

# Nitric Oxide and Septic Vascular Dysfunction

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**S**eptic shock is characterized by a systemic inflammatory response syndrome, hypotension with vasopressor-resistant systemic vasodilation, and development of multiple organ hypoperfusion and dysfunction (1). Organ failure in sepsis is thought to be mediated by a combination of direct parenchymal cytotoxicity and locally ineffective delivery or use of oxygen and nutrients (2). Sepsis is the leading cause of death in noncoronary intensive care units (1), while infectious disease, in general, is the third leading cause of death (after cardiovascular disease and cancer) in the United States (3). The mortality of patients with septic shock is approximately 50% (4,5), and it is an important cause of early (< 3 days) death in patients with severe sepsis (5). Sepsis leads to elaboration of a self-amplifying cascade of pro- or antiinflammatory cytokines and mediators, including a number of vasoactive substances (prostaglandins, nitric oxide [NO], endothelins, platelet activating factor [PAF], leukotrienes) (1).

NO appears to be an important mediator of impaired vascular responsiveness to vasoconstrictor agents in sepsis. Inhibition of NO synthesis improves vasopressor-responsiveness and increases blood pressure (BP) in most septic animal models and in humans; however, animal studies reveal numerous adverse effects of NO synthase (NOS) inhibition (6–10). This article reviews the role of NO in the pathogenesis of septic vascular dysfunction, focusing on the physiologic role of endothelial NO production in regulating blood flow distribution and tissue perfusion and the adverse effects of sepsis and of NOS inhibitors on endothelial function. Contributions of NO to the physiologic regulation of renal function are reviewed, and the renal effects of sepsis and NOS inhibition are discussed in detail. The effects of sepsis and NOS

inhibition in the pulmonary and splanchnic circulations are likewise reviewed. We discuss promising data suggesting that selective inhibition of the inducible NOS (iNOS) isoform favorably affects global and regional hemodynamics in animal experiments and improves short-term survival when compared with either standard (catecholamine) or isoform-nonselective NOS inhibitor support. Finally, we conclude that the balance between beneficial and deleterious effects of NO in human sepsis will ultimately determine whether NOS inhibitors become standard components of septic shock therapy.

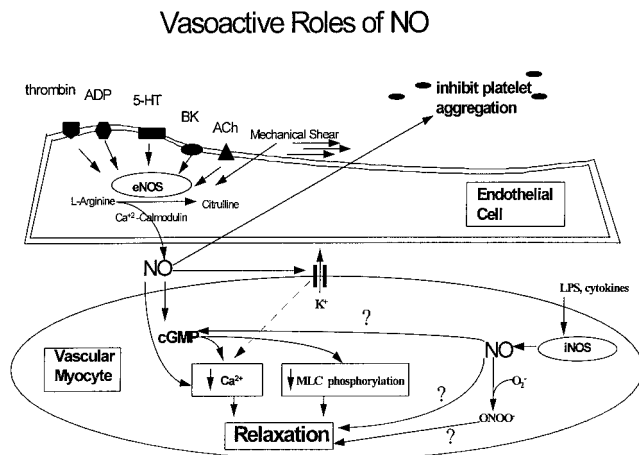
## Physiologic Roles of NO

The discovery of an endothelium-derived relaxing factor in 1980 and its identification as NO in 1987 together led to widespread interest in NO biology, culminating in the award of the 1998 Nobel Prize in Physiology and Medicine to Drs. Furchgott, Ignarro, and Murad (see references for a detailed review of these experiments) (11–14). NO serves multiple physiologic roles, including neurotransmission (central and peripheral), regulation of tissue perfusion, vascular tone and reactivity, platelet reactivity, renal volume control, and antimicrobial defense (11,14–19). NO is synthesized from the amino acid L-arginine by enzymes of the NOS family. Three NOS isoforms have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS (20). The former two isozymes are constitutively expressed and have physiologic roles; the last is usually present only after the induction by inflammatory stimuli. Most NO in the central nervous system and peripheral nerves is produced by nNOS, mediating the “nitridergic” component of peripheral nonadrenergic, noncholinergic neurotransmission, as well as contributing to central regulation of autonomic outflow to the cardiovascular system (11,15). nNOS is also present in the kidney, predominantly in the macula densa (see below) (21). Tight physiological regulation of eNOS modulates vascular tone, by releasing NO from the endothelium to counterbalance vasoconstrictor stimuli and accommodate

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**Figure 1.** The physiologic and pathophysiologic roles of nitric oxide (NO). Endothelial NO production is stimulated by numerous vasodilator agonists or shear stress. NO produced by constitutive endothelial NO synthase (eNOS) causes relaxation of adjacent vascular smooth muscle and vasodilation. NO also inhibits platelet aggregation and is thought to cause vasorelaxation primarily through guanylyl cyclase stimulation, cyclic guanosine monophosphate (cGMP) production, and decreased myocyte intracellular calcium availability and/or contractile apparatus calcium sensitivity (i.e., myosin light chain (MLC) phosphorylation). NO also causes vasorelaxation by potassium (K<sup>+</sup>)-channel activation, membrane hyperpolarization, and decreased intracellular calcium availability. Also depicted is the putative septic pathophysiologic mechanism whereby NO (and its oxidative breakdown product peroxynitrite, ONOO<sup>-</sup>), derived from inducible NO synthase (iNOS), directly causes vasorelaxation by effects on vascular smooth muscle (independent of the endothelium).

changes in blood flow. Accordingly, eNOS “knock-out” mice are routinely hypertensive (22). Multiple vasodilator stimuli (shear stress, vasodilator agonists) stimulate eNOS activity, resulting in the synthesis of NO from L-arginine; NO then diffuses to the adjacent smooth muscle layer and causes vasorelaxation (11,14) (Figure 1). The constitutive, physiologic nature of eNOS activity is most commonly inferred from effects of NOS inhibition in humans or laboratory animals, generally with guanidino-substituted analogues of L-arginine; inhibition leads to both systemic and pulmonary hypertension (23,24). NO, like prostacyclin, appears to be a major endothelium-derived regulator of vascular tone and tissue perfusion.

