprotein. Pathologic processes that alter endothelial function, such as hypertension and atherosclerosis, dramatically alter endothelial NO production resulting in diminishing the protective ability of NO.

In another pathologic state, NO activity may mediate the vascular consequences of hypoxia. The acute arterial pressor response stimulated by hypoxia is augmented by inhibitors of NOS in the pulmonary, coronary, and systemic circulations. In the coronary circulation, hypoxia has been identified as a primary stimulus for NO release. During hypoxia, pulmonary vessel release of NO is increased to modulate the pulmonary pressor response. These studies hint at a relation between available oxygen and NOS activity. In the presence of hypoxia, vascular tone becomes increasingly vasoconstrictive, leading to inadequate perfusion and potentiation of ischemic injury.[45] Interestingly, in the setting of renal failure, an endogenous NOS substrate inhibitor recently has been identified. Dimethylated arginine (DMA), a naturally occurring substrate analog, has been found in increased concentrations in patients with chronic renal failure, a condition associated with hypertension. Endogenous DMA usually is excreted unchanged. In chronic renal failure, however, DMA levels are elevated sufficiently to inhibit NO synthesis, and these increase in parallel with the serum creatinine. Dramatic decreases in DMA concentration follow hemodialysis. These findings suggest a link between the hypertension of chronic renal disease and NO synthesis.[46] Although a great deal of further research is warranted, NO plays a major role in the control of vascular tone, and mediating vascular reactivity to hypoxia and pro-atherosclerotic events. In addition, the alterations in vascular function that result from multiple pathophysiologic conditions can be explained, in large part, as a perturbation of basal NO production.

The antithrombotic properties of NO result in part from inhibition of platelet adhesion and aggregation. Basal endothelial synthesis of NO initiates a series of guanylyl cyclase-dependent steps that lead to the suppression of intraplatelet Ca^{2+} levels. Sudden elevations in intracellular Ca^{2+} with simultaneous stimulation of protein kinase C activity are prerequisites for platelet activation. Prostacyclin and NO act synergistically to inhibit aggregation and actively disaggregate platelets. Nitric oxide also inhibits platelet adhesion to collagen fibrils, endothelial matrix, and endothelial monolayers in a process independent of prostacyclin. In addition, it has become clear that platelets generate NO and that the L-arginine: NO pathway acts as an autocrine negative feedback mechanism to regulate platelet reactivity. Thus, platelet aggregation is regulated by intraplatelet NO as well as endothelial NO and prostacyclin.[47]

The cytokine-rich milieu that accompanies ischemia-reperfusion injury and sepsis can be associated with myocardial depression. Although a myocardial depressant factor has been invoked, identification of this compound has eluded investigators. In a model using the left ventricular papillary muscle in hamsters, Finkel and colleagues recently demonstrated that the negative inotropic effect of the cytokines TNF, IL-6, or IL-2 was blocked by L-NMMA. The addition of the true NOS substrate, L-arginine, with TNF and L-NMMA enhanced the neg-

ative inotropic effect seen with TNF alone. These investigators concluded that cytokine induction of endomyocardial iNOS, yielding increased NO production, resulted in negative inotropy. Thus, the regulatory effects of pro-inflammatory cytokines on myocardial iNOS expression may provide new approaches to therapy of myocardial stunning that can accompany sepsis or cardiopulmonary bypass.[48]

Induction of myocardial iNOS in the setting of pro-inflammatory cytokines suggests that iNOS present in endothelial cells, vascular smooth muscle, and macrophages also may play a role in hemodynamic derangements that accompany sepsis. Endotoxin and TNF have been deemed the central agents responsible for the cascade of events after septic shock. Septic shock is associated with the release of inflammatory cytokines and as such, may be mediated by increased iNOS activity. In a rat model, L-NMMA was found to prevent endotoxin-induced shock in a dose-dependent fashion. Although 30 mg/kg L-NMMA prevented endotoxin shock, 300 mg/kg L-NMMA accelerated and exacerbated endotoxin-induced hypotension.[49] Using a canine model of TNF-mediated hypotension, Kilbourn and associates demonstrated that L-NMMA completely reversed the 62-mm Hg decrease in mean arterial pressure induced by TNF. This effect occurred within 2 minutes and was sustained. Administration of the true substrate, L-arginine, resulted in restoration of hypotension. These researchers concluded that NO production mediates the hypotensive effect of TNF.[50] In a rabbit endotoxic shock model, Pastor and coworkers found that NOS inhibition was associated with increased mean arterial pressure caused by intense vasoconstriction as measured by significant decreases in aortic conductance. A significantly higher mortality was noted in the inhibitor group.[51] A published case report describes two patients with pressor-resistant septic shock. In both patients, intravenous infusions of L-NMMA (0.3 or 1.0 mg/kg) caused a rapid, albeit, short-lived increase in mean arterial pressure and systemic vascular resistance.[52] Geroulanos and colleagues also reported the use of L-NMMA in a patient with septic shock and multi-organ system failure. The administration of 7 mg/kg of L-NMMA was associated with normalization of blood pressure for 25 minutes.[53] Human trials of NOS inhibitors in septic shock are beginning to emerge. In a randomized, double-blind, placebo-controlled trial, 12 patients in septic shock received boluses of L-NAME (0.3 mg/kg, then 1 mg/kg) followed by an infusion at 1 mg/kg/hr for 6 hours. Mean arterial blood pressure and systemic vascular resistance increased an average of 10% and 50%, respectively; cardiac output decreased by as much as 30%. Elevations in pulmonary vascular resistance also were seen, although pulmonary artery pressures did not change significantly. After bolus administration, changes in cardiac output and systemic vascular resistance persisted for a period of 16 hours. Survival was not reported. These investigators have suggested that the combination of parenteral NOS inhibitors with inhaled NO may be more efficacious. These results, however, suggest that NOS inhibition may not be a panacea for the hemodynamic alterations of sepsis because decreases in cardiac output bring into question the adequacy of vital organ perfusion.[54] More investigation is required to delineate a role, if any, for NOS inhibitors in the treatment of septic shock because it remains to be determined whether these hemodynamic changes significantly improve morbidity or mortality. Preliminary evidence from human trials is not encouraging.

