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Nitric Oxide in Inflammation

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Overview of Nitric Oxide

Biosynthesis of Nitric Oxide

During the past few years, an enormous amount of research has been conducted on the vascular L-arginine/nitric oxide (NO) system. Alterations of NO production and/or bioavailability have been shown to occur both in experimental animal models (Osborne et al., 1989b; Tsao et al., 1990; Gauthier et al., 1995; Scalia et al., 1996) and in humans (Chester et al., 1990; Boger et al., 1997) in diverse settings such as hypertension, hypercholesterolemia, diabetes, ischemia–reperfusion, and heart failure.

Nitric oxide is synthesized by an enzyme termed *nitric oxide synthase* (NOS) that exists in three isoforms: endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS). All three isoforms of NOS convert the semiessential amino acid L-arginine to L-citrulline, thereby releasing NO (Moncada et al., 1991). Calcium-calmodulin and tetrahydrobiopterin are essential cofactors in this reaction (Gross and Levi, 1992; Klatt et al., 1995; Su et al., 1995). The substrate for NOS is the basic amino acid L-arginine, with a K_m (Michaelis constant) of approximately 5 μmol/L (Venema et al., 1996). L-arginine is synthesized as a product of the urea cycle and circulates in the blood in concentrations nearly equal to 100 μmol/L (Boger et al., 1997). In endothelial cells, however, the concentration of L-arginine has been estimated to be in the several hundred micromolar to the low millimolar range (Arnal et al., 1995). L-arginine is actively transported into the endothelium (Bogle et al., 1996) through a process subject to regulation by cytokines (Cendan et al., 1995). Even in the absence of extracellular L-arginine, the endothelium can resynthesize this amino acid from L-citrulline by using a recently described novel biosynthetic pathway (Hecker et al., 1995). Binding of calmodulin to eNOS and nNOS appears to act as a "molecular switch" to enable electron flow from flavin prosthetic groups in the NADPH reductase to heme, thereby facilitating the conversion of O_2 and L-arginine to NO and L-citrulline (Su et al., 1995). Calmodulin is incorporated into the molecular structure of iNOS.

Tetrahydrobiopterin is a critical cofactor for NOS and appears to contrib-

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ute to the ability of the enzyme to bind L-arginine. Interestingly, in the absence of tetrahydrobiopterin, the enzyme transfers electrons to molecular oxygen to produce the superoxide anion (Pou et al., 1992). Although several studies have suggested that levels of L-arginine, L-citrulline, and tetrahydrobiopterin may be deficient in acute and chronic inflammatory states (Cooke et al., 1992; Pieper, 1997; Stroes et al., 1997), future work to examine the manner in which various disease states affect the biosynthesis of NO is needed.

Physiological Versus Supraphysiological Concentrations of Nitric Oxide

Considerable research has focused on the physiological, biochemical, and molecular actions of NO in normal physiological processes and during pathological conditions. At low nanomolar concentrations, NO is cytoprotective in a number of experimental vascular diseases including ischemia–reperfusion injury (Aoki et al., 1990; Johnson et al., 1991; Siegfried et al., 1992a; Pabla et al., 1996), respiratory distress syndromes (Rossaint et al., 1993), intimal hyperplasia associated with vessel injury (Guo et al., 1995), atherosclerosis (Cooke et al., 1992), and vascular thrombosis (Groves et al., 1993). However, other experimental studies have suggested that high micromolar concentrations of NO are cytotoxic and contribute to cell injury in a variety of disease states including disorders of the lung (Wizemann et al., 1994), endotoxic and hypovolemic shock states (Nava et al., 1991), ischemia–reperfusion injury (Schulz and Wambolt, 1995), and anoxia–reoxygenation injury (De May and Vanhoutte, 1983). These cytotoxic effects of NO have been attributed mainly to the inducible form of NOS (iNOS), which is normally inactive, but can be induced by a variety of cytokines and other pathophysiologic agents including bacterial lipopolysaccharide (LPS) endotoxin (Cunha et al., 1994). Over a period of marked cytokine stimulation (i.e., 6–24 hours following stimulation of iNOS), suprapharmacologic concentrations of NO potentially may be produced, which are thought to contribute to cell and tissue injury (Robbins et al., 1994). More recently, it has been suggested that the purported toxic effects of NO are a result of the formation of a free radical species formed through the interaction between NO and superoxide, which has been reported to be peroxynitrite ($ONOO^-$; Pryor and Squadrito, 1995).

Biological Effects of Nitric Oxide

Nitric oxide is a gas [molecular mass, 30 daltons (D)] that is highly soluble in lipids and thus readily diffuses across cell membranes (Ignarro et al., 1987). Nitric oxide has a half-life of only 10 to 20 seconds in physiologic media, including blood (Palmer et al., 1987; Moncada et al., 1989). The vascular endothelium produces nitric oxide, which is basally released at concentrations of about 2 to 20 nmol/L. This nitric oxide diffuses to the subjacent vascular smooth muscle where it regulates vascular tone. However, the endothelium-derived NO also diffuses to the luminal surface of the endothe-

Table 22.1. Important Effects of Physiological Concentrations of Nitric Oxide

Preserves endothelial integrity
Dilates arterial blood vessels, participates in autoregulation of blood flow to vital organs
Inhibits leukocyte–endothelium interaction by suppressing expression of cell adhesion molecules
Inhibits platelet adherence and aggregation
Prevents microvascular fluid leakage
Maintains renal glomerular function
Promotes endothelial regeneration following injury
Inhibits vascular smooth muscle cell proliferation
No significant effects on myocardial contractility

lium where it exerts a number of important physiological effects (Table 22.1) including: (a) scavenging of superoxide radicals (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986); (b) inhibition of platelet adherence and aggregation (Radomski et al., 1987a, 1990); (c) modulation of endothelial layer permeability (Kubes and Granger, 1992); and (d) attenuation of leukocyte–endothelium interaction (Kubes et al., 1991; Davenpeck et al., 1994a).