### The Effects of Pathophysiologic NO Production on Vascular Contractility

The hemodynamic profile of septic (or “distributive”) shock is characterized by hypotension primarily caused by decreased systemic vascular resistance, accompanied (after volume resuscitation) by a relatively elevated cardiac output (in contrast to hypovolemic or cardiogenic shock) (1,6,11,14). Impaired venous return, which mandates aggressive initial volume resuscitation to unmask a hyperdynamic shock profile, is caused by a combination of increased capacitance

(septic venodilation) (25,26) and extravascular volume loss as a result of increased capillary permeability (a manifestation of septic endothelial dysfunction) (27-29). Septic endothelial injury and microvascular leakage is ameliorated by PAF antagonism, inhibition of leukotriene synthesis (27,28), and prevention of iNOS induction or action (27,29), suggesting a multifactorial pathogenesis. Several candidate mediators may contribute to septic vascular contractile dysfunction; one of these is NO, produced primarily by iNOS. Sepsis-induced myocardial dysfunction also contributes to septic hypotension and shock (30-32). The circulating septic “myocardial depressant factor” has yet to be identified; various inflammatory cytokines, iNOS-derived NO, and other mediators, such as PAF, may contribute to myocardial dysfunction (30-32).

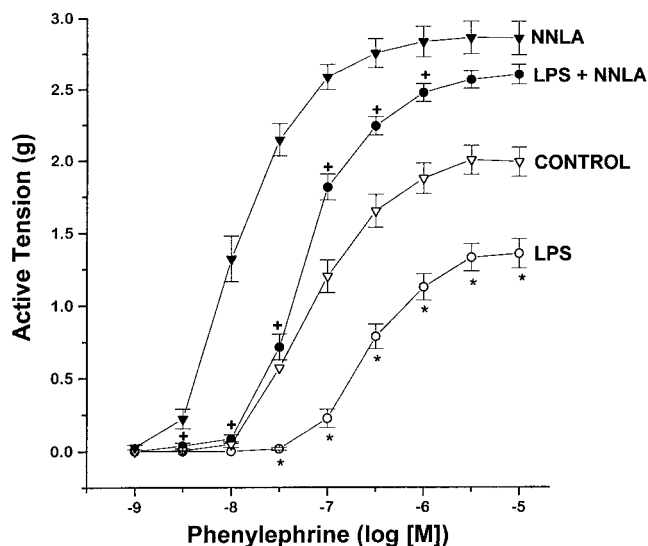
Several lines of evidence suggest that sepsis-induced NO plays a pivotal role in the pathogenesis of septic vascular hypocontractility and shock. iNOS is expressed in tissues of the immune system (leukocytes, macrophages), vasculature (myocytes, endothelium), kidney (mesangium, tubules), and other sites (pancreas, liver, enterocytes, airway, pneumocytes) after exposure to inflammatory stimuli such as endotoxemia or bacteremia (11,14,20,21,33). Plasma and urinary concentrations of NO breakdown products (primarily nitrite and nitrate) are elevated in septic animals and patients (6,11,33,34). Inhibitors of NOS or of guanylyl cyclase (e.g., methylene blue) augment vasopressor-responsiveness and limit hypotension in septic animals and in a small series of human subjects (6-11); in contrast, supplemental L-arginine exacerbates septic hypotension (34). Diminished norepinephrine contractions in isolated omental vessels from septic patients are restored by N<sup>ω</sup>-nitro-L-arginine-methyl ester (L-NAME) or methylene blue (35). Accordingly, pathologic NO synthesis is absent and hypotension is less severe after experimental sepsis in iNOS knockout mice (36). Such data, and analogy with exogenous nitrovasodilators, suggest that NO impairs vascular contraction, primarily by activating guanylyl cyclase and augmenting cyclic guanosine monophosphate (cGMP) levels (though other data suggest a role of potassium channel activation), leading to vascular hyperpolarization (37). It is then assumed that cGMP, acting through a specific protein kinase (or NO, acting in a cGMP-independent manner), impairs vascular contraction by decreasing myocyte free intracellular calcium concentration and/or by diminishing the calcium sensitivity of the contractile apparatus (12). Work from our laboratory (38,39) and by others (40) suggests that the latter mechanism is the more important of the two.

It should be noted that there are only limited clinical observations supporting the role of iNOS activation leading to septic vasodilation in humans; its role is largely inferred from animal studies. Some evidence

suggests a lesser role for NO in humans (33,41). Concerns include evidence of lesser NO production in septic humans compared with animals, the lack of a direct demonstration of iNOS induction in hypocontractile septic human vessels, a number of reports in which NOS inhibition raised BP without altering plasma nitrite-nitrate concentrations, and the continuing identification of NO-independent pathways contributing to septic vascular hypocontractility (33,41). NO-independent mediators, which have been shown in animals or humans to play a causal role in septic vascular hypocontractility, include activation of vascular potassium ( $K^+$ )-channels (leading to vascular smooth muscle hyperpolarization and relaxation) (42), vasopressin deficiency (43,44), vascular carbon monoxide production (45), and direct (NO- and carbon monoxide-independent) activation of guanylyl cyclase (46).