Another example of the role of NO in cytokine-induced shock comes from IL-2 immunotherapy for treatment of selected solid tumors. Kilbourn and colleagues tested the effect of L-NMMA in IL-2-induced hypotension. Dogs were given daily doses of concurrent IL-2 and L-NMMA. Therapy was well tolerated without changes in blood pressure, hepatic, renal, or hematologic parameters, and survival was 100% during the 5-day course of therapy. In the absence of L-NMMA, dogs became premorbid by day 4. In addition, lymphocyte-activated killer (LAK) cell activity was maintained in the presence of L-NMMA.[2] In selected human studies, levels of NO metabolites were found to be increased more than ninefold in patients receiving IL-2/LAK cell therapy, demonstrating the relevance of these canine studies to humans. Investigators have suggested that dose-limiting hypotension associated with IL-2 immunotherapy is NO-mediated.[55] A trial of NOS substrate inhibitors in IL-2 immunotherapy has been proposed, and it is anticipated that L-NMMA will decrease IL-2 toxicity, permitting use of increased doses and yielding improved response rates.

Although experimental evidence and scattered case reports invoke a role for NO in hypotension associated with cytokine-rich states, the cellular origin of the NO in this disease process is unknown. Potential sources include the endothelium, vascular smooth muscle, macrophages, and hepatocytes. Again, it remains to be shown that correcting hypotension affects cytokine induced organ dysfunction in a significant manner.

Elucidation of the role of NO in disease pathogenesis has resulted in attempts to modulate NO production for therapeutic effect. NO is the common final mediator for nitrovasodilators, which include sodium nitroprusside and nitroglycerin. Sodium nitroprusside is known to release NO directly, but the mechanism by which other organic nitrates activate guanylyl cyclase is controversial. There are two routes for degradation of nitrates, an enzymatic pathway yielding nitrites and a nonenzymatic pathway releasing NO. Both of these reactions require

the amino acid cysteine, but only nonenzymatic degradation yields biologically active NO. This dual pathway partially explains the phenomenon of clinical tolerance to nitrates. If cysteine is depleted in the enzymatic pathway, none remains to stimulate the nonenzymatic degradation that releases NO. Of note is the lack of cross tolerance to sodium nitroprusside, a direct NO donor.[56]

Within the clinical realm, nitrovasodilators have long been thought to differentially affect the arterial and venous circulations. Although L-NMMA infused into the brachial arteries of human volunteers decreased basal flow by 50%, sensitivity to exogenous NO is greater in the venous system.[57,58] This differential effect also has been noted in hamster and rabbit models.[59,60] Clinically, nitrovasodilators affect venous more than arterial circulations, a difference which may be the result of a lack of venous endothelial production of NO or the basal arterial release of NO.[61]

As noted above, multiple studies have shown impairments of relaxation to EDRF in atherosclerosis, hypertension, diabetes, and ischemia/reperfusion injury. L-arginine-rich diets prevent the development of hypertension in animals at risk, and L-arginine infusions cause rapid reductions of blood pressure in human subjects with essential hypertension.[62–64] In addition, NO may play a role in restenosis after angioplasty. In a model of balloon catheter-induced arterial injury, rabbits fed L-arginine before and after catheter injury exhibited a significant reduction in the intimal hyperplastic response. This effect was reversed in those rabbits given L-NAME.[65] Using an *in vitro* model, Durante et al. have shown that IL-1β-induced NO release by vascular smooth muscle cells is enhanced by plasmin and attenuated by thrombin.[66] In another model, they demonstrate that conditioned media from collagen-aggregated platelets inhibit vascular smooth muscle release of NO induced by IL-1β.[67] These data indicate that endogenous release of NO is involved intimately with the fibrinolytic and thrombotic cascade associated with endothelial injury. Endogenous NO may act as an anticoagulant in the maintenance of vascular homeostasis and in the setting of endothelial injury by interacting with the fibrinolytic system to enhance thrombolysis and inhibit thrombus formation. Therefore, augmentation of NO release or administration of nitrovasodilators or S-nitrosothiols may increase the efficacy of thrombolytic therapy.