Against this backdrop of important effects of physiological concentrations of NO, effective NO levels are decreased in a variety of circulatory disorders, including myocardial ischemia–reperfusion (Tsao and Lefer, 1990; Pearl et al., 1994), circulatory shock and trauma (Lefer and Lefer, 1993; Scalia et al., 1996), hypercholesterolemia and atherosclerosis (Freiman et al., 1986; Osborne et al., 1989a). The decrease in nitric oxide levels occurs in the early stages of hypercholesterolemia before the development of atherosclerotic plaques (Freiman et al., 1986; Lefer and Ma, 1993; Scalia et al., 1998), and is clearly due to reduced basal release of NO (Lefer and Ma, 1993) in addition to diminished agonist-mediated NO release. It is therefore not surprising that replacement therapy to restore the NO deficit has been considered in several of these disease states.

Nitric Oxide Synthases

Nitric oxide is synthesized in mammalian cells by a family of three NO synthases (NOS; Moncada and Palmer, 1991). It is not known whether additional mammalian NOS isoforms exist, but the failure of homology-based molecular cloning approaches to identify novel NOS cDNA makes it unlikely that newly discovered members of the mammalian NOS gene family will bear significant structural similarity to the current trio of isoforms. A widely accepted nomenclature, which will be used in this chapter, identifies the three mammalian enzyme isoforms as nNOS, iNOS, and eNOS, reflecting the cell of origin for the original protein and cDNA isolates (Moncada et al., 1997). The human genes for the NOS isoforms are categorized in order of their isolation and characterization. Thus, the human genes encoding nNOS, iNOS, and

Table 22.2. Isoforms of Nitric Oxide Synthase (NOS)

Isoform	Regulation	Molecular Mass	Tissue/Cell
I (nNOS)	Ca^{2+}-calmodulin	160 000	Brain Skeletal muscle
II (iNOS)	Unknown, inducible by cytokines	130 000	Macrophages Vascular smooth muscle Cardiac myocytes Glial cells
III (eNOS)	Ca^{2+}-calmodulin	135 000	Endothelial cells Platelets Cardiac myocytes

eNOS are termed *NOS 1, NOS 2,* and *NOS 3,* respectively. Table 22.2 summarizes the main characteristics and tissue distribution for the three isoforms of NOS.

The eNOS and nNOS forms of the enzyme are important physiologically in maintaining a variety of homeostatic responses. The nNOS isoform of the enzyme is important as a neurotransmitter in the gastrointestinal tract (e.g., gastric emptying, defecation) and in central nervous system integration. The iNOS isoform of the enzyme is normally inactive, or functioning at low levels of expression, but can be induced by a variety of cytokines and bacterial endotoxin. However, the same NOS isoform may play entirely distinct biological roles when expressed in different tissues, and must not be assumed that pathways outlined in one tissue necessarily pertain when the same isoform is expressed in a different cell. For example, differential tissue-specific splicing of nNOS mRNA generates structurally distinct protein molecules when the enzyme is expressed in neurons versus skeletal muscle (Silvagno et al., 1996).

Physiological Roles of Nitric Oxide Relevant to Inflammation

Injury or activation of the endothelium changes its regulatory functions and results in abnormal endothelial function. The dysfunction of the endothelium has been functionally defined as an imbalance between relaxing and contracting factors, between procoagulant and anticoagulant mediators, or growth-inhibiting and growth-promoting substances. The endothelium undergoes phenotypic modulation in response to injury or activation. A role of the endothelial cells in cardiovascular diseases might then be dependent on these phenotypic alterations, rather then on actual damage per se.

One of the important early events in the pathophysiology of endothelial dysfunction is the loss of ability to release endothelium-derived NO (Lefer et al., 1991). There are several varieties of acute and chronic circulatory dis-

eases that produce a marked degree of endothelial dysfunction characterized by a significant reduction of endothelium-derived NO. These include ischemia–reperfusion of heart, kidney, splanchnic organs, and brain, in which a rapid increase in oxygen-derived free radicals is a major factor contributing to loss of effective concentrations of NO over a period of about 2.5 to 5 minutes (Tsao and Lefer, 1990; Tsao et al., 1990). Hemorrhage and traumatic shock also result in reduced NO within 5 minutes (Scalia et al., 1996), whereas congestive heart failure, atherosclerosis, and hypertension produce a gradual loss of NO over a period of weeks or months (Cohen et al., 1988; Freiman et al., 1996).

Effects on Vascular Smooth Muscle

Nitric oxide produced by the vascular endothelium regulates vascular tone by relaxing vascular smooth muscle cells (Furchgott and Vanhoutte, 1989). This effect of NO was shown to be inhibited by hemoglobin, methylene blue, and other agents such as dithiothreitol and hydroquinone and to be mediated by stimulation of guanylate cyclase with the consequent elevation of intracellular cyclic guanosine monophosphate (cGMP; Moncada et al., 1991). Endothelium-dependent relaxation, which has been shown in many vascular preparations, including veins, arteries, and microvessels, occurs in response to a variety of humoral agents, such as acetylcholine, adenine nucleotides, thrombin, substance P, the calcium ionophore A23187, and bradykinin (Moncada et al., 1991). Other stimuli such as hypoxia, increase in flow, and electrical stimulation, can also cause endothelium-dependent relaxation of vascular tissue in vitro. It has been extensively demonstrated that a continuous release of NO maintains dilator tone, thus regulating systemic blood pressure and endothelial function (Moncada et al., 1991). It is likely that NO-dependent vasodilator tone is locally regulated and, as such, is one of the simplest and yet most fundamental adaptive mechanisms in the cardiovascular system. Therefore, it is possible that the loss of NO-mediated vasodilator tone is at least as important in essential hypertension, atherosclerosis, and in vasospastic phenomenona as those observed in the irreversible phases of circulatory shock.

Inotropic Actions on Cardiac Muscle

Over the past 5 years, the explosion of reports on the role of nitric oxide in the regulation of cardiac energetics has generated much discussion. As in the case of vascular physiology and biology, the discovery and characterization of myocardial NO-dependent signaling pathways have clarified many aspects of specific signal transduction cascades in cardiac muscle while generating apparent paradoxes and new hypotheses to be tested.

A number of cellular constituents of cardiac muscle can express iNOS in response to LPS and specific cytokines, including the endothelium and smooth muscle of the cardiac microvasculature, the endocardial endothelium, tissue macrophages, and cardiac myocytes (Kelly et al., 1996). However,

the physiological roles and pathophysiological consequences following induction of this high-output NOS in these cells are less clear. The most convincing evidence for an important pathophysiological role for iNOS to date has come from experimental animal models of systemic inflammatory response syndrome (Ungureanu-Longrois et al., 1995) and cardiac allograft transplantation (Worrall et al., 1996). However, Thoenes et al. (1996) could find no evidence of increased iNOS expression in patients with heart failure from specimens obtained postmortem, although iNOS was present in the hearts of most of the patients who succumbed to systemic sepsis.

