Role of Nitric Oxide in Inflammation and Tissue Injury during Endotoxemia and Hemorrhagic Shock

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Since the discovery that nitric oxide (NO) accounts for the biologic activity of endothelial-derived relaxing factor, a torrent of research over the last decade has focused on its role, protective or detrimental, in myriad pathophysiologic conditions. Recently, increasing attention has focused on NO as a possible mediator of the severe hypotension and impaired vasoreactivity characteristic of circulatory failure. Given the ubiquitous and complex role of NO in biologic systems, inhibition of NO synthesis in experimental and clinical studies of shock has yielded mixed, sometimes contradictory, results. Although overproduction of NO in the vasculature may result in systemic vasodilation, NO synthesis has also clearly been shown to have a beneficial role in regulating organ perfusion and mediating cytotoxicity. In this review, the pathophysiologic importance of NO in septic shock and hemorrhagic shock is discussed, and novel therapeutic strategies for manipulation of NO formation are examined. — Environ Health Perspect 106(Suppl 5): 1139–1143 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl5/1139-1143/shah/abstract.html

Key words: nitric oxide, inducible nitric oxide synthase, sepsis, hemorrhagic shock

Introduction

Since the first report that nitric oxide (NO) can account for the biologic activity of endothelial-derived relaxing factor, increasing evidence has accumulated over the last decade that NO plays an important role in various biologic systems (1,2). NO is formed from the amino acid L-arginine via the enzyme nitric oxide synthase (NOS). Three distinct isoforms of NOS are known to exist; two were originally described as constitutive and one inducible (3). The constitutive enzymes, neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are primarily calcium–calmodulin dependent, although eNOS does exhibit calcium-independent activity under certain conditions. The inducible NOS (iNOS or NOS2) is fully active at physiologic calcium levels. When stimulated, nNOS and eNOS intermittedly produce small (picomolar) amounts of NO over short time intervals. Their main function is as cell-signaling mediators of physiologic processes such as neurotransmission, regulation of local blood flow, and blood pressure. In contrast, iNOS generates large (micromolar) quantities of NO over extended periods of time during host defense and immunologic reactions (3,4). Though typically absent in resting cells, iNOS induction has been shown to occur in response to immunologic stimuli such as cytokines and microbial products, and in response to hypoxia (5). This upregulation of iNOS has been demonstrated in myriad cells, exerting either a cytoprotective or cytotoxic effect (4). However, vascular smooth cells can also express iNOS, as can the endothelium, leading to overproduction of NO in the circulation (6). Intense recent research has focused on NO as a possible mediator of the characteristic hemodynamic features of circulatory failure (7,8). Indeed, several experimental and clinical studies have suggested NOS inhibition might have therapeutic potential in circulatory shock, and other studies have clearly demonstrated the beneficial nature of iNOS expression in modulating tissue perfusion and mediating cytotoxicity (9,10). In this review, we discuss the pathophysiologic importance of iNOS expression in circulatory shock and also examine novel therapeutic strategies for manipulation of NO formation.

Role of NO in Sepsis

The possible involvement of the L-arginine–NO pathway in both the vascular and cellular processes seen in sepsis has been supported by numerous in vitro and in vivo studies (4). iNOS appears to be expressed in a wide array of cell types during sepsis, including immune cells (such as macrophages, neutrophils, T lymphocytes), as well as cells outside the classical immune system (for example, hepatocytes, Kupffer cells, vascular smooth muscle cells, endothelial cells, and fibroblasts). Expression of iNOS is regulated, both positively and negatively, by a number of mediators present during infection and inflammation. The main stimuli for iNOS induction include lipopolysaccharide (LPS), interferon-γ, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α; inhibitory cytokines, such as transforming growth factor-β, IL-4 and IL-10, as well as glucocorticoids, can prevent this induction. The expression of iNOS in response to these agents differs among cell types, but a maximal inducing effect is generally obtained by the combination of microbial products and cytokines acting synergistically (4). iNOS activity is also regulated by substrate and cofactor availability. Tetrahydrobiopterin (BH4), an essential cofactor for the enzyme, is coincided with iNOS in cytokine-stimulated vascular smooth muscle cells (11).