The amount of NO produced in any single organ system or pathologic state may determine whether it is protective or toxic. Although small amounts are necessary for homeostasis, large amounts, such as those produced on activation of the inducible isoform of NOS, are cytotoxic.[68] The ability to produce large amounts of NO may be an important line of defense against cellular invaders or tumor cells. In fact, NOS inhibition prevents lysis of tumor cells by macrophages that express the inducible form of NOS and produce large amounts of NO. Inhibiting only the inducible form of NOS probably would allow the constitutive release of protective levels of NO while preventing the consequences of massive overproduction.

PULMONARY SYSTEM

Pulmonary vascular and parenchymal production of NO has received increasing attention as its functions within the systemic vasculature are identified. Like macrophages elsewhere, rat pleural and alveolar macrophages produce NO in response to endotoxin and cytokines. However, several pulmonary-specific, NO-mediated regulatory mechanisms exist. In addition to the classic cholinergic bronchoconstrictor and adrenergic bronchodilator neural mechanisms, evidence supports the existence of a nonadrenergic, noncholinergic (NANC) neural system. The inhibitory (brochodilator) component of NANC responses are believed to be mediated partially by vasoactive intestinal peptide. Within guinea pig airways, a component of the NANC inhibitory response is attenuated in a concentration-dependent manner by L-NMMA.[45] Li and colleagues found that L-NMMA and L-NAME both reduce NANC-stimulated relaxation of guinea pig airway tracheal smooth muscle.[69] In the human airway, NO was found to mediate a component of the inhibitory response elicited by NANC nerves. These data suggest that NO is a neurotransmitter involved in bronchodilatation in human airways.[70]

Nitric oxide-containing vasodilators and bioactive NO adducts have been studied as potential bronchodilatory agents in humans. Vasodilators such as isosorbide dinitrate and nitroglycerin act as nonspecific smooth muscle relaxants. Nitrates have been found to be effective relaxants of bovine airway smooth muscle *in vitro*.[71] Intravenously administered nitroglycerin relaxed tracheal smooth muscle in nonasthmatic anesthetized humans.[72] The efficacy of sublingual nitrates in the treatment of asthma remains controversial. Bioactive NO adducts such as S-nitrosothiols relax precontracted guinea pig tracheal rings and human peripheral bronchi, whereas inhaled S-nitrosothiols induce prompt and sustained bronchodilation.[73,74] These investigations indicate that NO or an NO-like product plays a role in NANC-mediated tracheal smooth muscle relaxation in a number of different species.

Although it is tempting to invoke basal release of NO by pulmonary endothelium as the primary mediator of pulmonary vascular tone in man, it is unclear whether this is true. *In vitro* experiments with vascular rings from multiple species demonstrate that inhibition of NO pro-

duction by mechanical or biochemical means consistently results in greater responses to vasoconstrictor stimuli. However, in isolated perfused rat lungs, inhibition of NO production by substrate analogs has no effect on resting perfusion pressure. In contrast, methylene blue, an inhibitor of soluble guanylyl cyclase, significantly increases pulmonary vascular resistance in perfused lungs from humans. In the setting of acute hypoxic pulmonary vasoconstriction, inhibition of NO synthesis markedly enhances the pressor response to acute hypoxic challenges. Alternatively, in pulmonary hypertension secondary to chronic hypoxia, NO-dependent relaxation of pulmonary artery rings from humans undergoing heart-lung transplantation and isolated perfused lungs from rats is markedly attenuated. Data suggest that NO synthesis or release is impaired in chronic hypoxia with resultant pulmonary hypertension.[75]