Because of this limited availability of information, much of our understanding of the inotropic effects of NO on cardiac myocytes is based on actions of pharmacological NO donors or agents that mimic some actions of cGMP. Nitric oxide donors do not appear to exert any significant effect on myocardial contractility up to and including rather high concentrations. Only when cardiac muscle is stimulated with β-adrenergic agents, do nitric oxide donors exert a negative inotropic effect, and that is small, averaging only 5%–7% decrease from control values (Brady et al., 1992; Balligand et al., 1993). Nitric oxide donors fail to exert a significant negative inotropic effect in isolated cardiac myocytes, or in isolated ventricular papillary muscles even at concentrations far above those that result in maximal vasorelaxation of vascular rings isolated from the same animal (i.e., 500 nmol; Weyrich et al., 1994). Nitric oxide donors also fail to induce a significant inotropic effect even in the intact animal (Pennington et al., 1979; Lefer et al., 1993; Crystal, and Gurevicius, 1996). Moreover, NOS inhibitors exacerbate the cardiodepression observed in dogs subjected to cardiac stunning (Hasebe et al., 1993). These and other data using authentic NO gas in isolated cardiac preparations fail to show a significant direct cardiodepressant effect of NO. This is not surprising because the high myoglobin levels in cardiac myocytes sequester the NO before it is able to exert any significant inotropic effect on the heart.

Effects on Leukocyte–Endothelium Interaction

Effective NO levels are decreased in a variety of acute inflammatory disorders of the cardiovascular system, including myocardial ischemia–reperfusion, circulatory shock, trauma, hypercholesterolemia, and atherosclerosis. Under these conditions, circulating leukocytes marginate, adhere to the microvascular endothelium, and emigrate through the endothelial monolayer where many of them infiltrate into the interstitial space, and migrate toward the source of inflammation (Fig. 22.1).

The first clear demonstration that nitric oxide can regulate leukocyte–endothelium interaction is the now classic study of Kubes et al. (1991), who showed that NOS inhibition enhanced leukocyte adherence to the mesenteric vascular endothelium in the cat, which could be blocked by a monoclonal antibody directed against the β_2 integrins. This was a seminal finding that pointed toward nitric oxide deficiency as being important in the pathophysiology of inflammation. Further studies demonstrated that this inhibitory effect on leukocyte behavior by NO has several components. First, nitric

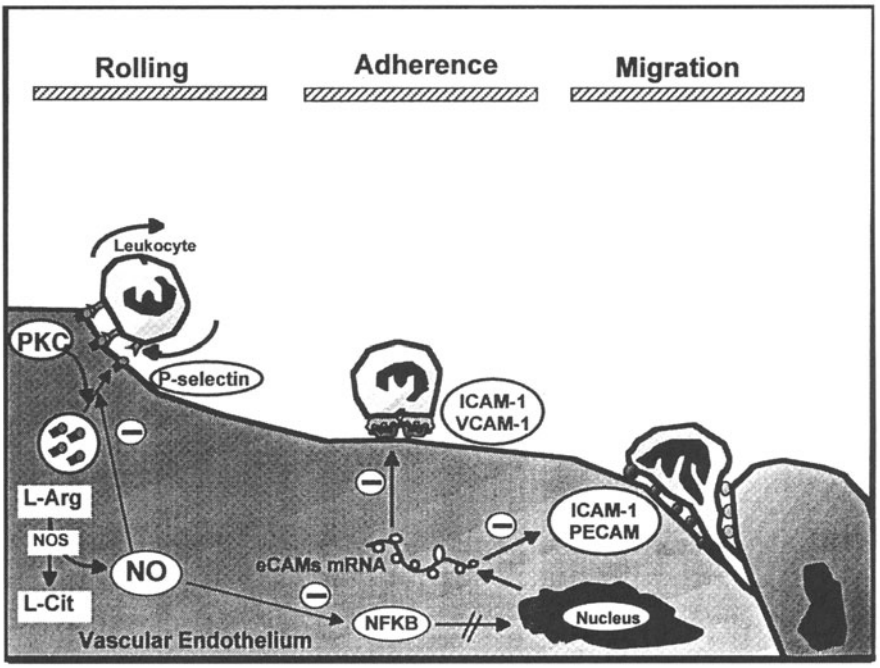

Fig. 22.1. Neutrophil–endothelial cell interactions in the microvasculature: Under normal conditions nitric oxide (NO) generated from L-arginine within the endothelial cell controls leukocyte rolling, adherence, and transmigration, thus protecting against inflammatory insult. Physiological NO levels in the endothelium modulate expression of cell adhesion molecules and the nuclear transcription factor (NF-κB); activation of NF-κB results in increased surface expression of endothelial cell adhesion molecules (eCAMs). Increased expression of P-selectin (P-sel) on the endothelial cell surface promotes PMN rolling via its high affinity P-selectin glycoprotein ligand-1 (PSGL-1) on the neutrophil (PMN) surface. Increased intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression regulates firm adhesion. Finally, ICAM-1 and PCAM permits PMN transmigration across the vascular endothelium (PKC = protein kinase C; L-Arg-L-arginine; L-Cit = L-citrulline).

oxide markedly attenuates leukocyte rolling along the endothelium by inhibiting the expression of P-selectin on the vascular endothelium (Davenpeck et al., 1994b). Second, nitric oxide inhibits the firm adherence of leukocytes to the endothelium (Gauthier et al., 1994, 1995), partially by inhibition of ICAM-1 and VCAM-1 expression (De Caterina et al., 1995). Down-regulation of these cell adhesion molecules by NO occurs through inhibition of protein kinase C activation (Murohara et al., 1996) and by prevention of activation of the nuclear transcription factor NF-κB (De Caterina et al., 1995), which usually induces expression of the mRNA for these adhesion molecules. Third, NO inhibits leukocyte action by inhibiting the cytoassembly of NADPH

oxidase (Clancy et al., 1992), thereby attenuating the release of superoxide radicals by activated leukocytes, particularly granulocytes (Moilanen et al., 1993).