Although NO is a simple molecule, its widespread production in sepsis, coupled with its effects on a variety of intracellular and extracellular target molecules, results in a complex array of biologic roles (10). The

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Abbreviations used: BH4, tetrahydrobiopterin; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; HS, hemorrhagic shock; IL, interleukin; iNOS, inducible nitric oxide synthase; LNAME, nitro-L-arginine methyl ester; L-NIL, L-N-iminoethyl-L-lysine; L-NMMA, N-nitro-L-arginine; LPS, lipopolysaccharide; nNOS, neuronal nitric oxide synthase; "NO, nitric oxide; NOS, nitric oxide synthase; O2\textsuperscript{-}, superoxide anion radical; OONO\textsuperscript{-}, peroxynitrite; TNF, tumor necrosis factor.
interaction of 'NO with the metalloproteins in a number of key enzymes can modulate their activity. Many of the signaling actions of 'NO are mediated by soluble guanylate cyclase. By binding the iron on the heme component of soluble guanylate cyclase, NO is able to activate the enzyme leading to cyclic guanosine monophosphate (cGMP) formation. Increased cGMP levels account for several of the important cellular actions of 'NO, including smooth muscle relaxation, platelet aggregation and adherence, as well as neutrophil chemotaxis. However, through its disruption of iron–sulfur clusters in essential energy-generating enzymes involved in mitochondrial electron transport, glycolysis, and the Krebs cycle, 'NO can adversely affect cellular metabolism. Furthermore, high concentrations of 'NO, as produced by induced macrophages, can directly interfere with DNA in target cells, resulting in fragmentation. A combination of these effects is thought to account for the cytotoxic role of 'NO against microbial proliferation during infection (10,12).

Another critical reaction that 'NO undergoes during inflammation is with the superoxide anion radical (O$_2^-$), yielding peroxynitrite (OONO$^-$$^-$$^-$). OONO$^-$ is a potent oxidant that can decay under acidic conditions to produce a powerful hydroxyl-like free radical (13). This reaction between 'NO and O$_2^-$ can have a protective or damaging consequence, depending on the individual sites and rates of production of the free radicals, and the redox status of both the generating cells as well as the target cells. OONO$^-$ formation can initiate adverse effects such as lipid peroxidation of membranes, and modification of structural proteins through nitration of tyrosine residues (14). Indeed, increased levels of 3-nitrotyrosine have been detected in the lungs of patients with sepsis and animals with acute lung injury. However, OONO$^-$ can also S-nitrosylate glutathione and other thiol-containing substances to form S-nitrosothiols, which have marked cardioprotective and cytoprotective effects (15).

In vivo, substantial data, in both animals and patients, have shown enhanced 'NO formation in sepsis. In rats, LPS administration produced widespread iNOS expression in various tissues, including the vessel wall, with attenuation of vascular responsiveness. In a porcine model of sepsis, endotoxin-induced hypotension was reversed by the NOS inhibitor, nitro-L-arginine methyl ester (L-NAME) (16). In surgical patients with documented sepsis, high levels of nitrite and nitrate, the metabolic end products of 'NO, correlated with endotoxin levels and low systemic vascular resistance (17); furthermore, in patients with advanced cancer receiving immunotherapy, a marked elevation in serum nitrate levels was observed after IL-2 administration, which corresponded with toxic hemodynamic changes (18).

Therapeutic Implications

Induction of iNOS within the vascular wall, both in smooth muscle cells and the endothelium, has been implicated in the characteristic circulatory changes seen in sepsis. The resulting increase in 'NO production can have profound effects on hemodynamic stability by inducing vasorelaxation, which leads to a decrease in systemic vascular resistance and hypertension. This has led to numerous experimental and clinical studies investigating the inhibition of 'NO synthesis to restore arterial blood pressure in sepsis (19). However, given the complex physiologic effects of 'NO, including the potentially beneficial nature of iNOS expression on tissue perfusion, cytoprotection, and immunoregulation, the results of these studies have been mixed.

The majority of these investigations have achieved 'NO inhibition using l-arginine analogs. Numerous l-arginine analogs, which act by competitively inhibiting NOS, have been used to unravel the functions of 'NO as well as potential therapeutic agents. The majority of these analogs act in a nonspecific manner, that is, inhibition of both constitutive and inducible isozymes, with different inhibitors varying in potency with regard to different isozymes (19). Despite improvements in systemic blood pressure with NOS inhibitors, the majority of clinical reports published have shown adverse effects on cardiac output and impaired oxygen delivery (19,20). Experimental studies using NOS inhibitors have also revealed ambivalent findings. Although several reports in animal models of sepsis have demonstrated an improvement in blood pressure, blocking NOS activity decreases renal blood flow, increases capillary leak and intestinal damage, and exacerbates pulmonary hypertension (21,22).

The liver has long been recognized as having an important role in the hemodynamic, metabolic, and inflammatory responses to sepsis. Systemic blockade of 'NO synthesis worsens hepatic injury in endotoxemia (23). We have previously demonstrated that this damaging effect of NOS inhibition was, in part, mediated by oxygen radicals and platelet deposition, suggesting a cytoprotective role of 'NO in preventing microvascular thrombosis and as a free radical scavenger (24). In addition, 'NO has a protective role in hepatic microcirculatory dysfunction during sepsis through its effect on leukocyte adherence to sinusoidal walls (25). Furthermore, 'NO may also protect against circulatory vasocostricators during inflammation, as enhanced 'NO synthesis counteracted phenylephrine-induced increases in intrahepatic resistance in endotoxin-treated rats (26). Finally, we have recently demonstrated that different types of NOS inhibitors resulted in detectable apoptosis in the liver following LPS injection (27). This increase in apoptosis was present even with l-N-iminoethyllysine (L-NIL), a rather specific inhibitor of iNOS, revealing another important protective role of 'NO as an antiprototic agent in sepsis.