The source of NO contributing to septic vascular hypocontractility varies according to the duration of experimental sepsis. Endotoxin (lipopolysaccharide [LPS]) administration itself produces the systemic effects typical of the sepsis syndrome, including (as a function of dose) shock and death (6-10,47,48). Early endotoxemia is characterized by hypotension which is responsive to NOS inhibition, at a time (1-2 h) when iNOS is not yet induced (49). This early phase of septic vascular hypocontractility seems to be mediated in part by eNOS, acutely releasing endothelial NO (50). Bradykinin and other kinins are the putative intermediate stimuli for eNOS activation (PAF and endothelin are other potential contributors) (51-53). Kinin-elicited endothelial prostacyclin release may also contribute to early septic vascular hypocontractility (51). Later (after 4-6 h), inhibitors of the NO-cGMP pathway continue to improve septic vascular hypocontractility, independent of endothelium or eNOS, and raise systemic BP (Figure 2) (6-11,54-56). Early coadministration (with LPS) of dexamethasone, to prevent iNOS induction, also ameliorates septic vascular hypocontractility and systemic hypotension during this later phase of sepsis (57). Thus it appears that initial septic hypotension is mediated primarily by eNOS-derived NO, with nonendothelium-derived NO assuming a greater role in mediating septic vascular hypocontractility after iNOS induction in the vascular wall and elsewhere.

Experimental design significantly influences findings and conclusions regarding the predominant mediators of septic vascular dysfunction. The stimulus for experimental sepsis may alter the response to therapy. LPS-induced tolerance is less protective against subsequent sepsis caused by cecal ligation and perforation (peritonitis) than by endotoxemia (58). Species differences importantly alter the response to sepsis and its therapy, and many investigators believe that studies in higher mammals (dogs, sheep, pigs, primates) are more relevant to human sepsis than the less expensive approach of studying septic rodents (10).



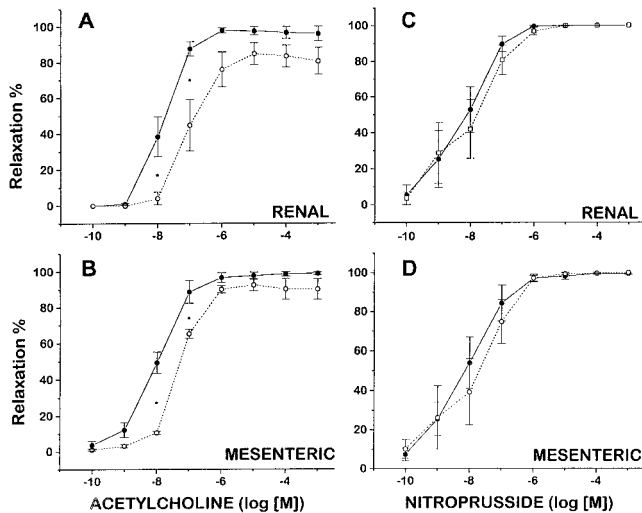
**Figure 2.** Vasoconstrictor concentration-response curves for phenylephrine (PE) in control (triangles) and lipopolysaccharide (LPS)-treated (circles) rat aortic rings with intact endothelium in the absence (open) and presence (closed) of  $N^G$ -nitro-L-arginine (NNLA, 100  $\mu$ M).  $n = 7$  for each group. Standard error bars are shown. \* denotes a value that differs significantly between LPS and LPS + NNLA ( $P < 0.05$ ). + denotes a value that differs significantly between LPS + NNLA and control + NNLA. Contractile responses to PE were markedly reduced in aortae from LPS-treated rats (open circles) compared with vessels from sham-treated controls (open triangles). NNLA largely, but not completely, eliminated the LPS-induced contractile defect, shifting the PE dose-response curve to the left in aortae from both LPS- (closed circles) and sham-treated (closed triangles) rats. Adapted from Umans JG, Wylam ME, Samsel RW, Edwards J, Schumacker PT. Effects of endotoxin in vivo on endothelial and smooth-muscle function in rabbit and rat aorta. *American Review of Respiratory Disease* 1993;148:1638-4, ©Official Journal of the American Lung Association.

The choice of vascular/hemodynamic variables studied may also affect conclusions regarding the pathogenesis of septic vascular dysfunction; for example, endothelium-dependent vasorelaxation depends more on a hyperpolarizing vasodilator called "EDHF" than on NO in many resistance vessels compared with larger conductance arteries (60).<sup>1</sup> The isoform specificity of the NOS inhibitor used (eNOS versus iNOS) and the timing of the intervention (see above) may in turn alter the response to NOS-cGMP inhibition.

## The Effects of Sepsis on Physiologic NO Functions: Impaired Vasorelaxation and Organ Hypoperfusion in Sepsis

In addition to impairing vasoconstriction, sepsis diminishes agonist-stimulated vasorelaxation (54,61-64)

<sup>1</sup> Umans JG. Endothelium-dependent relaxation of rat mesenteric arteries mediated by nitric oxide charybdotoxin-sensitive K channels [abstract]. *J Am Soc Nephrol* 1992;3:556.