The initial step in leukocyte recruitment during the inflammatory response is leukocyte rolling along the endothelium of postcapillary venules (Fig. 22.1). Leukocyte rolling is largely mediated by P-selectin, a member of the selectin family of adhesion glycoproteins. P-selectin is believed to be one of the earliest endothelial cell adhesion molecules involved in leukocyte recruitment to the site of inflammation. Davenpeck et al. (1994b) extensively studied the interrelationship between NO and P-selectin and its correlation to ischemia–reperfusion phenomena. They demonstrated that ischemia–reperfusion of the mesenteric circulation, a condition that has been shown to reduce dramatically endothelial NO, resulted in a rapid increase in leukocyte rolling and adherence to the venular endothelium within the first 30 minutes following reperfusion. This clearly indicates that the critical reduction in NO release 30 minutes after reperfusion is correlated with an increased upregulation of P-selectin on the endothelium (Davenpeck et al., 1994b). Moreover, Gauthier et al. (1994) showed that in the same model of ischemia–reperfusion of the rat mesenteric circulation, infusion of a nitric oxide donor markedly attenuated postreperfusion rolling and adherence of leukocytes to the venular endothelium. This clearly demonstrated that restoration of physiological NO levels in the systemic circulation during microcirculatory perturbations results in a reduced leukocyte–endothelial cell interaction with amelioration of the associated circulatory shock. In order to investigate this interrelationship between NO and adhesion molecules further, Davenpeck et al. (1994b) also showed that exposure of the rat mesenteric microvasculature to NOS inhibitors mimics many of the effects of ischemia–reperfusion; the main results from that study are depicted in Figure 22.2. The number of adherent leukocytes in the microvasculature is very low during normal physiological conditions. However, exposure of the rat mesentery to increasing concentrations of L-N^G-nitroarginine methyl ester (L-NAME) resulted in a consistent and concentration-dependent increase in leukocyte adherence. Equimolar concentrations of L-arginine, but not D-arginine, were able to overcome L-NAME–induced leukocyte adherence, thus suggesting the specific NO-mediated mechanism of L-NAME–induced leukocyte–endothelium interaction. In this regard, leukocyte adherence could be completely abolished by administration of a monoclonal antibody directed against P-selectin. Furthermore, immunohistochemical localization of P-selectin was upregulated in the rat mesenteric microvasculature following direct inhibition of NO synthase by L-NAME, confirming the important interaction between P-selectin and nitric oxide.

Similar results were obtained with leukocyte adherence. The expression of immunoglobulin superfamily members (i.e., intercellular adhesion molecule-1, or ICAM-1 and vascular cell adhesion molecule-1, or VCAM-1) on the microvascular endothelium has been recently correlated to inhibition of nitric oxide synthesis and release from endothelial cells during hypercholesterolemia (Scalia et al., 1998), and in experimental models of inflammation

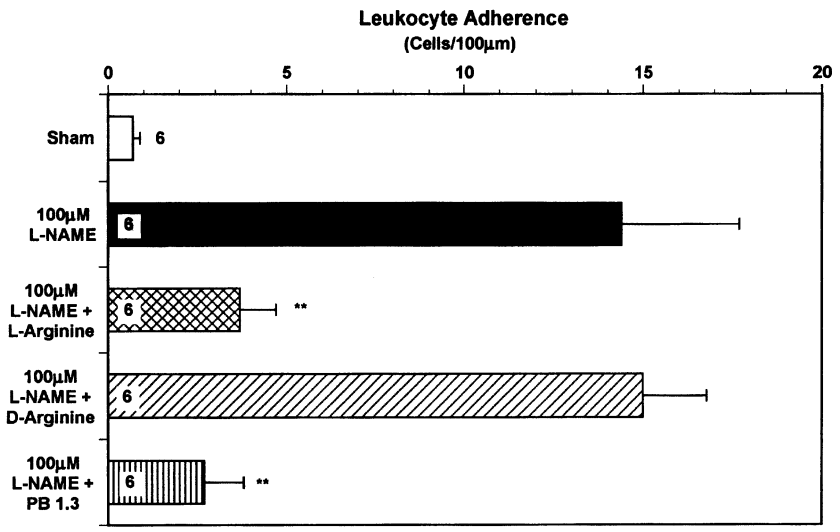

Fig. 22.2. L-NAME–induced inhibition of nitric oxide synthase increases leukocyte adherence along the venular endothelium of the rat mesenteric microcirculation. This phenomenon was inhibited by equimolar concentration of L-arginine and was also blocked by the systemic administration of an antibody against P-selectin (PB 1.3). Therefore, loss of endogenous nitric oxide release up-regulates leukocyte adherence, the prelude to leukocyte infiltration into inflamed tissues (from Davenpeck et al., 1994b).

induced by superfusion of the rat mesentery with either L-NAME, thrombin, or hydrogen peroxide (Scalia et al., 1997; Scalia and Lefer, 1998). In particular, ICAM-1 expression on endothelial cells was found to be up-regulated during induction of hypercholesterolemia in animals fed a high cholesterol diet (Gauthier et al., 1995; Scalia et al., 1998). More recently, nitric oxide was also found to be able to reduce cytokine-induced expression of another cell adhesion molecule characteristic of endothelial activation during atherogenesis (i.e., VCAM-1; De Caterina et al., 1995). Nevertheless, in chronic gastrointestinal inflammatory states, high concentrations of NO may be proinflammatory by being converted to nitrogen dioxide (NO_2) or nitrogen trioxide (N_2O_3; Miller and Sandoval, 1999).

Adhered leukocytes exit the microcirculation and emigrate into the extravascular space (Fig. 22.1). The process of extravasation requires a complex interaction between leukocyte adherence glycoproteins and their counterreceptors on the venular endothelial cells in response to chemotactic stimuli present within inflamed tissues. This process has also been shown to be modulated by NO released by the vascular endothelium, as confirmed by the increase in extravasated leukocytes in mesenteric tissue of L-NAME–superfused rat mesenteries (Scalia and Lefer, 1998).

Finally, selective gene deletion has been accomplished for all three iso-

forms of NOS (see Table 22.2). Recently, microvascular responses to thrombin stimulation have been studied in eNOS and nNOS gene-deleted mice (Lefer et al., 1999). Both eNOS and nNOS gene-deleted mice showed marked increases in both leukocyte rolling and adherence in thrombin-stimulated mouse peri-intestinal venules. These increased leukocyte–endothelium interactions were on the order of three to seven-fold, and were in large part due to up-regulation of P-selectin on the venular endothelium.

