REVIEW ARTICLE

Nitric Oxide Synthase Inhibition in Sepsis? Lessons Learned from Large-Animal Studies

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Nitric Oxide (NO) plays a controversial role in the pathophysiology of sepsis and septic shock. Its vasodilatory effects are well known, but it also has pro- and antiinflammatory properties, assumes crucial importance in antimicrobial host defense, may act as an oxidant as well as an antioxidant, and is said to be a "vital poison" for the immune and inflammatory network. Large amounts of NO and peroxynitrite are responsible for hypotension, vasoplegia, cellular suffocation, apoptosis, lactic acidosis, and ultimately multiorgan failure. Therefore, NO synthase (NOS) inhibitors were developed to reverse the deleterious effects of NO. Studies using these compounds have not met with uniform success however, and a trial using the nonselective NOS inhibitor N^G-methyl-L-arginine hydrochloride was terminated prematurely because of increased mortality in the treatment arm despite improved shock resolution. Thus, the issue of NOS inhibition in sepsis remains a matter of debate. Several publications have emphasized the differences concerning clinical applicability of data obtained from unresuscitated, hypodynamic rodent models using a pretreatment approach versus resuscitated, hyperdynamic models in high-order species using posttreatment approaches. Therefore, the present review focuses on clinically relevant large-animal studies of endotoxin or living bacteria-induced, hyperdynamic models of sepsis that integrate standard day-to-day care resuscitative measures.

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Our understanding of nitric oxide (NO) has increased dramatically over the years. Originally regarded simply as a vascular relaxant factor (1–3), NO has since gained a reputation as a fascinating molecule with multiple, complex roles within many biological systems. In the last two decades, it has become clear that in the pathogenesis of sepsis and septic shock, NO may act both as friend and foe (4), with both direct and indirect deleterious and beneficial effects (Table 1) (5). NO has well known vasodilatory effects in sepsis (6), and its pro- and antiinflammatory, as well as its oxidant and antioxidant properties (7) and role as a "vital poison" for the immune and inflammatory network (8), have been the source of fascination, confusion, and controversy.

Large amounts of NO and peroxynitrite (ONOO-), among other factors, are implicated as mediators for the late phase of hypotension, vasoplegia, cellular suffocation, apoptosis, lactic acidosis, and multiorgan failure in septic or endotoxic shock (9). An understanding of the biology of NO has also provided a new therapeutic target in the management of sepsis. Nitric oxide synthase (NOS) inhibitors were developed to target this molecule and reverse its deleterious effects (Table 2) [(110)]. However, studies with the use of NOS inhibitors have not met with uniform success. There are ample data available on NO effects and NOS inhibition in sepsis based on *in vitro* and *in vivo* experiments (10,11). However, most of the *in vivo* data are available from rodent shock models, and studies on humans are limited. Recently, a phase II randomized, double-blind, placebo-controlled study using the nonselective NOS inhibitor N^G-methyl-L-arginine hydrochloride (546C88; L-NMMA) provided data on improved shock resolution (12), increased vascular tone,

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Table 1. Possible Protective and Deleterious Effects of Nitric Oxide

Direct effects	
Protective	Deleterious
⟨ Macro- and microcirculatory blood flow	⟨ Cytotoxic
⟨ Inhibition of leukocyte and platelet aggregation	⟨ Vascular dysregulation
⟨ Host defense	
Indirect effects	
Protective	Deleterious
⟨ Scavenging of oxygen radicals	⟨ Formation of peroxynitrite

Adapted from Groeneveld et al. (5).

Table 2. Drugs Used in Experimental Medicine to Inhibit Nitric Oxide Synthase (NOS) Activity or NO Effect

- NOS synthesis inhibitor
  - Corticosteroids
- NOS dimerization inhibitor
  - BBS-2 (selective iNOS inhibitor)
- Substrate (L-arginine) competitive analogues
  - Aminoacid derivates (nonselective NOS inhibitors)
    - L-arginine derivates (nonselective NOS-inhibitors)
      - N-nitro-L-arginine-methyl-esther L-NAME
      - N^G-methyl-L-arginine hydrochloride (546C88, L-NMMA)
      - N−-nitro-L-arginine (L-NNA)
      - N−-amino-arginine (L-NAA)
    - acetamidin containing arginin analogues (selective iNOS-inhibitors)
      - L-N6-(1-iminoethyl)-lysine (L-NIL)
      - (S)-2-amino-(1-iminoethylamino)-5-thioheptanoic acid (GW274150)
      - ONO1714
    - indazoles (only in vivo selective nNOS inhibitors)
      - 7-nitroindazole (7-NI)
  - Non-aminoacid derivates (selective iNOS inhibitors)
    - guanidines
      - aminoguanidine (AG)
      - 2-mercaptoethylguanidine (MEG)
    - isothioureas
      - S-methylisothiourea (SMT)
      - aminoethyl-isothiourea (AE-TIU)
    - bis-isothioureas
      - N-3-aminomethyl-benzylacetamidine (1400W)
- NO scavangers
  - cell-free hemoglobin