**Figure 3.** Percent relaxation to acetylcholine (ACh) after application of an EC<sub>50</sub> concentration of PE (i.e., half-maximal contraction) is plotted as a function of ACh concentration. Concentration-response curves of renal (A) and mesenteric (B) arteries from lipopolysaccharide-treated dogs (open circles) were shifted significantly to the right, compared with those from sham-treated dogs (closed circles). Standard error bars are shown.  $n = 5$  for each vessel. \* denotes values that differ significantly from corresponding control values. In contrast, percent relaxation to nitroprusside (which acts directly on vascular smooth muscle, independent of endothelium) is unimpaired in renal (C) and mesenteric (D) arteries from lipopolysaccharide-treated dogs (open circles), compared with those from sham-treated dogs (closed circles). Adapted from Wylam ME, Samsel RW, Umans JG, Mitchell RW, Leff AR, Schumacker PT. Endotoxin *in vivo* impairs endothelium-dependent relaxation of canine arteries *in vitro*. *American Review of Respiratory Disease* 1990;142:1263-7, ©Official Journal of the American Lung Association.

(Figure 3). This effect of sepsis operates through impairment of agonist-dependent and -independent endothelial calcium mobilization (65,66), and subsequent diminution of NO release (67-69). Despite globally diminished systemic vascular resistance, focal vasoconstriction occurs in septic shock, most notably in the mesenteric (70,71), pulmonary (72-76), and renal circulations (77,78). In each of these vascular beds, NO appears to play an important role in maintaining tissue perfusion during sepsis, primarily by counterbalancing sepsis-induced vasoconstrictor influences. A detailed examination of the manifestations of septic vascular dysfunction in the mesenteric, pulmonary, and renal circulations graphically illustrates the complex issues involved in the use of inhibitors of the NO-cGMP pathway for therapy of septic shock. In addition, effects on the coronary (63), cerebral (79,80), and other circulatory beds (which will not be further discussed in this review) may also be significant considerations. Finally, the role of NO in the pathogenesis of impaired tissue oxygen extraction in sepsis is also incompletely understood. Apart from the effects of septic cardiovascular dysfunction on oxygen delivery and distribution, there is experimental evidence to

suggest that NO might indirectly improve tissue oxygenation by enhancing oxygen release from hemoglobin in the periphery (81), but also directly impair mitochondrial oxygen uptake at the tissue level (82). We and others found that NOS inhibition failed to normalize splanchnic (56) or global (56,83) oxygen extraction in endotoxemic dogs, although the use of nonselective NOS inhibitors may have adversely affected blood flow distribution, masking any potential benefit.

### Mesenteric/Splanchnic Circulation

The intestinal mucosa is vulnerable to hypoperfusion-induced ischemic injury, because countercurrent blood flow in villous capillaries can lead to shunting of oxygen when delivery is impaired. We and others have shown that perfused capillary density in canine intestinal mucosa is decreased during endotoxemia (70) and that nonselective NOS inhibition can worsen splanchnic perfusion in this model, resulting in intestinal and/or hepatic ischemic injury (56,71,84-89). Similarly, therapy with either L-arginine (NOS substrate) or exogenous NO donors can ameliorate septic splanchnic hypoperfusion (89-91). Conversely, other reports suggest that prevention of NO synthesis, either by pretreatment with dexamethasone or by using NOS inhibitors, ameliorated LPS-induced intestinal mucosal injury and/or bacterial translocation *in vivo* (92) and *in vitro* (93). Experimental design variables may be responsible for some of the apparent inconsistencies in this literature; for example, whereas early nonselective NOS inhibition worsened intestinal microvascular leakage in endotoxemic rats, later therapy (3 h onward) was protective (29,85,86). The concerning implications of these data for rational use of NOS inhibitors in septic shock are clear because even subclinical intestinal ischemia and mucosal injury may be lethal, given the role of the gut mucosal barrier and the hepatic reticuloendothelial system in preventing secondary sepsis and multiple organ system failure caused by translocation of luminal bacteria and endotoxin to the systemic circulation (2).

### Pulmonary Circulation

In contrast to the systemic vasculature, where basal tone maintains systemic arterial pressure and perfusion, the pulmonary circulation is a relatively low pressure and low resistance system. NO exerts a tonic vasodilator effect in the pulmonary circulation, just as it does systemically (24); this effect appears, in part, mediated through secondary prostaglandin release (94). In contrast with effects in the systemic vasculature, endotoxemia fails to grossly impair vasoconstrictor-responsiveness in the pulmonary circulation. Rather, it limits vasodilator agonist-stimulated pulmonary arterial relaxation, leading to pulmonary hypertension

in contrast to systemic hypotension (72). Pulmonary hypertension develops despite iNOS induction in pulmonary artery myocytes (73) and appears as a result of augmented vasoconstrictor influences, including increments of both thromboxane and endothelin (74,75,95,96).

Nonselective NO inhibition worsens septic pulmonary hypertension (76), diminishing cardiac output (30). Selective iNOS inhibition (with aminoguanidine) in endotoxemic rats only mildly increased pulmonary hypertension, but markedly increased pulmonary vascular sensitivity to either angiotensin II (AII) or hypoxia (hypoxic pulmonary vasoconstriction [HPV]) (73). Fischer et al. (97) found that nonselective NOS inhibition likewise improved HPV and decreased shunt in septic sheep. Currently, it appears that iNOS induction protects against septic pulmonary hypertension, but does so at the expense of impaired HPV and ventilation-perfusion matching, resulting in increased shunt. As in the systemic vasculature, endotoxin also impairs pulmonary vascular contractility in a NO-independent manner (38,39,97,98), so that even selective iNOS inhibition is unlikely to entirely normalize HPV and ventilation-perfusion matching in sepsis. It is unclear what role impaired endothelium-dependent relaxation plays in the pathogenesis of septic pulmonary hypertension or the precise contribution of eNOS antagonism to the adverse effects of NOS inhibition in this setting (99). Nevertheless, it is important to appreciate that even selective iNOS inhibition may augment sepsis-induced pulmonary hypertension (by withdrawing a protective vasodilator response), leading to myocardial dysfunction and worsening shock, despite initially raising systemic arterial pressure.