These findings, taken together, clearly point to a dynamic interaction between endothelium-derived nitric oxide and leukocyte–endothelial cell interaction in vivo. Reduced nitric oxide release from the microvascular endothelium, during abnormal pathological conditions, leads to increased adhesion molecule expression on the endothelial cell surface. This phenomenon plays a significant role in the margination and emigration of leukocytes from the blood stream and their subsequent accumulation into injured tissues.

Antiplatelet Effects of Nitric Oxide

One important anti-inflammatory property of NO is inhibition of platelet aggregation (Radomski et al., 1987a). NO inhibits platelet aggregation via a cGMP-dependent mechanism (Mellion et al., 1981). Together with prostacyclin, NO inhibits platelet aggregation and disaggregates platelets, suggesting that the release of NO by the vascular endothelium plays an important homeostatic role by maintaining thromboresistance of the endothelium.

In addition, NO inhibits platelet adhesion to collagen fibrils, endothelial cell matrix, and endothelial cell monolayers (Radomski et al., 1987b, 1987c). In contrast, prostacyclin has only a weak inhibitory effect on platelet adhesion (Higgs et al., 1978). This suggests a key role for NO in the process of platelet adhesion and repair of the vessel wall during physiological and pathophysiological conditions. In this regard, a recent study has reported that treatment of isolated platelets with nitric oxide donors attenuates expression of P-selectin induced by activation of protein kinase C (Murohara et al., 1996). Thus, NO exerts antiplatelet functions by both blocking platelet aggregation and inhibiting P-selectin expression on the platelet cell surface.

Nitric Oxide and Transcription Factors

Although it is clear that nitric oxide exerts significant anti-inflammatory effects in the intestinal microvasculature and epithelium, the precise mechanisms of these effects are not fully elucidated. The up-regulation of E-selectin, ICAM-1, and VCAM-1 in endothelial cells requires de novo synthesis of proteins, and therefore requires about 2 hours. De Caterina et al. (1995) recently showed that, in cytokine-stimulated human saphenous vein endothelial cells, several NO donors inhibit VCAM-1 expression by 35%–55%, and also reduce E-selectin and ICAM-1 expressions to a lesser extent under similar conditions. In the case of VCAM-1, nitric oxide attenuated VCAM-1 gene transcription, in part, by inhibiting NF-κB.

In cultured human endothelial cells, L-NAME markedly up-regulated P-selectin mRNA and protein expression (Armstead et al., 1997b), which led to significantly increased leukocyte adherence to cultured human endothelial monolayers. Moreover, this up-regulation of P-selectin could be reversed by co-incubation with the nitric oxide donor, SPM-5185 (Armstead et al., 1997b), but not by its non-NO donating control compound lacking the NO moiety. One of the messengers in the up-regulation of P-selectin, and other endothelial cell adhesion molecules in some species is NF-κB (De Caterina et al., 1995). Recently, NF-κB up-regulation (i.e., binding of a P-selectin–specific NF-κB element to nuclear extracts) was found in organs of rats subjected to traumatic shock (Armstead et al., 1997a). The mechanism of this enhanced P-selectin mRNA appears to relate to IκB-α promoter activity (Spiecker et al., 1998) because nitric oxide donors reduced endothelial cell adhesion molecule expression up to 70% through induction of IκB-α promoter activity. IκB-α is a functional inhibitor of NF-κB, and thus effectively diminishes P-selectin and other adhesion molecules' (e.g., ICAM-1) adhesive action on endothelial cells.

Actions of Supraphysiologic Concentrations of Nitric Oxide

Although NO exerts a variety of important homeostatic effects at nanomolar concentrations, NO is purported to exert cytotoxic effects at high concentrations (i.e., in the high micromolar range). These effects are somewhat controversial because it is not clear whether (a) these concentrations can ever occur in vivo, (b) all of these cytotoxic effects are detrimental to the host organism, and (c) the data pointing to cytotoxic effects of NO really are direct effects of NO, or are due to the actions of other substances. We will review the salient features of this area relevant to the role of NO in inflammation.

Antimicrobial Actions of Nitric Oxide

Nitric oxide was originally discovered in biological systems from two very different directions. The first insight was as an endothelium-derived relaxing factor (EDRF) by pharmacologists (Furchgott and Zawadski, 1990). However, in a parallel manner, NO was discovered by microbiologists as an endogenous bactericidal agent (Hibbs, 1991; Nathan and Hibbs, 1991). Thus, NO released by certain lymphocytes and macrophages can act as an anti-infective agent by killing invading bacteria. This works very well in murine leukocytes, but human leukocytes produce much lower levels of NO, which puts in doubt the clinical relevance of this mechanism in humans (Denis, 1991). The antimicrobial effects of NO appear to include a variety of viruses as well as bacteria and fungi (Mannick et al., 1994). In this case, both latent and lytic viruses, normally suppressed by endogenous NO, can reactivate and reinfect when NO synthesis is inhibited by con-

ventional NOS inhibitors (i.e., L-NMMA). This raises an important question regarding the clinical usefulness of NOS inhibitors in situations like septic shock.

Potential Role of Nitric Oxide in Septic or Endotoxic Shock

The potential role of NO in endotoxemic or septic shock has been, and continues to be, controversial. Bacterial endotoxin is known to stimulate iNOS in vascular smooth muscle cells and in macrophages, generating large amounts of NO (Shears and Billiar, 1995). Whether these high levels actually occur in vivo is problematic and unproven at present. In fact, there is abundant evidence that vascular NO levels assessed by measuring endothelium-derived nitric oxide is uniformly reduced following endotoxemic shock (Lefer, 1998). If nitric oxide levels markedly increase in endotoxemic or septic shock, they would have to be elevated by three or four orders of magnitude in order to induce shock. It is highly unlikely that localized NO would spill over into the circulation, an event that is a prerequisite for the propagation of the hypotension occurring in septic shock (Lefer, 1998).

One of the main pillars of the hypothesis that NO is a key mediator of endotoxic or septic shock is that inhibition of nitric oxide synthases is beneficial in these forms of shock states. This topic has received considerable attention by many investigators since the provocative report of Petros et al. in 1991. That group treated two patients in septic shock with an NOS inhibitor. Of the two shock patients receiving L-NMMA, one lived and one died, rendering this study rather inconclusive because the usual mortality rate of septic shock is about 50%. Kilbourn and Griffith (1992) advanced the hypothesis that inhibition of NOS is a potentially important treatment in endotoxemic or septic shock, although others (Wright et al., 1992) have cautioned that inhibition of both constitutive and inducible NOS during endotoxemia may be undesirable.