After Vincent et al. (11) and Domenico (110).

and reduced cardiac index and oxygen delivery (13); however, the subsequent phase III trial was terminated prematurely because of increased mortality in the treatment arm (14). The accompanying editorial to these studies pointed out the importance of nonspecific versus specific NO inhibition, the right amount of NO inhibition, the issue of hemodynamics-directed therapy versus accompanying biological effect, and the importance of study design in sepsis (15). Thus, this area of investigation continues to be dogged by controversy and uncertainty.

Part of the problem is that data generated from unresuscitated, hypodynamic rodent models of sepsis using a pretreatment approach are not clinically applicable, unlike data from resuscitated, hyperdynamic septic models in pigs, dogs, sheep, baboons, etc., using posttreatment approaches (16,17). The purpose of this review is to examine some of the recent data available on NOS inhibition in animal models of sepsis and septic shock and thus to elucidate on the issue of why this approach has not reached clinical application. We will focus mainly on clinically relevant, large-animal studies of endotoxin or living bacteria-induced, hyperdynamic models of sepsis that integrate standard day-to-day care resuscitative measures.

NO Biology

NO is synthesized by the NOS family of enzymes, which includes the calcium-dependent, constitutive (cNOS) isoforms (neuronal [nNOS], NOS-1, and endothelial [eNOS], NOS-3) and the calcium-independent, inducible isoforms (iNOS; NOS-2) (18). In addition, a special form located in mitochondria in different organs (mitochondrial NOS [mtNOS]) has been described (19).

nNOS-derived NO acts as a neurotransmitter, plays an important role in autonomic outflow to the cardiovascular system, and is present in the kidney, skeletal muscle, myocardium, and pancreas. eNOS-derived NO plays an important role in the regulation of regional vascular tone and blood flow and is also present in some immune cells. iNOS is expressed in immune cells, erythrocytes, vascular smooth muscle, kidney, pancreas, liver, and lung (8). The original concept of the solely inducible role of iNOS has been challenged by the observation that iNOS messenger RNA and protein are constitutively expressed in the heart (but not in the liver) and that lipopolysaccharide challenge even decreases this myocardial iNOS expression (and induces the protein expression in the liver) (20).

The constitutive NOS forms are responsible for a constant, low rate of NO production, which can be increased acutely (in the nano-molar range) for a short time as part of the acute inflammatory response. The inducible form is expressed on demand and requires several hours to be activated, depending on the organ and the species; however, once expressed, it produces

micromolar amounts of NO. Interestingly, under conditions of reduced substrate (like L-arginine) or cofactor availability, it can also produce superoxide (21), underscoring the marked interaction between NO and reactive oxygen species (ROS). The isoforms may interdependently regulate their activity by their NO production (22).

NO has an extremely short half-life (8–9 s) *in vivo* and rapidly degrades to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). However, it readily reacts with thiol groups and with molecular oxygen or superoxide to form $ONOO^-$, which results in DNA and membrane damage (23). The chemical reaction between NO, superoxide, and molecular oxygen constitutes a super system by which energy metabolism in cells and tissues is regulated in a site-specific manner depending on the relative concentrations of these three radical species (24).