Simultaneous administration of inhaled NO ameliorated pulmonary hypertension caused by NOS inhibition in several animal sepsis models (100-104). Inhaled NO likewise diminishes pulmonary hypertension and right ventricular dysfunction as result of sepsis alone in animals (105-107). However, in one study, the majority (60%) of patients with septic shock and acute respiratory distress syndrome (without added NOS inhibitors) failed to respond to inhaled NO (108). These limited results suggest other combination strategies to ameliorate septic pulmonary hypertension, such as endothelin (109) or thromboxane (75,110) antagonism, might also be required as adjunctive therapy with NOS inhibition.

### Renal Circulation

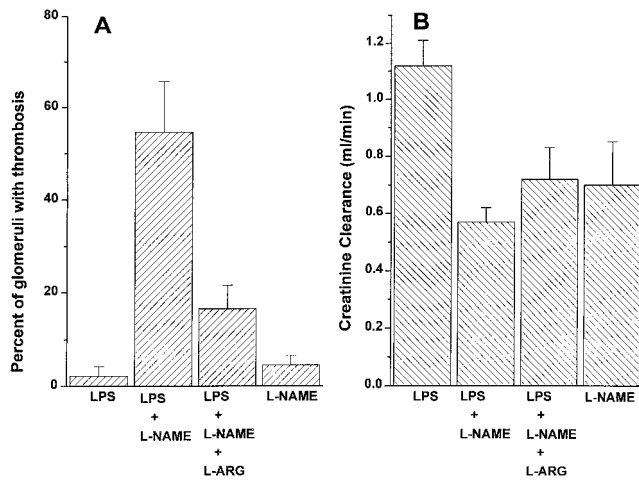
All three NOS isoforms are expressed constitutively in the kidney (11,18,21,111,112). The macula densa (a chloride-sensing nephron segment distal to the thick ascending limb of the loop of Henle) is the principal site of renal nNOS expression (21); other locations include renal nerves and a variety of vascular and

epithelial sites. Increased chloride delivery past the loop of Henle results in afferent arteriolar vasoconstriction caused by a process termed tubuloglomerular feedback, which normally serves to defend intravascular volume by limiting glomerular filtration rate (GFR) when salt excretion is excessive. This afferent arteriolar vasoconstriction is normally blunted by NO, locally synthesized in the macula densa (113), such that NOS inhibition might lead to unopposed afferent vasoconstriction, impaired GFR, and even acute renal failure (ARF) in selected circumstances.

eNOS is expressed in glomerular capillaries, afferent and efferent arterioles, and throughout the renovascular endothelium; it modulates vasoconstrictor influences both in the glomerular mesangium and the renal vasculature. NO is selectively synthesized in the *afferent* (but not *efferent*) arteriole during AII-induced vasoconstriction. The resulting selective efferent arteriolar vasoconstriction caused by AII can serve to maintain intraglomerular capillary hydrostatic pressure, and thus, GFR, in states of diminished renal perfusion, such as volume depletion, heart failure, and shock.

The kidney is one of the few sites where iNOS is constitutively expressed, absent any known inflammatory or immune stimulus; sites include distal segment of proximal tubule, medullary thick ascending loop of Henle, collecting duct, and a few vascular locations. The physiologic role of renal iNOS probably involves adaptation of tubular sodium excretion to match intake, thus maintaining sodium balance and volume homeostasis. During endotoxemia or sepsis, renal iNOS expression is massively increased, predominantly in the glomerular mesangium and renal tubular epithelium; the functional significance of this effect is unclear, as will be discussed below (38).

Systemic nonselective NOS inhibition causes hypertension in healthy animals (and human subjects), along with (in glomerular micropuncture studies) marked increases in afferent and efferent arteriolar tone, decreased glomerular plasma flow, and relatively preserved GFR (because of increased BP and increased efferent tone) (18,112,114). During local (intrarenal) NOS inhibition, which avoids the autoregulatory increase in renovascular resistance caused by hypertension during systemic NOS inhibition, a lesser increase in renovascular resistance is observed, and (in cortical nephrons) afferent but not efferent arteriolar tone increases. The vasoconstrictor system(s) responsible for the increase in efferent arteriolar tone seen during systemic but not local NOS inhibition is not known, but there is some evidence of roles of endothelin and AII in these phenomena (114). If endothelin indeed maintains efferent arteriolar tone during systemic NOS inhibition in humans, then the potentially



**Figure 4.** A, Administration of lipopolysaccharide (LPS) to rats resulted in minimal thrombosis, unless  $N^{\omega}$ -nitro-L-arginine-methyl ester (L-NAME; a nonselective NOS inhibitor) was administered in addition. Coadministration of L-arginine (L-ARG) (NOS substrate) decreased the extent of glomerular thrombosis induced by the combination of LPS and L-NAME. L-NAME alone causes glomerular thrombosis to a degree similar to LPS alone. B, The creatinine clearance measurements corresponding to the glomerular thrombosis scores in each group shown in A. Creatinine clearance was not significantly different from control in rats treated with LPS alone. L-NAME treatment decreased the glomerular filtration rate, significantly in combination with LPS. L-ARG partially restored the glomerular filtration rate in combination with LPS and L-NAME. Adapted with permission from Reference 119.

attractive combination of endothelin and NOS inhibition to treat septic shock with pulmonary hypertension (see above) might be complicated by decreased intraglomerular hydrostatic pressure, diminished GFR, and ARF.