Given the role of NO in the modulation of pulmonary vascular tone, inhaled NO is undergoing clinical investigation. Inhaled NO offers the advantage of low toxicity with selective pulmonary vasodilatation. It dilates the vasculature of ventilated alveoli with resulting improvement in ventilation-perfusion matching and is inactivated rapidly by hemoglobin. In animal studies of pulmonary hypertension induced by hypoxia, thromboxane, or heparin-protamine interactions, inhaled NO in concentrations of 5 to 80 ppm produced pulmonary vasodilatation that was rapid and reversible without systemic sequelae.[76] In recent clinical studies with inhaled NO, Pepke-Zaba et al. demonstrated selective pulmonary vasodilator effects in eight patients with primary pulmonary hypertension. Inhaled NO (40 ppm) decreased pulmonary vascular resistance by an average of 30% without changing systemic vascular resistance. In contrast, prostacyclin produced dose-related decreases in both pulmonary vascular resistance and systemic vascular resistance.[77] Similar findings have been noted in patients with congenital heart disease.[78] In persistent pulmonary hypertension of the newborn, inhaled NO therapy (20 ppm) improved oxygenation without altering systemic blood pressure.[78] Frostell et al. described the effects of inhaled NO during hypoxia in human volunteers. In the absence of hypoxia, inhaled NO failed to alter pulmonary or systemic hemodynamics. On the other hand, inhalation of 12% O_2 significantly increased both pulmonary vascular resistance and pulmonary artery pressure, whereas addition of 40 ppm NO decreased pulmonary vascular resistance and pulmonary artery pressure to control levels without changing systemic hemodynamics.[79] These findings corroborate animal data showing that inhaled NO produces rapid and selective pulmonary vasodilatation during such acute hypertension, but is without effect in the absence of pulmonary hypertension. Other pathophysiologic states appropriate for inhaled NO include adult respiratory distress syndrome (ARDS) and congenital diaphragmatic hernia.[76] Initial reports suggest that inhaled NO (18 to 36 ppm) may prevent the development of adult respiratory distress syndrome. In affected patients treated with inhaled NO for 7 days, sustained improvement in lung function was produced.[7] Toxicity of inhaled NO remains an unresolved issue. The potential for NO to induce pulmonary toxicity in states of ongoing oxidative stress or newborn lungs is unknown. The reactivity of the NO metabolite, NO_2, is an additional consideration. Kinetic analysis of NO_2 formation indicates that inhaled NO therapy may be accompanied by potentially toxic concentrations of NO_2. Animal studies, however, have found no toxic effects from inhalation of 10 to 40 ppm of NO for periods up to 6 months; toxic effects of inhaled NO in humans remain the object of investigation.[76]

GASTROINTESTINAL SYSTEM

In the gastrointestinal system, evidence suggests that NO regulates mucosal blood flow, mucosal protection, hemodynamic responses to liver disease, hepatocyte synthetic function, and relaxation of the muscularis. Neuronal mediation of intestinal muscle relaxation has been attributed to the presence of a NANC neurotransmitter. A number of candidates have been proposed including adenosine triphosphate and vasoactive intestinal peptide, but evidence is conflicting for both cases. Recently, NO has emerged as a possible component of a nonpurinergic, nonpeptidergic system that mediates smooth muscle inhibition in the gastrointestinal system. In an *in vitro* model, electric field stimulation of NANC nerves from rat gastric fundus and dog ileocecal junction resulted in the release of a substance that caused relaxation of vascular smooth muscle. That this was inactivated by hemoglobin and an substrate inhibitor of NO synthesis suggests that the substance was NO. Immunohistochemical staining has localized NOS to cells in the myenteric plexus and neuronal processes in the rat duodenum. Exogenous NO-induced relaxation similar to that seen with NANC nerves has been found in longitudinal and circular muscle strips from the lower esophageal junction, small intestine, and internal anal sphincter from a variety of animal species. Furthermore, inhibition of NO synthesis and inactivation of NO by hemoglobin *in vitro* attenuated the relaxation associated with NANC nerves in systems such as guinea pig colon, canine ileum, human jejunum, and human colon.[80]

The clinical implications of these findings still are largely obscure. It has been speculated that alteration in density of NO-producing nerves or alteration in smooth muscle sensitivity to NO may have a role in some neuromuscular disorders. Examples might include the non-

peristaltic contractions noted in aganglionic segments of Hirshsprung's disease, and in achalasia and "nutcracker" esophagus. The relaxed lower esophageal sphincter seen with gastroesophageal reflux may reflect increased NO synthesis or end organ sensitivity to NO. An imbalance between excitatory and inhibitory neuronal activity could result in conditions such as chronic intestinal pseudo-obstruction.[80] In a recent clinical report using immunohistochemical staining, Vanderwinden et al. found an absence of NOS in the enteric nerve fibers of infants treated for hypertrophic pyloric stenosis, whereas infant control subjects showed dense staining for NOS. This suggests that the absence of a putative inhibitory neurotransmitter in the pylorus results in the clinical syndrome of hypertrophied pyloric musculature and gastric outlet obstruction.[81]

The interplay between gastrointestinal mucosal blood flow and the development of mucosal erosions or ulcers has led to investigation of potential vasodilator function of NO in the maintenance of mucosal integrity. In the rat stomach, L-NMMA reduces gastric mucosal blood flow, indicating the role of NO in the regulation of basal gastric mucosal vascular tone. In addition, increases in mucosal blood flow induced by bradykinin are endothelium-dependent, and NO inhibitors decrease pentagastrin-mediated increases in gastric mucosal blood flow.[82,83] This would imply that NO also regulates agonist-mediated increases in blood flow. Interestingly, both topical and intravenous administration of NO donors, such as nitroprusside, reduce the severity of ethanol-induced hemorrhagic damage.[84] Conversely, inhibition of NO synthesis with concomitant administration of indomethacin results in substantial mucosal injury. Additional results indicate that endogenous NO may interact with prostacyclin and vasodilatory neuropeptides to regulate gastric mucosal integrity.[85] The mechanism underlying the mucosal protective effects of NO is unknown. Investigators invoke vasodilatation or inhibition of platelet aggregation, but NO also may regulate epithelial cell function. Current data, however, is derived entirely from animal models, and as such, the role of NO in human gastrointestinal mucosal blood flow is not well defined.