Measurement of NO Production

The extremely short half-life of NO makes direct measurement difficult; however, an intravascular catheter-type of NO sensor has been developed (25). Plasma and body fluid concentrations of the NO end products nitrite, nitrate, and total nitrate+nitrite (NOx) have been used as surrogates of NO production (26), but their concentrations do not always mirror the rate of formation of NO, and there is some evidence that plasma nitrite mainly reflects constitutive NOS activity in mammals (27). Furthermore, NO production rates show huge differences between humans, large animals, and rodents (16,17). During sepsis or endotoxemia, NOx levels are largely increased in rodents, whereas data in higher-order species or in humans show a marked variation from no change to significant increase (28,29). In support of this phenomenon are data from models of well-resuscitated porcine endotoxemia, where plasma nitrate levels did not register any change after 9 h but NO production rate increased four times, as derived from the dilution of the plasma isotope-enrichment of ^{15}N-labeled nitrate (30). This concept was corroborated by findings from another study in which decreased arginine concentration and increased total body arginine turnover matched the increased NO production (as measured by conversion of $^{15}N_2$-arginine to ^{15}N-citrulline) during 24 h of porcine endotoxemia, whereas NOx concentrations remained constant (31). However, in a prospective study of humans with septic shock, increased plasma NOx concentrations were demonstrable, which correlated directly with endotoxin concentrations and inversely with systemic vascular resistance (32). The amount of exhaled NO in the expired gas, another surrogate for NO production (26,27), also showed a marked interspecies difference in concentrations (33). In addition, the measurement of exhaled NO may be markedly affected by changes in alveolar ventilation and cardiac output (34), as well as airway pathology (35). From the above data, it can be seen that the available methods for estimating NO production are limited in accuracy, in clinical applicability, and in the ability to provide a real assessment of rate of NO synthesis.

This problem may have also contributed to the fact that NO-blocking therapies might not result in uniform success. In the above-mentioned clinical studies (12–14), surrogate variables, mainly arterial hypotension despite adequate intravascular volume resuscitation and increased cardiac output, had been used instead of direct measurement of NO formation. However, the huge interindividual difference in baseline plasma NOx levels between values well within the normal range and supranormal levels (13) suggests that in some of the treated patients, endogenous NO release might have returned to the normal range, and thus, the administration of NOS inhibitors possibly suppressed the NO formation required for whole-body homoeostasis.

Pathophysiology of NO in Septic Shock

Circulation/Heart. The cardiovascular effects of NO, and consequently iNOS, inhibition are complex. NO plays a significant role in coronary vasomotion (e.g., basal epicardial coronary vasomotor tone and flow-induced dilation) and influences the regulation of coronary blood flow during physiological conditions (36). During endotoxemia, NO is an important player in both the immediate and delayed vascular hyporeactivity, even without major iNOS induction (28), which may be restored with NOS inhibition with Nω-nitro-L-arginine-methyl-esther (L-NAME) (37,38). Javeshghani et al. (39), using L-NAME, have also shown increased calcium-dependent NOS activity in the systemic vasculature, even after 2 h of endotoxemia, whereas iNOS activity did not change over time. This study also demonstrated the negative inotropic effects of L-NAME, despite its ability to partially restore the pressor response of norepinephrine.

The role of NO in septic myocardial dysfunction is as complex and controversial (reviewed in (40)) as in heart failure (41). However, excess formation of NO resulting from iNOS activation is assumed to sustain the ability of the left ventricle to fill during diastole, e.g., to support diastolic function because of maintenance of adequate relaxation and to increase myocardial perfusion caused by coronary vasodilatation, such as in late myocardial ischemic preconditioning (40). Indirect evidence may be derived from the fact that survival of septic episodes was better in patients presenting with ventricular dilation, which suggests that this sepsis-associated increase in left ventricular end-diastolic volume is an adaptive response that allows maintenance of adequate cardiac output because of

increased preload under conditions of compromised contractility (42). Finally, inhaled NO, commenced 30 min after an endotoxin challenge in pigs, maintained the end-systolic elastance and prevented left ventricular impairment (43). However, cardiodepressant activity of proinflammatory cytokines seems to involve iNOS, both in the early stage, as a β_1 adrenoreceptor-independent mechanism, and in the late stage, caused by an iNOS-dependent β_1-adrenergic signal transduction defect (42). In fact, exposure of rat cardiomyocytes to sera from patients with acute septic shock resulted in similarly depressed contractility as incubation with various proinflammatory cytokines. NOS inhibition with L-NMMA restored contractility to control levels (44). Furthermore, intracoronary infusion of the NO donor sodium nitroprusside impaired systolic pressure development despite improved diastolic relaxation and distensibility (45). Finally, iNOS blockade beneficially influenced systolic function, e.g., cardiac contractility. In fact, in a sheep model of combined burn and smoke inhalation injury, combined iNOS inhibition and peroxynitrite blockade with mercaptoethylguanidine prevented both early (immediate) and late (24 h) cardiac depression and the development of hemoconcentration and increase of NOx concentrations (46).