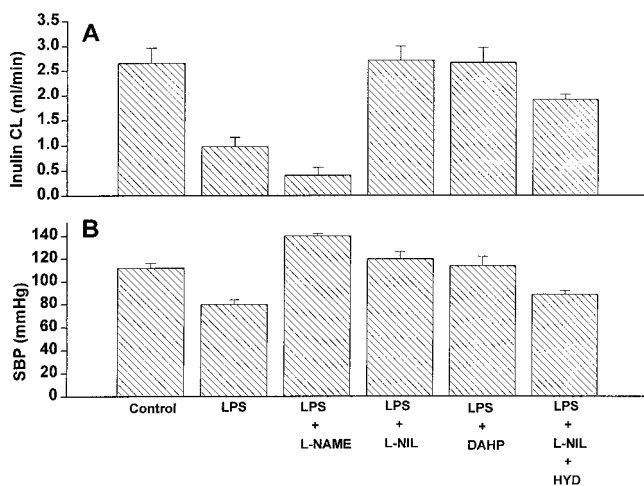
The contribution of septic endothelial dysfunction to impaired organ perfusion and the potential for NOS inhibition to aggravate organ hypoperfusion, despite apparently favorable systemic hemodynamic effects, is most graphically illustrated in the renal circulation. There has been longstanding interest in the pathophysiology of endotoxin- and sepsis-induced ARF. Increased renal vascular resistance, depressed renal blood flow and GFR (77,78,115,116), and occasionally the development of glomerular thrombosis (117,118) are established features of septic ARF, most extensively studied in the rat endotoxemia model. Thrombosis is particularly pronounced when pharmacologic NOS inhibition accompanies LPS administration (119–121); addition of NOS substrate (L-arginine) (119) or exogenous nitrovasodilators (120) to the combination of endotoxemia and the NOS inhibitor L-NAME substantially prevents glomerular thrombosis (Figure 4). However, coadministration of unrelated vasodilators such as hydralazine or atrial natriuretic peptide (another cGMP-stimulating vascular and mesangial relaxing agent), fails to prevent L-NAME-induced thrombosis in endotoxemic rats (120). This illustrates the importance of considering sepsis-induced

endothelial dysfunction in the design of rational therapies for septic shock.

Three complementary mechanisms, reduced blood flow, a hypercoagulable state, and a procoagulant endothelial phenotype, likely act in concert to favor thrombotic ARF, despite amelioration of hypotension by L-NAME, in the endotoxemic rat model. Increased renal vascular resistance, which stands in marked contrast to systemic vasodilation, disproportionately decreases renal blood flow during sepsis or endotoxemia (116). Septic renal vasoconstriction depends on contributions by the renal sympathetic nerves, by humoral mediators (e.g., norepinephrine, Ang II, vasopressin, and endothelin), and by local production of endothelin, thromboxane  $A_2$ , and leukotrienes (77,78,116).

Physiologic NO synthesis in the renal microvascular and glomerular endothelium is important in maintaining tonic renal vasodilation and perfusion in health (111,112). Such NO (and prostanoid) endothelial vasodilator mechanisms would be even more important to oppose the local renal vasoconstriction which attends septic shock. Unfortunately, sepsis likely impairs vasodilator agonist-induced NO release by the renal microvascular endothelium, similar to previous observations in the systemic vasculature (54). Inhibition of eNOS by nonselective NOS inhibitor therapy, when superimposed on this background of an already impaired endothelium-dependent renovascular and glomerular vasorelaxation, might readily precipitate glomerular thrombosis and ARF. In this regard, both the timing of intervention and the relative eNOS-iNOS specificity of the inhibitor appear critical. Indeed, several reports appear inconsistent with the thrombotic ARF observed in the L-NAME rat model. For example, L-NAME improves both creatinine clearance and urine output in sheep when given as a bolus 24 h into a 32-h *Escherichia coli* infusion (122). Even in the rat, L-NMMA ( $N^{\omega}$ -monomethyl-L-arginine), started 60 min before a 30-min LPS infusion and continued throughout the experiment, attenuated the loss of GFR at 3–6 h after LPS administration (123). L-NMMA, which is less eNOS-selective than L-NAME, likewise did not appear to have adverse renal effects in septic sheep (124) or dogs (125), suggesting that limited eNOS inhibition may be key to maintaining renal function during NOS inhibition in sepsis. It is also possible that the benefit of nonselective NOS inhibition is achieved through secondary prevention of vascular and tissue damage by iNOS-derived NO and its reactive metabolites (see below) (99,126), rather than through hemodynamic effects. These competing mechanisms may lead to the apparently inconsistent observations from several studies of nonselective NOS inhibition.

Selective iNOS inhibition, using either L- $N_6$ (1-iminoethyl)lysine or 2,4-diamino-6-hydroxy-pyrimidine, increased BP, maintained GFR, and did not lead to glomerular thrombosis in endotoxemic rats (126,127)



**Figure 5.** Inulin clearance (CL; glomerular filtration rate [GFR]) (A) and systemic blood pressure (SBP) (B) in endotoxemic rats in the presence or absence of isoform-nonspecific  $N^{\omega}$ -nitro-L-arginine-methyl ester (L-NAME) versus inducible isoform-specific ( $L-N_{\epsilon}$ - (1-imino-ethyl)lysine [L-NIL], 2,4-diamino-6-hydroxy-pyrimidine [DAHP]) NOS inhibitors. A, Administration of LPS to rats decreased GFR, an effect worsened by addition of L-NAME. In contrast, L-NIL or DAHP restored GFR in endotoxemic rats to control values. L-NIL was still partially protective against endotoxemic impairment of GFR when hydralazine was additionally titrated to decrease arterial blood pressure to levels comparable to LPS rats. B, The systemic arterial blood pressure measurements corresponding to the inulin clearance data in each group shown in A. Blood pressure was significantly diminished from control values in rats treated with LPS alone. L-NAME increased blood pressure above control values in endotoxemic rats. L-NIL or DAHP each restored blood pressure to control values in endotoxemic rats. Titration of hydralazine achieved hypotension comparable to LPS alone in endotoxemic rats treated with L-NIL. Adapted with permission from Reference 126.