Nitric oxide also has been implicated in the maintenance of microvascular integrity of the intestinal mucosa after challenge with endotoxin. In the rat, intravenous endotoxin results in acute intestinal vascular damage, vasocongestion, and plasma exudation into the intestinal lumen.[86] Platelet-activating factor and thromboxane A_2 are released endogenously in response to endotoxin and are key mediators of these hemorrhagic lesions.[80] Recent evidence suggests that NO may protect against the sequelae of endotoxin-induced shock. Pretreatment with L-NMMA enhances endotoxin-induced intestinal damage and plasma leak in the rat model.[86] Co-administration of a bioactive NO adduct attenuates this damage. Multiple studies have duplicated these findings in rat and canine models.[80,87] It is hypothesized that NO aids in the maintenance of microvascular integrity and flow in the setting of endotoxin administration, although the mechanism remains obscure. An intriguing line of investigation follows the observation that NO can modulate leukocyte-endothelial interactions. Neutrophil adhesion to vascular endothelium and emigration from the vasculature (diapedesis) is inhibited by NO.[88] Inhibition of NO also leads to increased expression of CD11/CD18 on neutrophils.[37] Endothelial expression of the adhesion molecules, ICAM-1 and ELAM, is upregulated in the presence of NOS inhibitors.[89] Basal production of NO is critical in the regulation of proteins deemed crucial for cell-cell interaction and exerts a tonic effect on leukocyte adhesion to the endothelium. These data suggest that NO may regulate molecular components of leukocyte sequestration and activation and as a result, may play a role in ischemia-reperfusion injury and endotoxin-induced shock. Others have speculated that NO interacts with the superoxide anion to produce a less toxic species. In an observation combining free radical production and leukocyte function, Kubes et al. found that inhibition of NO synthesis resulted in increased superoxide formation and mast cell-mediated leukocyte adhesion to postcapillary venules.[90] Thus, the mechanism for the mucosal protective effect of NO in endotoxin-induced shock is unknown, but may involve free radical detoxification, leukocyte adhesion properties, and microvascular hemodynamic regulation. Further research may provide insights into the pathogenesis of disease, such as inflammatory bowel disease, ischemia-reperfusion injury, and infectious colitis. Additional interventions using bioactive NO adducts may prove to be clinically useful.

An association between the hyperdynamic circulation of cirrhosis and increased NO production has been proposed. This hyperdynamic state is characterized by increased cardiac output, increased heart rate, decreased blood pressure and decreased systemic vascular resistance. It has been suggested that this state is the result of decreased sensitivity to endogenous vasoconstrictors or increased activity of endogenous vasodilators. Although a number of putative vasodilators have been suggested, a dominant compound has not been identified. Recently, NO has been suggested as the primary endogenous vasodilator responsible for this altered hemodynamic state.[80] Increased levels of endotoxin have been detected in the circulation of cirrhotic patients and could serve as the induction agent for iNOS expression. Elevated levels of endotoxin occur in the absence of obvious sepsis and are thought to result from porto-systemic shunting. Short-term administration of L-NNA in a rat model of cirrho-

sis and portal hypertension attenuated the hyperdynamic splanchnic and systemic hemodynamic profile.[91] Using a model of partial portal vein ligation in the rat, Groszman et al. demonstrated that intravenous L-NNA reduced porto-systemic shunting without reducing portal pressure, indicating that NO also may regulate collateralization of the splanchnic circulation.[92] Other investigators have found that the sodium avidity associated with cirrhosis and resulting in ascites can be prevented by systemic administration of NOS substrate inhibitors.[1] Available animal data suggest that cirrhosis is akin to a state of NO excess. Certainly, portal-systemic shunting, portal hypertension, and sodium re-absorption associated with cirrhosis is responsive to administration of NOS inhibitors but again, the source of NO in this disease state is unknown.