Cobb and Danner (1996) reviewed the endotoxic shock literature related to the effects of NOS inhibitors on hemodynamics and survival in large animals and humans; of the 26 animal and 4 human studies, only 5 actually measured survival (i.e., dog and pig studies). Two of those studies showed that NOS inhibitors actually reduced survival and 3 showed no change in survival rates. Furthermore, deleterious effects of NOS inhibitors were found to contribute to increased mortality in endotoxemic rabbits (Pastor et al., 1994) and endotoxemic mice (Minnard et al., 1994). NOS inhibitors have deleterious hemodynamic effects in several species including dogs, sheep, pigs, and humans (Cobb and Danner, 1996). This relates to the vasoconstrictor effect of NOS inhibitors; of the 28 studies in which systemic vascular resistance was measured, 26 reported a marked increase in vascular resistance and 2 found no change (Cobbs and Danner, 1996). Coupled with cardiac output measurements in 29 studies, 22 showed a significant decrease and 7 exhibited no change in cardiac output during endotoxic shock. An increase in cardiac output—an essential compensatory response in circula-

tory shock states like endotoxemic shock—has never been observed following administration of a NOS inhibitor.

These latter findings suggest that by shutting off endogenous production of NO by the endothelium, one causes marked vasoconstriction, increasing arterial blood pressure at the expense of blood flow. NOS inhibitors also aggravate pulmonary hypertension and reduce oxygen delivery to the tissues. It is clear that circulatory shock is a consequence of a sustained and marked reduction in blood flow to the vital organs, which if not reversed, usually leads to cardiovascular collapse (i.e., circulatory shock) and eventually to death (Lefer, 1994). Thus, reducing cardiac output even further with NOS inhibitors is counterproductive in endotoxemic and other forms of circulatory shock.

As a further cautionary note, recent studies have shown that in iNOS-deficient mice, there is a significantly enhanced leukocyte–endothelium interaction that promotes local tissue inflammation (Hickey et al., 1997). This is consistent with the report that iNOS exerts vasculoprotective effects in the coronary circulation of pigs (Fukumoto et al., 1997). Thus, the increase in iNOS activity in sepsis may actually be a compensatory effect due to the decrease in eNOS activity.

In summary, there is reasonable doubt that NOS inhibition can significantly protect in endotoxic shock. Moreover, it is very difficult to obtain a selective iNOS inhibitor that does not also inhibit eNOS. The selectivity is blurred at doses that are necessary to dramatically inhibit iNOS. In fact, a recent class of "selective" iNOS inhibitors was found to be cardiotoxic and had to be discontinued as potential drug candidates (Southan et al., 1995). Moreover, when NOS inhibitors block nitric oxide production, they also expose the host to latent virus infections that can be extremely dangerous (Mannick et al., 1994) because nitric oxide functions normally to attenuate viral infections.

Peroxynitrite as a Toxic Mediator of Nitric Oxide Effects

Nitric oxide and superoxide radicals can interact to form the highly reactive species, peroxynitrite ($ONOO^-$; Radi et al., 1991; Pryor and Squadrito, 1995), which is reported to be cytotoxic at high concentrations (Radi et al., 1991). Peroxynitrite has been invoked as a mediator of myocardial and parenchymal cell injury resulting from ischemia–reperfusion or shock states (Szabo et al., 1995; Yasmin et al., 1997). These investigators found that concentrations of 30–100 μmol $ONOO^-$ cause myocardial injury in isolated perfused rat hearts subjected to ischemia–reperfusion, and that nitrotyrosine, a purported "footprint" of peroxynitrite, is detectable in these rat hearts as evidence of the presence of peroxynitrite.

There are several serious problems with accepting peroxynitrite as a major mediator of inflammatory states like reperfusion injury. First, circulating concentrations of NO are normally 1–10 nmol/L (Kelm and Schrader, 1990), and $ONOO^-$ levels, even at maximal iNOS activation, are only in the low

micromolar range (i.e., 0.4–5.0 μmol; Grisham et al., 1998). These findings, coupled with the ultrashort half-life of $ONOO^-$ (< 1 second) make it highly unlikely that $ONOO^-$ occurs in vivo at concentrations above 1–2 μmol. In fact, when measured by electron spin resonance (ESR) techniques, peroxynitrite levels in the ischemic–reperfused rat heart were only 100 nmol (Wang et al., 1996). Second, NO and superoxide form peroxynitrite only when they both exist in close proximity at equimolar concentrations. When there is a significant imbalance between these two reactants, the excess reactant exerts a feedback inhibition that curtails $ONOO^-$ formation (Miles et al., 1996). Moreover, one cannot measure authentic peroxynitrite in vivo. One must resort to immunohistochemical measurement of the so-called footprint of $ONOO^-$, nitrotyrosine. Unfortunately, nitrotyrosine can be formed from a variety of substances other than $ONOO^-$, including chloride ions, hypochlorous acid, myeloperoxidase, and other nitrogenous radicals (Eiserich et al., 1996, 1998). Even more troubling, Pfeiffer and Mayer's (1998) landmark study showed that peroxynitrite failed to nitrate tyrosine at physiological pH, casting grave doubts about nitrotyrosine as a valid marker of peroxynitrite at all. Physiologically relevant concentrations of $ONOO^-$ (i.e., in the range of 400 nmol to 2 μmol) actually protect against myocardial ischemia–reperfusion injury in the rat (Lefer et al., 1997) and the cat (Nossuli et al., 1997). Moreover, these concentrations of $ONOO^-$ significantly attenuate leukocyte–endothelium interactions both in vitro and in vivo (Lefer et al., 1997; Nossuli et al., 1997). Finally, $ONOO^-$ in vivo releases NO in solution as an NO donor (Nossuli et al., 1998). Recently, Balazy et al. (1998) showed that $ONOO^-$ and glutathione (GSH) can react to generate S-nitroglutathione ($GSNO_2$), which decomposes to generate nitric oxide. Furthermore, NO can be transnitrosated onto carrier proteins in plasma, which acts as an NO carrier, thus transporting and protecting the circulating NO until it is released from the S-nitrosated carrier molecule (Stamler et al., 1992). Stamler et al. suggested that S-nitrosothiol protein adducts act as circulating carriers of NO. This mechanism could explain the cytoprotective and beneficial effects of $ONOO^-$ in vivo. Thus, a significant body of evidence exists that seriously questions the role of peroxynitrite as a mediator of ischemia–reperfusion injury or circulatory shock.