(Figure 5). In contrast, nonselective (iNOS and eNOS) inhibition with L-NAME raised BP but caused ARF, as reported previously (119). These data focus attention on several issues which must be clarified in order to rationally target NOS inhibition to ameliorate septic shock without causing focal tissue hypoperfusion. Specific iNOS inhibition appeared to preserve glomerular endothelial vasodilator function (assessed by agonist-stimulated cGMP production) despite endotoxemia, suggesting that iNOS-derived NO (and probably peroxynitrite) from the systemic circulation and renal vasculature (mesangium and myocytes) may be cytotoxic to glomerular endothelium (99,126).

The antithrombotic effect of NO may also be an important factor in preventing glomerular thrombosis, particularly in the procoagulant milieu of LPS-induced endothelial activation (16,17,53) combined with hypoperfusion, local vasoconstriction, and disseminated intravascular coagulation. In fact, Westberg et al. (120) found that platelets from rats who received both LPS and L-NAME were too tightly aggregated to separate for cGMP measurements, whereas those from rats receiving the combination LPS, L-NAME, and nitroglycerine were not aggregated. NO production

by mesangial iNOS may therefore have an important protective role in preventing septic glomerular thrombosis and ARF. Conversely, this same iNOS-derived NO may contribute to renal ischemia by causing systemic hypoperfusion, and cause renal cytotoxicity directly through effects on the renal endothelium (99,126,128,129) and tubules (91). These data further highlight the difficulties inherent in designing, evaluating, and clinically applying inhibitors of the NOS-cGMP system for hemodynamic support in septic shock.

## Effects of NOS-cGMP Inhibition in Sepsis

### Nonselective NOS Inhibition

Early-generation NOS inhibitors were primarily guanidino-substituted analogs of L-arginine, which variably inhibit all three NOS isoforms by competition at the substrate binding site. Other approaches to inhibition of the NOS-cGMP pathway have included "downstream" interventions, such as guanylyl cyclase inhibition (methylene blue) or NO scavenging (cross-linked hemoglobin preparations). Selectivity and potency data (comparing 50% inhibitory concentration) are derived from a number of experimental preparations, including purified NOS enzyme preparations, intact or lysed cell preparations (cytokine/LPS-stimulated macrophages for iNOS, endothelial cells for eNOS), *in vitro* bioassay with blood vessels, and whole animal experiments. Comparisons between the two most widely-studied NOS inhibitors suggest that L-NAME is primarily eNOS-selective, whereas L-NMMA shows no marked preference for iNOS, eNOS, or nNOS (9). It is important to interpret selectivity data within the context of the preparation studied, however, because experimental variables may significantly alter conclusions; for example, L-NAME is over an order of magnitude less potent toward iNOS than  $N^{\omega}$ -nitro-L-arginine in cell preparations lacking esterase activity (required to de-esterify the inhibitor's carboxyl group) (9).

Given the wealth of information regarding the occurrence of endothelial activation and dysfunction in sepsis, the role of eNOS in maintaining tissue perfusion in the face of tonic vasoconstrictor influences and the increased importance of this protection in septic shock, it is not surprising that the results of nonselective NOS inhibition in treatment of experimental sepsis have been decidedly mixed. Despite universally raising BP, global and regional (see above) tissue perfusion have been adversely affected in several models, and mortality has been increased accordingly in many, but not all, cases (6-10). For example, the early finding that  $N^G$ -methyl-L-arginine restored BP in endotoxemic dogs (130) led to subsequent studies in

which L-NMMA alone ( $1\text{--}10\text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$ ) (6,131) or in combination with catecholamine support (132) not only failed to increase survival in this model, but at the highest dose tested ( $20\text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$ ) led to increased mortality (6,131). Similarly, other studies have suggested that adverse outcomes with nonselective NOS inhibition are to some extent dose-dependent, so that small doses of nonselective agents may be beneficial (133), whereas large doses of even a more selective agent may begin to importantly inhibit eNOS (134). It is unclear whether species effects or other experimental variables explain the contrasting beneficial effect in porcine abdominal sepsis (135) of a dose of L-NMMA ( $10\text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$ ) which was ineffective in canine endotoxemia (6,128,129). The timing of NOS inhibition therapy may also be an important determinant of its therapeutic index, because the relative contribution of reversible NO-mediated effect to septic vascular hypocontractility may decline during prolonged sepsis. Additionally, individuals with more severe septic endothelial injury and dysfunction are more likely to suffer adverse consequences of NOS inhibition throughout the course of sepsis. Whatever the pros and cons of the various nonselective NOS inhibitors, the common observation of adverse effects of nonselective NOS inhibition in experimental sepsis has led to the development of iNOS-selective agents, with the expectation that unfavorable hemodynamic effects might be minimized.

### *iNOS-Selective Inhibitors*

Numerous iNOS-selective inhibitors are currently in development (7–9,126,127,136), and preclinical data suggest potential advantages over the nonselective L-arginine analogs used in the majority of previous studies. Some of these newer NOS inhibitors are based on amino acids other than arginine (L-citrulline, L-lysine), and have been shown to afford greater isoform selectivity against nNOS (S-ethyl- and S-methylthiocitrulline), or iNOS (L- $N_6$ -[1-imino-ethyl]lysine). A number of nonamino acid-based NOS inhibitors are also in development, such as guanidines and S-substituted isothiouras; for example, aminoguanidine was the first relatively iNOS-selective inhibitor identified, and it has achieved beneficial effects in experimental sepsis (see below).