Data from human studies corroborate the animal findings. In patients with cirrhosis, Guarner and colleagues found significantly increased serum levels of nitrate and nitrite NO metabolites that correlated with elevated serum levels of endotoxin. Administration of an oral nonabsorbable antibiotic, colistin, resulted in decreased levels of both endotoxin and nitrite/nitrate. They postulated that circulating endotoxin induces iNOS activity, resulting in the characteristic hemodynamic findings of cirrhosis.[91] Midgley et al. have reported the use of methylene blue in a patient with severe hepatic failure and hypotension. Methylene blue selectively inhibits the action of the target molecule of NO, soluble guanylyl cyclase. Intravenous bolus injection of methylene blue increased the systemic blood pressure for a period of 60 minutes.[93] In another human study, administration of a single dose of molsidomine, an NO donor, caused a significant and sustained decrease in portal venous pressure. Intrinsic hepatic clearance of indocyanine green was unchanged in the presence of molsidomine, indicating preserved hepatic clearance function.[80] If additional evidence supports the hypothesis that endotoxin induction of iNOS results in the hyperdynamic circulation of cirrhosis, blockade of this pathway may alleviate or reverse associated complications, such as ascites, edema, and hepatorenal syndrome. Selective intestinal decontamination, glucocorticoids, or NOS substrate inhibitors may prove useful as therapy in this disease state.

In the setting of multi-organ system failure, patients often manifest hepatocellular dysfunction, such as elevated bilirubin and decreased serum albumin. Although the mechanism is unclear, it has been suggested that hepatocyte dysfunction is the result of cytokine-induced mediator release by macrophages or Kupffer cells. Nitric oxide has been targeted as a regulatory mediator. Hepatocytes contain iNOS and produce large amounts of NO after endotoxin and cytokine stimulation. In *in vitro* and *ex vivo* models, cytokine-mediated production of NO stimulates soluble guanylyl cyclase and increases extracellular levels of cyclic guanosine monophosphate (GMP). In isolated rat hepatocytes, synthesis of NO profoundly decreases total protein synthesis in a post-translational, cGMP-independent manner. Furthermore, in hepatocytes exposed to pharmacologically synthesized NO and in Kupffer cell-hepatocyte cocultures, NO inhibits activity of several mitochondrial electron transport enzymes, including *cis*-aconitase, NADH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase, in a concentration-dependent manner. In addition, hepatocyte production of NO decreases glyceraldehyde-3-phosphate dehydrogenase enzyme activity, possibly by a process of S-nitrosation of essential active site thiol groups.[80] The potential hepatoprotective or hepatotoxic effects of NO, however, have yet to be clarified. In an *in vivo* model of endotoxin-induced murine hepatocyte injury, inhibition of NO synthesis was markedly hepatotoxic, and histologic examination of the liver revealed intrahepatic thrombosis. Co-administration of superoxide dismutase and deferoxamine reduced the extent of hepatocyte injury, implicating oxygen-derived free radicals in the process. Conversely, increased NO production was hepatoprotective.[94] However, the cellular source of NO mediating this protective effect is unknown. Kuo and coworkers have demonstrated that inhibition of cytokine-mediated NO synthesis depletes hepatocyte stores of reduced glutathione, an essential free radical detoxification system. In a model of acetaminophen-induced hepatocyte injury, inhibition of NO synthesis by the competitive substrate, L-NMMA, potentiated acetaminophen hepatotoxicity.[95] Although a great deal of experimentation continues, evidence suggests that hepatocyte NO production may be hepatoprotective in states of free radical production, such as ischemia-reperfusion injury, liver allograft rejection, and drug-toxicity.

Additional functions have been discovered for NO in the pancreas. In the prairie dog, NO regulates sphincter of Oddi basal myogenic function by inhibitory neural pathways.[96] Recently, these findings have been duplicated in human subjects undergoing endoscopic retrograde cholangiopancreatography. (A. Slivka, Brigham and Women's Hospital, personal communication, June 1994). Evidence also is accruing for a toxic effect of NO on pancreatic islets in type I diabetes mellitus. Concomitant treatment of mice with low-dose streptozotocin and NOS inhibitors significantly attenuated the resultant hyperglycemia.[97] Furthermore, in murine induction models of diabetes, inhibition of iNOS significantly prolonged the time to onset of diabetes.[98] On a cellular level, exposure of islets to an NO donor resulted in cell lysis in a time- and concentration-dependent manner.[99] Finally, investigators have shown that IL-1 suppression of insulin

production is NO dependent.[97] Thus, current data indicate that although low levels of NO regulate islet insulin production, high levels are toxic.

CENTRAL NERVOUS SYSTEM

Interest in the role of NO in central nervous system physiology emerged with the identification of a cerebellar isoform of cNOS.[100] In parallel with *in vivo* and *in vitro* functional studies, the cell biology of NO in the central nervous system has been examined. The human neuronal cNOS gene has been localized to chromosome 12. Its protein product is associated with the soluble cellular fraction, in contrast to the endothelial isoform of cNOS.[26,101] Recent evidence indicates that iNOS does not occur in neurons.[102] Neuronal cNOS copurifies to homogeneity with NADPH diaphorase, which occurs in 2% of cerebral cortical neurons. Studies using NADPH diaphorase staining suggest that NOS is present in dendrites and axon terminals associated with the microvasculature in brain parenchyma, although the limitations of this staining technique must be recognized because the two enzymatic activities are not necessarily colocalized.[101,103] Given the presence of NOS in central nervous system endothelial, neuronal, and glial cells, the potential roles of NO in cerebral blood flow, formation of memory, and neurotoxicity must be examined. Central nervous system-specific manifestations of disease states, such as hypertension, subarachnoid hemorrhage, ischemia-reperfusion, hypercapnia-mediated alterations in blood flow, Huntington's chorea, and Alzheimer's disease, have been considered as examples of aberrant NO physiology.[68]