These seemingly divergent results with NOS inhibitors suggest that although overproduction of 'NO in the vasculature contributes to the vasodilation seen in septic shock, iNOS expression during inflammation also represents a beneficial, adaptive response in some organ systems. Moreover, different tissues can react distinctly to the effects of 'NO cytotoxicity. In this setting, global nonselective inhibition of NOS, including the potentially undesirable consequences of eNOS inhibition, would be harmful. If confirmed, this would suggest that use of isoform-specific inhibitors of NOS within the vascular bed would be more appropriate. Moreover, thorough pharmacokinetic studies of these agents are warranted to ensure their safe and effective use. A recent study reported that S-methyl-isothiourea, a relatively selective inhibitor of iNOS activity, decreased pulmonary leak and improved survival in endotoxia (28). However, because of the tissue-protective and antiapoptotic effects of 'NO, even selective iNOS inhibitors may be detrimental in certain tissues during sepsis. In the future, combining the salutary effects of site-specific local donors that exploit the cytoprotective actions of 'NO with specific agents that combat the deleterious hypotensive and tissue-damaging effects of 'NO overproduction may be needed to treat septic shock (10). In this regard, inhaled 'NO gas has shown promise as a selective pulmonary vasodilator in patients.
with pulmonary hypertension associated with sepsis (29). Any 'NO gas reaching the bloodstream is quickly inactivated by binding to hemoglobin, thereby limiting adverse dilatatory effects in the systemic circulation. Furthermore, we have recently reported the efficacy of a liver-selective 'NO produg in blocking TNF-α-induced apoptosis and toxicity in the liver with little effect on systemic blood pressure (30). This produg is selectively metabolized to biologically active 'NO in hepatocytes only, resulting in elevation of hepatic cGMP levels.

Another approach to reducing 'NO bioavailability may be the use of 'NO scavengers. By impeding the reaction between 'NO and reactive oxygen species, 'NO scavengers may reduce the tissue-damaging consequences of free radical formation without overinhibition of eNOS or iNOS. There has been considerable interest recently in the use of cell-free hemoglobin in septic shock. The pressor response seen with cell-free hemoglobin has been attributed to 'NO scavenging by the heme (31).

Role of 'NO in Hemorrhagic Shock

Physiologic studies of hemorrhagic shock (HS) have shown that in the face of severe, prolonged hypovolemia, the neuroendocrine responses characteristic of compensated shock begin to fail and a compensated state develops (32). This compensatory phase is characterized by peripheral vasodilatation, capillary leak, and hyperactivity to pressor agents (33). Recent studies have focused on 'NO as a possible mediator of decomposition, with increases in iNOS activity being reported in several organs after prolonged hemorrhagic shock (34). In addition, inhibition of 'NO formation after hemorrhage not only increased arterial pressure but also improved renal blood flow, glomerular filtration rate, and short-term survival (35,36). This has led to speculation that 'NO production has a harmful effect during HS and that NOS inhibitors may provide therapeutic benefit. However, there is conflicting evidence suggesting 'NO synthesis has a protective function. Nonspecific 'NO blockade increased shock-induced hepatic injury, an effect that was reversible with l-arginine (37). A recently published study reports that the presence of an NOS inhibitor, L-NAME, during resuscitation from hemorrhage prevented the restoration of hepatic arterial blood flow, suggesting a role for 'NO-mediated vasodilatation in preventing hepatic ischemia during HS (38). In addition, the administration of 'NO donors has been beneficial in models of traumatic shock, hemorrhagic shock, and mesenteric ischemia-reperfusion injury (39). These latter studies suggest diminished 'NO production occurs with hemorrhage. These findings are consistent with those in trauma patients, where nitrite and nitrate levels were reduced for prolonged periods after injury (17,40). This impairment of 'NO production in victims of hemorrhagic hypotension may be due to impairment of eNOS, and indeed, several investigators have demonstrated decreased vasodilatory activity in vascular rings taken from hemorrhaged animals in response to agonists that stimulate endothelial 'NO production (41). Furthermore, we recently showed that vascular decomposition is not due to excessive 'NO production by the induced enzyme (42). Rats subjected to HS were observed as they progressed from compensated shock to decompensated shock and ultimately irreversibility. No iNOS expression could be detected until the very late irreversible phase of HS. The hemodynamic instability associated with decomposition occurred well before NOS induction, indicating other factors or vasoactive mediators must be involved in decomposition (42).