Based on encouraging data from animal studies, iNOS-selective inhibitors are likely to have more favorable hemodynamic effects as pressors in human septic shock. We (55) and others have demonstrated the iNOS-selectivity of L-canavanine, which causes favorable global and regional hemodynamic effects in animal septic shock; unlike L-NAME (26), L-canavanine augmented venous return (137) and improved both end-organ function (138,139) and tissue

adenosine triphosphate stores (139). Finally, iNOS-selective inhibitors have achieved vasopressor effect equivalent to norepinephrine therapy in animal septic shock, but have done so with improved end-organ function (134,136,138–141) and survival (136,141). Included in these data are some direct comparisons of nonselective and selective NOS inhibitors: both L-canavanine (138) and aminoguanidine (140,141), but not L-NAME, improved end-organ function and survival in endotoxemic rodents. Combined with the positive renal effects of selective iNOS inhibition described above, these encouraging animal studies support the concept that short-term (3–16 h) use of iNOS inhibitors may represent an effective and potentially advantageous approach to pressor support in septic shock; longer term (days) efficacy and survival benefit have not been evaluated in animal studies.

### *Protective Effects of NO in Sepsis*

Although it can be anticipated that iNOS-selective inhibitors will favorably affect global and regional perfusion in human septic shock, it must be remembered that morbidity and mortality in these subjects will also be determined by the contributions of other beneficial (142,143) and harmful effects of iNOS-derived NO and its breakdown products (peroxynitrite, etc.). The therapeutic utility of any NOS inhibitor may be confounded by beneficial effects of NO in sepsis, somewhat analogous to the disappointing sepsis experience with anticytokine therapies. NO has both antimicrobial/immunomodulating (144,145) and antithrombotic (16,17) effects and may have organ-specific protective effects in sepsis (6,84). NO also specifically inhibits other sepsis/stress-induced pathways that are potentially cytotoxic, such as the heat shock response (146,147). Conversely, prevention of NO and peroxynitrite-induced cytotoxicity and tissue injury (128,129,148), along with favorable hemodynamic effects, may outweigh the loss of the various beneficial effects of NO in sepsis. Host comorbidities (such as chronic pulmonary hypertension, mesenteric or renal vascular disease) or the infectious agent underlying sepsis (19,143) may also limit the tolerability of NOS inhibition therapy, even with iNOS-specific agents. Interestingly, although iNOS-knockout mice are resistant to the hemodynamic disorders and lethality of sepsis, they also exhibit impaired wound healing and defense against *Listeria* infection (36,143). NO and a number of its oxidation products (e.g., peroxynitrite) exhibit antimicrobial activity, particularly against intracellular pathogens (mycobacterium tuberculosis, leishmania, toxoplasma, salmonella), but also against more routine nosocomial pathogens such as *E coli* and *Staphylococcus aureus* (19). Such data further underline the complex consequences of iNOS inhibition in sepsis and the potential for NOS inhibition to contribute to

adverse outcomes despite apparently favorable early hemodynamic effects.

### *NO-cGMP Inhibition in Human Sepsis*

Published therapeutic experience with inhibitors of the NO-cGMP pathway in human septic shock has so far been restricted to nonselective agents. Kilbourn et al. (149) have obtained beneficial hemodynamic responses without observing adverse effects using L-NMMA to treat cancer patients with the sepsis-like syndrome of interleukin-2-induced hypotension and shock. Published data in septic shock *per se* include a number of case reports or small series of patients unresponsive to standard fluid and vasopressor therapy (150-156) and a few small studies of patients requiring inotropic and/or vasopressor support (157-161). Despite early promise (143,161,163),<sup>2</sup> a recent randomized, controlled, multicenter Phase III clinical trial of NOS inhibition with L-NMMA (Glaxo Wellcome® 546C88) in human septic shock was halted in early 1998 before completion, when interim analysis revealed a significant increase in mortality in the L-NMMA group compared with placebo (164). Data (also reported only in abstract form) from an earlier Phase II study of this agent in 312 subjects had included earlier resolution of shock and decreased vasopressor requirements in L-NMMA-treated subjects (163), with no adverse effects on gross measures of renal, hepatic, or other end-organ functions.<sup>3</sup> It is interesting to note, however, that worsening pulmonary hypertension was documented in a subset of patients from the Phase II study (166). Recent human data using L-NAME for septic shock therapy confirm the capacity of nonselective NOS inhibitors to precipitate detrimental pulmonary hypertension while raising systemic arterial pressure (152,153). Hopefully, avoidance of such potential confounders through the use of iNOS-selective agents (with or without combination therapies such as inhaled NO, or pharmacologic antagonism of vasoconstrictors such as endothelin) will yield improved outcomes in future studies of NOS inhibition in septic shock. Pending results of preclinical and clinical trials of numerous agents currently in development, it remains to be seen whether use of iNOS-selective inhibitors or other agents targeting this pathway will prove a useful strategy for treatment of septic shock.

<sup>2</sup> Grover R, Zaccardelli D, Collice G, et al. Cardiovascular effects of 546C88 in human septic shock [abstract]. *Intensive Care Med* 1995;21:S21.

<sup>3</sup> Guntupalli K, Grover R, Jeffs R, et al. Effects of 546C88 on selected indices of organ function in patients with septic shock [abstract]. *Intensive Care Med* 1995;21:S21.

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