Nitric oxide is a potent dilator of cerebral blood vessels *in vivo* and *in vitro* and is produced by the endothelium but not the vascular smooth muscle of cerebral arteries.[102,104,105] Similar to that of the systemic vasculature, endothelium-dependent relaxation of cerebral arteries to a variety of agonists, including acetylcholine, serotonin, substance P, and oxytocin, requires NO formation.[102] In addition, multiple investigators have established a role for NO in regulation of basal cerebral vascular tone, although there may be a differential response within large *versus* small vessel networks.[102,106,107] Surprisingly, a proportion of this NO is not of endothelial origin, but is derived from glia or neurons. Impaired endothelium-dependent relaxation of the cerebral arteries is noted in the presence of hypertension, hypercholesterolemia, subarachnoid hemorrhage, and ischemia.[102] However, the vessels maintain their vasodilatory responses to exogenous NO and nitroprusside. Investigators invoke a potential differential alteration in NO *versus* endothelium-derived contracting factor release.[108,109] After subarachnoid hemorrhage, relaxant response of cerebral arteries is impaired. Because free hemoglobin avidly binds NO and inhibits smooth muscle relaxation, it is thought to play a major role in the vasoconstriction following subarachnoid hemorrhage.[110] Another potential etiology is hemoglobin-mediated generation of superoxide with its consequent inactivation of NO and peroxynitrite generation. In a similar manner, production of superoxide is thought to mediate the impaired endothelial relaxant response of cerebral arteries after ischemia-reperfusion.[102,111] The role of NO in cerebral autoregulation, however, is unknown.

Neuronal NO also contributes to regulation of vascular tone. Activation of the glutamate receptor has been found to be a major stimulus for neuronal NO synthesis.[112] Experiments that demonstrate an increased cGMP response that is attenuated by NOS inhibitors and enhanced by L-arginine have established NO to be a transduction mechanism of the glutamate receptor.[68,112] Local application of the excitatory amino acids—glutamate, aspartate and NMDA (N-methyl-D-aspartate)—results in arteriolar dilatation via NO synthesis.[102] In addition, NO from neuronal sources mediates local cerebral blood flow in response to seizures and cortical depression.[102] Nitric oxide has been implicated in memory formation as the result of long-term potentiation of synaptic transmissions after specific receptor stimulation, and *in vivo* experiments have demonstrated that inhibition of NO synthesis impairs learning behavior.[113-116] Nitric oxide also may have a role in olfaction, nociception, and vision.[68] Finally, NO is produced by glial cells (astrocytes, microglia, and oligodendrocytes).[117] In the setting of global ischemia and reperfusion, elevated levels of NADPH diaphorase are noted in astrocytes, suggesting increased NO production.[118] Although, the functional consequences of glial cell production of NO are unknown, it has been suggested that glia may serve as cellular stores for L-arginine.[117]

The contribution of NO to cerebral blood flow after hypercapnia/hypoxia and ischemia-reperfusion deserves additional comment. Although increased cerebral blood flow after hypoxia is not NO dependent, several studies have implicated NO in the vasodilatation that accompanies hypercapnia.[102,119] Although the exact mechanistic details are unknown, increases in cerebral blood flow after hypercapnia are attenuated but not ablated by NOS inhibitors.[107,120,121] Activity of NOS inhibitors is maximal at a pCO_2 of 50 to 60 mm Hg and abolished at a $pCO_2 > 100$ mm Hg.[107] The cellular source of the NO in hypercapnia is unknown. Current consensus is that endothelium and vascular smooth muscle are not the primary sources of NO in this setting.[102] In the setting of cerebral ischemia, the NO concentration increases within minutes, followed by a gradual decline whereas during reperfusion, NO levels again increase.[122] A recent

study has demonstrated increased expression of cerebral endothelial cNOS after ischemia.[123] Theoretically, NO may offer both beneficial and detrimental effects in the setting of cerebral ischemia-reperfusion. Although maintenance of cerebral blood flow, inhibition of platelet function, inhibition of leukocyte adhesion, and blockade of excessive NMDA receptor activity are potentially protective, the interaction between NO and superoxide, with its consequent oxidative injury, is an additional consideration. Attempts to dissect the protective and toxic effects of NO have resulted in divergent conclusions. Repeated administration of NOS inhibitors after induction of cerebral ischemia reduces infarct volume and cerebral edema in several *in vivo* studies.[124,125] In newborn rats, dosage of an NOS inhibitor was protective for a period of 15 hours before initiation of ischemia by carotid occlusion.[126] *In vitro* models have shown that NO donors mimic the excitotoxicity associated with cell death in ischemic brain injury and that NOS substrate analogs block this effect.[102,127,128] These data imply that the extent of neuronal death in ischemic brain injury may be attenuated by NOS inhibitors.[1] However, other investigators have observed no effect or a detrimental effect on ischemia-induced cerebral injury after NOS inhibition.[102,107] Although the exact physiology remains to be clarified, it is suggested that NO may be beneficial in the period immediately after initiation of cerebral ischemia, but may be neurotoxic after many hours to days. Additional considerations relevant to the neurotoxicity or neuroprotective properties of NO are the redox state of NO and the relative contributions of iNOS and cNOS. Additional study is required.