Nitric Oxide Modulating Agents in Inflammatory States

Nitric oxide can be applied or administered in several forms and by several routes. One should be aware that although NO has a half-life of about 5 to 10 seconds at physiological pH and temperature (Moncada et al., 1991), NO can be infused so that its effects can be sustained for hours or days. In the case of carotid artery endothelial denudation and subsequent restenosis of the artery, a nitric oxide donor was infused intravascularly for 7 days by an osmotic pump implanted in the neck of the rat (Guo et al., 1994). Under those conditions, the nitric oxide donor, but not a control molecule having the same organic backbone but lacking the NO moiety, prevented restenosis

Table 22.3. Methods of Administering Nitric Oxide to Intact Animals

Form of Administration	Advantages	Disadvantages
NO (breathing)	Authentic NO; regionally active	Dose must be carefully titrated
NO (in solution)	Authentic NO; can be injected at site desired	Transient
NO donors (e.g., nitroglycerin, sodium nitroprusside)	Eliminates problems with handling of gas; readily absorbed	Some induce tolerance; nonlinear release of NO
NO precursors (L-arginine, tetrahydrobiopterin)	Can be ingested in diet or given intravenously	Effective only if precursor activity is low
NO enhancers (e.g., SOD)	Reduces the inactivation of NO	Is not specific for NO
NOS gene transfection	Can exert a long-term effect	Difficult to transfect effectively

Note. NO = nitric oxide; NOS = nitric oxide synthase; SOD = superoxide dismutase.

of the artery and promoted endothelial regulation (Guo et al., 1994, 1995). These findings show that continuous administration of very low amounts of NO can be physiologically relevant.

Table 22.3 summarizes the potential forms of administration of NO in vivo. As can be seen, NO can be administered in a variety of forms including authentic NO gas and organic nitrates that release NO in solution. The different classes of nitric oxide donors and their biological effects have recently been reviewed (Lefer and Lefer, 1994). One can also promote the production of endogenous NO or retard the inactivation of endogenous NO. This can be done by administering the precursor of NO (i.e., L-arginine), or an essential cofactor (i.e., tetrahydrobiopterin, TB_4, or by utilizing recombinant human superoxide dismutase (rhSOD), which scavenges superoxide radicals and thus retards the inactivation of NO by this oxygen-derived free radical. Also, one can transfect blood vessels in vivo with the gene for eNOS and allow the endothelium to produce and release endogenous NO.

Nitric Oxide Gas

The first study to show that NO could protect against an inflammatory disorder like reperfusion injury was conducted by Aoki et al. (1990), who infused authentic NO gas into cats subjected to occlusion of the splanchnic circulation for 120 minutes. The NO gas was dissolved in physiologic saline and infused close to the site of ischemia starting just prior to reperfusion. Nitric oxide markedly attenuated the postreperfusion circulatory collapse, retarded plasma proteolysis, and inhibited the formation of a cardiotoxic peptide known as myocardial depressant factor (MDF Lefer, 1987). These salutary effects of physiologic levels of NO led to a significant improvement in survival time and severity of splanchnic ischemia shock. This report was closely followed by a study using authentic NO in feline myocardial ischemia

reperfusion (Johnson et al., 1991). In these experiments, NO gas in solution was infused intravascularly just prior to coronary artery reperfusion, continuing over the entire 4.5-hour reperfusion period. The NO solution was also bioassayed on isolated cat aortic rings for vasorelaxant activity to ensure that the NO that was infused throughout the reperfusion period was biologically active. The beneficial effects of the NO infusion included a marked attenuation of cardiac necrosis and reduced neutrophil infiltration into the ischemic–reperfused myocardium (Johnson et al., 1991). Control solutions without NO were ineffective against these indices of cardioprotection. Thus, authentic NO in solution protected in two inflammatory situations.

In addition to using NO dissolved in aqueous media, NO gas can be inhaled. Zapol and colleagues (Frostell et al., 1991) pioneered the use of inhaled NO at low concentrations (i.e., 20–80 ppm) as a selective pulmonary vasodilator in hypoxic pulmonary hypertension. The beneficial effects of NO occurred in the absence of any systemic hemodynamic effects. Furthermore, inhaled NO attenuates leukocyte adherence in distant nonischemic microvascular beds during regional ischemia–reperfusion in the cat (Fox-Robichaud et al., 1998), clearly an anti-inflammatory effect. Thus, inhaled NO gas may be a useful means for delivering physiologically useful concentrations of NO to the heart and lungs, and perhaps other more distant organs. Presumably, the NO nitrosates plasma proteins and is transported in that manner to the site of action (Stamler et al., 1992). This may account for the effects of NO beyond the lungs.

Nitric Oxide Donors

A wide variety of nitrogenous compounds can release NO in solution. These NO-generating compounds are commonly known as NO donors. Virtually all of them are organic nitrates, but a substance as simple as sodium nitrite $NaNO_2$ at acidic pH (i.e., pH 2.0) can spontaneously release significant quantities of free nitric oxide in solution. The major classes of organic NO donors are the sydnonimines (e.g., SIN-1), cysteine-containing NO donors (e.g., SPM-5185), the NONOates (e.g., DEA/NO, SPER/NO), and of course the well-known organic nitrates widely used clinically for many years (e.g., nitroglycerin, sodium nitroprusside). The use of these NO donors in cardiovascular disease was reviewed in Lefer and Lefer (1994).