The role of iNOS activity in HS remains unclear, but recent studies suggest that iNOS upregulation plays an important role in the inflammatory response that occurs in sustained HS followed by resuscitation. Using either the selective inhibitor L-NIL or iNOS knockout mice, we found that iNOS inhibition or deficiency not only prevented the upregulation of the inflammatory cytokines IL-6 and granulocyte colony-stimulating factor following resuscitation from HS but also produced a marked reduction in lung and liver injury (43). Furthermore, the activation of the proinflammatory transcriptional factors nuclear factor kappa B and signal transducer and activator of transcription 3 was also reduced, suggesting iNOS upregulation has a key role in proinflammatory signaling and the subsequent activation of inflammatory cascades. Though the basis of such a 'NO-signaling pathway needs further clarification, several recent studies have implicated a possible redox-sensitive mechanism. 'NO activates the critical signaling enzyme p21ras through S-nitrosylation. This activation is enhanced by depletion of glutathione, the major antioxidant present in cells (44). In HS, tissues are subject to redox stress by hypoxia and oxygen radical formation, suggesting this 'NO-p21ras interaction may be an important cell-signaling mechanism in HS and resuscitation.

Therapeutic Implications

Just as in sepsis, these apparently discrepant findings would suggest that indiscriminate use of nonselective NOS inhibitors as an adjuvant therapy in the treatment of HS is harmful. Although NOS inhibition has improved hemodynamics in several models of HS (35,36), various examples of organ dysfunction have also been seen with their use (37,38). It may be that better understanding and characterization of the pharmacokinetics and selectivity of NOS inhibitors is needed prior to their use in HS. As an example, a recent study showed that although N-monomethyl-l-arginine (L-NMMA), a nonselective inhibitor of NOS, resulted in improved blood pressure in rats subjected to hemorrhagic insult, high-dose L-NMMA caused a marked reduction in cardiac output and stroke volume, with increased damage in various organs—deleterious effects that were avoided with low-dose L-NMMA administration (45). Furthermore, in rats subjected to HS, L-NIL-based resuscitation reduced liver damage compared to nonselective NOS inhibitors (46).

Vascular quenching of 'NO using scavengers may again provide an alternative to NOS inhibition as a means to achieve the goal of reducing 'NO levels. Use of 'NO scavengers after HS and resuscitation may serve to supplement a possibly depleted antioxidant defense system and limit the harmful effects of free radicals such as OONO− and hydroxyl radicals. Removal of 'NO by this method is complicated by the extreme rapidity of the reaction between 'NO and O2− (47). We have seen promising preliminary results with use of an iron-based 'NO scavenger. In rats subjected to HS, 'NO scavenger-based resuscitation improved liver injury and short-term survival compared to standard volume resuscitation (46).

Conclusion

In this review the emerging importance of 'NO and iNOS expression in the pathophysiology of circulatory shock has been examined. Although overproduction of 'NO may play a role in the hemodynamic
changes seen in both septic shock and hemorrhagic shock, iNOS upregulation clearly also has a beneficial protective role in several organ systems. In conditions where excess NO production results in maladaptive damaging consequences with disruption of homeostasis, the therapeutic strategy should be to remove this surplus NO without adversely affecting the cytoprotective actions of NO. Clearly, interfering with the physiologic and microcirculatory role of eNOS through nonselective, global inhibition of NOS is undesirable in shock (Table 1). Though considerable progress has been made over the last decade, further improving our understanding of the pathophysiology involved in these processes and learning more about the complex and diverse actions of NO will help in developing more coherent, efficacious therapeutic interventions.

Table 1. Effects of nitric oxide in endotoxemia and hemorrhagic shock and proposed therapeutic strategies for manipulation of nitric oxide production.

|                  | Endotoxemia | Hemorrhagic shock |
|------------------|-------------|------------------|
| **Effects of NO by eNOS** | Beneficial—maintains perfusion; cytoprotective       | Beneficial—maintains perfusion; cytoprotective       |
| **iNOS**         | Beneficial and toxic—depending on site of production and microenvironment | Beneficial and toxic—can induce tissue damage and promote inflammation with sustained shock |
| **Therapeutic strategy** | Avoid | Avoid |
| **Inhibition of eNOS** | Possibly desirable—to reduce cytotoxicity and combat hypotension | Possibly desirable—to limit exaggerated inflammatory response and development of multiple organ dysfunction syndrome |
| **NO scavengers** | Probably desirable—quench extracellular NO without inhibition of eNOS or iNOS; supplement antioxidant defenses | Probably desirable—quench extracellular NO without inhibition of eNOS or iNOS; supplement antioxidant defenses |
| **NO donors**    | Possibly desirable—site-specific donors without adverse systemic side effects; limited availability | Possibly desirable—site-specific donors without adverse systemic side effects; limited availability |

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