In endotoxin-challenged dogs, treatment with the nonselective NOS inhibitors L-NMMA or Nω-nitro-L-arginine restored systemic vascular resistance but substantially decreased cardiac output and femoral arterial blood flow (47). In another study, different doses of L-Nω-amino-arginine (L-NAA) increased systemic and pulmonary vascular resistance but decreased heart rate, cardiac output, as well as systemic oxygen delivery and uptake and were also associated with increased mortality. Concomitant supplementation with L-arginine did not change these results (48). Finally, in a porcine model of endotoxemia, nonselective NOS inhibition with L-NMMA maintained arterial blood pressure at preendotoxin values concomitant with increased systemic vascular resistance and decreased cardiac output but did not influence oxygen transport (49,50).

In a model of porcine endotoxemia specifically designed to compare effects of a nonselective (L-NAME) NOS inhibitor and a more selective (S-methylisothiourea [SMT]) iNOS inhibitor, L-NAME resulted in an earlier and more extended decrease in cardiac output compared with SMT or placebo during a 5-h observation period. L-NAME did not affect left ventricular function, whereas SMT improved it, as shown by decreased end-systolic and end-diastolic volumes. At the same time, only L-NAME was detrimental to the right ventricle, as indicated by an increase in both end-systolic and end-diastolic volumes. In the control groups (i.e., without endotoxin challenge) of this model, both L-NAME and SMT caused left ventricular dilation and decreased cardiac output, but only L-NAME was also detrimental to right ventricular dilation. Importantly, there were no significant changes in the plasma NOx concentrations during the experiment in any group (51).

The myocardial effect of L-NAME is independent of its vasoconstrictor effect, as shown by Cohen et al. (52) in healthy anesthetized dogs, in which the profound dose-dependent myocardial depression was unrelated to increased afterload, coronary vasoconstriction, or myocardial ischemia. Thus, as shown in endotoxemic dogs, increased right ventricular afterload may assume importance in this context (53,54). In fact, pulmonary hypertension was a major complication of nonselective NOS inhibition in the above-mentioned Phase II and III clinical trials using L-NMMA. L-NMMA also decreased cardiac output and systemic oxygen delivery and increased systemic and pulmonary vascular resistances and mean arterial blood pressure; however, oxygen uptake remained unchanged in chronically instrumented sheep before and after inducing bacterial sepsis by infusion of *Pseudomonas* IV. The vasoconstrictor effect on the carotid and aortic blood flow was observed both in nonseptic and septic states, whereas superior mesenteric and renal arterial blood flows were not affected (55).

Selective iNOS inhibition corrected the sepsis-induced hypotension in most of the studies by increasing systemic vascular resistance without a deleterious effect on cardiac output. In several studies, N-3-aminomethyl-benzylacetamidine (1400W) restored mean arterial blood pressure but did not affect cardiac output or oxygen exchange in hyperdynamic porcine endotoxemia, whereas NO concentrations in the exhaled air decreased in the treatment group (56,57). The highly selective iNOS inhibitor L-N6-(1-iminoethyl)-lysine (L-NIL) exerted similar macrohemodynamic properties as 1400W in a hyperdynamic model of long-term porcine bacteremia (58), and in unanesthetized sheep, another specific iNOS-blocker, the cyclic amidine derivative, ONO1714, did not modify systemic blood pressure or cardiac output during endotoxemia, whereas it blunted the endotoxin-induced increase of NOx in plasma (59). Finally, after combined burn and smoke inhalation injury, BBS-2, a novel, highly selective iNOS-synthesis (dimerization) inhibitor, did not affect systemic blood pressure but reversed sepsis-induced myocardial contractile depression, as indicated by preserved left ventricular stroke work and stroke volume, although cardiac output was not affected during a 48-h observation period. The increased NOx levels in the cardiac tissue observed in the control group were significantly attenuated by the treatment so that the authors concluded that iNOS significantly contributed to myocardial dysfunction caused by burn and smoke inhalation (60).

In conclusion, whereas NO *per se* is beneficial for the heart, in large amounts it has detrimental effects on the systemic circulation because of profound vasodilation. Nonselective NOS inhibition clearly promotes myocardial depression in various models of sepsis, whereas selective iNOS inhibition seems to support systolic function under these conditions because of improved contractility (40). Nevertheless, given the protective effect of iNOS-derived NO on diastolic function and coronary perfusion (40), the role of myocardial iNOS is still to be determined (61).