The earliest study showing that an NO donor is effective in reperfusion injury is that of Johnson et al. (1990) who employed acidified $NaNO_2$ in myocardial ischemia–reperfusion. The $NaNO_2$ significantly attenuated reperfusion-induced myocardial necrosis and cardiac infiltration of neutrophils demonstrating an anti-inflammatory effect. $NaNO_2$ also inhibited cat platelet aggregation in vitro. Later studies were conducted employing two sydnonimine NO donors including a control substance (i.e., the same organic backbone of the NO donor molecule minus the NO moiety). These NO donors (i.e., SIN-1, C87-3754) both attenuated cardiac necrosis by 65%–70%, confirming their effectiveness and supplying proof of concept. Importantly, the cardioprotective effect of these agents occurred at infusion rates

that did not affect systemic hemodynamics. Furthermore, both SIN-1 and C87-3754 preserved the coronary endothelium, significantly attenuating the endothelial dysfunction observed in untreated ischemic–reperfused cats (Siegfried et al., 1992b). These NO donors also inhibited the inflammatory actions of activated PMNs by preventing their release of superoxide and its subsequent inactivation of endothelium-derived NO (Moilanen et al., 1993). These results are consistent with data obtained with a cysteine-containing NO donor employed in myocardial ischemia–reperfusion injury (Siegfried et al., 1992a).

In addition to myocardial ischemia–reperfusion, NO donors are effective in splanchnic ischemia (i.e., splanchnic artery occlusion/reperfusion, SAO/R) and in total body ischemia–reperfusion (i.e., hemorrhage–reinfusion). In the cat, the sydnonimine NO donor C87-3754 significantly protected against the lethality of SAO/R. Survival rate increased significantly from 0% in untreated SAO/R cats to 75% in cats given C87-3754 (Carey et al., 1992). Moreover, the plasma activities of two pro-inflammatory agents, cathepsin D (a lysosomal protease) and myocardial depressant factor (MDF; a cardiotoxic peptide; Carey et al., 1992), were significantly inhibited by the active NO donor. The results were confirmed by Gauthier et al. (1994), who reported that S-nitroso-N-acetylpenicillamine (SNAP), but not its NO-depleted form, exerted important salutary effects in SAO/R in rats. These beneficial effects included attenuating leukocyte–endothelium interaction at the microcirculatory level. SNAP, a well-known NO donor, had previously been shown to have potent antineutrophil effects (Ma et al., 1993), and to protect rats subjected to severe hemorrhage–reinfusion, resulting in shock (Symington et al., 1992). In the case of hemorrhagic shock (Symington et al., 1992; Kurose et al., 1994), SNAP increased survival from 0% to 88% and markedly attenuated PMN infiltration into splanchnic organs as well as attenuating endothelial dysfunction. This is consistent with NO donors, which have been shown to reduce microvascular protein leakage in splanchnic ischemia–reperfusion injury (Kurose et al., 1994).

These findings suggest that subvasodilator doses of NO donors are effective against reperfusion injury, and that major mechanisms of the tissue protection in reperfusion injury are endothelial preservation and reduced leukocyte–endothelium interaction (Siegfried et al., 1992a,b; Lefer et al., 1993). Moreover, these mechanisms are consistent with the results obtained using authentic NO in these same disease states (Lefer and Lefer, 1993).

Nitric Oxide Precursors

The substrate for NO biosynthesis, the amino acid L-arginine, has also been studied in different inflammatory states. L-arginine, but not its stereoisomer D-arginine, markedly attenuated myocardial necrosis in cats and dogs subjected to myocardial ischemia–reperfusion (Nakanishi et al., 1992; Weyrich et al., 1992). In both cases, L-arginine was infused intravascularly just prior to reperfusion and continued for several hours. In addition to the cardioprotection afforded by L-arginine, neutrophil infiltration into the reper-

fused myocardium was attenuated. Both studies also showed that L-arginine preserved coronary endothelial function, suggesting an effect on the vascular endothelium via generation of NO synthesis. This beneficial effect probably is mediated by antineutrophil effects on the dysfunctional endothelium because L-arginine infused into isolated rat hearts perfused with PMNs preserved left ventricular function and coronary flow in global ischemia–reperfusion (Pabla et al., 1996). Thus, L-arginine preserves the vascular endothelium, suppresses leukocyte–endothelium interaction, attenuates PMN infiltration, maintains cardiac integrity (i.e., reduces necrosis) and preserves cardiac contractility in the setting of myocardial ischemia–reperfusion. Similarly, tetrahydrobiopterin (TB_4) a cofactor in NO biosynthesis, also protects in myocardial reperfusion injury in the dog (Tiefenbacher et al., 1996).

In addition to myocardial ischemia–reperfusion, L-arginine infusion reduces reperfusion injury in rabbit skeletal muscle (Huk et al., 1997), in rat liver (Nilsson et al., 1997), and in rat skin (Cordeiro et al., 1997). Thus, L-arginine exerts cytoprotective effects in a wide variety of tissues when given parenterally just before reperfusion. Another advantage of L-arginine is that it can be given orally. This has been achieved in hypercholesterolemic rabbits by oral feeding of L-arginine (Cooke et al., 1992), which improved endothelial function and retarded atherogenesis over several months. More recently, humans ingesting L-arginine orally for 6 months experienced improved coronary vascular endothelial function associated with ameliorated symptoms of coronary artery disease (Lerman et al., 1998). These findings support the value of chronically enhancing endogenous NO production in coronary vascular disorders and other inflammatory diseases.

Nitric Oxide Snythase Gene Transfection

An interesting and new approach to augmenting physiologic quantities of NO in vivo is to transfect the gene encoding the endothelial form of nitric oxide synthase (eNOS). This has been achieved in the rat carotid artery subjected to intimal injury (von der Leyen et al., 1995). Transfection of this gene significantly attenuated the restenosis of the rat carotid artery and maintained vascular homeostasis in this important blood vessel. Thus, gene transfection may ultimately become a therapeutic modality to enable NO-deficient vessels to regain their NO synthetic capacity.

Summary

In summary, basal NO produced by the vascular endothelium in physiologic amounts results in circulating levels of 1–10 nmol/l and acts as a very important homeostatic agent. Nitric oxide formed by eNOS exerts a variety of anti-inflammatory effects including inhibition of leukocyte–endothelium interaction, attenuation of platelet aggregation, reduction in microvascular fluid leakage, and quenching of superoxide radicals.

Higher concentrations of NO in the low micromolar range can occur during activation of iNOS. Although high levels of NO are purported to be toxic to certain cells, micromolar concentrations of NO exert important bactericidal and antiviral effects that are important in preventing systemic and local infections. Indeed, NO formed from iNOS can replace the lost or reduced NO usually observed in inflammatory states like reperfusion injury and circulatory shock. Thus, more recent evaluation of the role of higher levels of NO tend to assign some positive value to these NO concentrations rather than the more classical negative view of higher NO concentrations.

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