INFLAMMATION AND IMMUNE REGULATION

The production of NO by iNOS in macrophages and lymphocytes suggests a role in the regulation of immunity and inflammation. Peripheral blood monocytes, alveolar macrophages, and neutrophils also have been shown to synthesize NO.[68,129] Inhibition of lymphocyte proliferative responses and antimicrobial and tumoricidal activity seen after induction of iNOS are thought to result from inhibition of enzymes in the electron transport chain and Krebs cycle, formation of OONO— and inhibition of DNA synthesis.[68,129] Nitric oxide synthesis is a necessary component of nonspecific defense mechanism for a number of pathogens—*Cryptococcus neoformans, Schistosoma mansoni, Trypanosoma brucei, Toxoplasma gondii, Mycobacterium, Leishmania* and *Plasmodium.*[130–136] Until recently, the pathway for induction of iNOS was presumed to be initiated by macrophage cytokine elaboration or lipopolysaccharide from gram-negative bacteria. However, Kremsner and coworkers have shown that malarial antigen could also induce iNOS expression.[137] The state of present knowledge suggests that nonspecific immunity is mediated by iNOS. Therefore, nonreticuloendothelial cells, such as hepatocytes, and vascular smooth muscle cells, which contain iNOS, may play a heretofore unrealized role in immunity. Specifically, the lung and liver, acting as immunologic filters, are anatomically well situated to act in this capacity.

Investigators have examined the effect of NO in lymphocyte proliferation. Observations indicate that the allogeneic immune response, as determined by the mixed lymphocyte reaction, involves NO synthesis.[138] Nitric oxide and the S-nitrosothiol, S-nitroso-glutathione inhibit T lymphocyte DNA synthesis by an IL-2 independent mechanism.[138] Conversely, administration of the NOS substrate inhibitor, L-NMMA, results in a robust response in mixed lymphocyte reaction.[139] In models such as the rat-sponge-matrix, rat heterotopic heart, orthotopic liver, and small bowel transplants, increased NO metabolites were found in allogeneic grafts when compared with syngeneic grafts.[140] In addition, increased NO production was found in the animals during rejection and graft-*versus*-host disease.[141] Monitoring of NO levels has been suggested as a clinical diagnostic device for initiation of intervention in transplantation management. In chronic inflammatory processes, NOS inhibitors ameliorate injury in such diverse processes as chronic ileitis, arthritis, and immune-complex mediated vascular injury in lung and dermal tissue, whereas synovial fluid from patients with active osteoarthritis contains elevated levels of NO metabolites.[68,129] Additional immunologic or inflammatory conditions that may involve aberrant NO production include type I diabetes mellitus and aortic aneurysm formation.[97,129]

ADDITIONAL CONSIDERATIONS

Nitric oxide, acting as a vasodilator and smooth muscle relaxant, has been implicated in human penile erectile function.[142] Electrically evoked *in vitro* relaxation of the corpus cavernosum is prevented by NOS inhibitors and mimicked by NO donors.[143,144] Trabecular smooth muscle relaxation occurs in association with an elevation in intracellular cGMP. Immunohistochemical staining has identified NOS in the autonomic innervation of the pelvis and penis.[145] In addition, injection of NO donors into the corpus cavernosum of human volunteers and patients with erectile dysfunction has confirmed its role in producing and potentiating penile erection.[146,147]

CONCLUSION

Nitric oxide is a deceptively simple free radical with a multitude of tissue-specific functions. The current state

of knowledge indicates major regulatory roles for NO in the cardiovascular, pulmonary, gastrointestinal, hepatobiliary, immune, and central nervous systems. As increased research into the field continues, NO undoubtedly will be found to have additional homeostatic roles. However, pathophysiologic states characterized by excessive NO production also exist. With the development of bioactive NO-adducts and tissue-specific NOS inhibitors, pharmacologic intervention in the clinical arena may soon occur. Nitric oxide biologic activity can be described as homeostatic at a certain basal level, but an excess or lack of NO contributes to a variety of physiologic derangements. When the clinician is faced with these disease states, additional consideration of the potential role of NO will be necessary. As the future brings increased pharmacologic manipulation of NO production, appropriate application will require a working knowledge of the role of NO in the specific disease state.

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