Lung

The role of NOS is controversial in acute lung injury because both detrimental and beneficial effects of NO have been described (62–64). Also, inhaled NO may be beneficial as a selective pulmonary vasodilator and thus improve gas exchange because of a reduced intrapulmonary shunt fraction (65). In addition, inhaled NO reduced transvascular albumin flux in patients with acute respiratory distress syndrome (ARDS) (66) caused by a decrease of the so-called "effective pulmonary capillary pressure," i.e., the microvascular hydrostatic pressure (67), which resulted from a preferential reduction of the venous component of the pressure decrease over the pulmonary vascular bed. Nevertheless, multicenter trials of inhaled NO were successful in infants but did not affect outcome in adult ARDS patients (68). However, endogenous NO overproduction in the lung leads to a loss of hypoxic pulmonary vasoconstriction, resulting in increased ventilation-perfusion mismatch and consequently worsening of hypoxemia. This effect was partially blunted by NOS inhibition (69), although NO scavenging with pyridoxilated hemoglobin had no effect (70). In addition, NO-derived $ONOO^-$ also contributes to alveolar capillary damage and pulmonary edema. As mentioned earlier, in case of L-arginine depletion, iNOS may produce superoxide and, in fact, exogenous L-arginine ameliorated burn- and smoke-induced pulmonary injury (21).

Soejima et al. (71) extensively investigated the effect of NOS inhibition in a chronically instrumented and resuscitated ovine model of acute lung injury induced by endotoxemia, bacteremia, or smoke inhalation combined with 40% grade III skin burn or bacterial instillation into the airways. NO synthesis was upregulated in these experiments, as indicated by increased plasma NOx concentration, which was associated with increased microvascular permeability and pulmonary edema. The combined NOS inhibitor and ONOO- blocker mercaptoethylguanidine prevented these effects when administered 1 h after the burn and smoke injury. L-NAME given 24 h after starting long-term, hyperdynamic ovine endotoxemia increased both mean pulmonary arterial blood pressure and effective pulmonary capillary pressure, whereas vascular permeability did not change (72).

Recently, the use of the superselective iNOS synthesis (dimerization) inhibitor BBS-2 was shown to improve pulmonary gas exchange, lung compliance, and airway obstruction and attenuated the increase in tracheal blood flow, lung lymph flow, and capillary leakage. Because BBS-2 treatment influenced neither mean pulmonary artery blood pressure nor effective capillary pressure, the authors concluded that its beneficial effect was not caused by differences in hydrostatic pressures but was probably caused by the inhibition of $ONOO^-$ formation (63).

In endotoxemic sheep, pretreatment with ONO1714, another specific iNOS inhibitor, did not affect pulmonary hemodynamics but significantly reduced lung lymph filtration, improved oxygenation, and prevented the increase in NOx concentration during 5 h of observation (59). The effect of ONO1714 pretreatment on hypoxic pulmonary vasoconstriction using repeated hypoxic challenges was also investigated: this compound significantly (albeit incompletely) restored the hypoxia-related pulmonary vascular hyporesponsiveness and also prevented the increase in NOx levels (73).

Finally, in a sheep model of acute lung injury caused by smoke inhalation and subsequent bacterial instillation into the airway, the effects of nNOS inhibition with 7-nitroindazole (7-NI), nonselective NOS inhibition with L-NMMA, and iNOS inhibition with aminoguanidine (AG) were compared. 7-NI improved pulmonary gas exchange caused by a decreased shunt fraction, attenuated airway obstruction, and decreased tracheal blood flow and pulmonary edema when compared with the controls. L-NMMA also improved oxygenation, whereas AG did not show any significant benefit. The increase in NOx levels was inhibited only by 7-NI during the first 12 h of the experiment, suggesting a major role of nNOS in early NO release and a later activation of other NOS isoforms (64).

In summary, NO plays a major role in acute lung injury, and the inhibition of different NOS isoforms (especially iNOS) seems to be beneficial to lung function and capillary permeability.

Gut and Liver

The hepatosplanchnic region is thought to play a major role in the development of multiorgan dysfunction during sepsis (74). Experiments *in vitro* (75) and *in vivo*, both using pharmacologic interventions (76,77) and genetic depletion (78), showed that excess NO release resulting from iNOS activation was associated with markedly impaired gut barrier function and aggravated morphologic injury. Restoration of cellular energy metabolism caused by the normalization of mitochondrial respiration (79) seems to assume major

importance in this context. By contrast, data from higher-order species are by no means as unequivocal; in pigs, for example, both cNOS and iNOS are present in the gut mucosa (80). In a porcine study, jejunal luminar mucosal NO production remained constant during bacterial sepsis despite increased blood flow (81), and another study reported decreased NO formation in conjunction with reduced portal venous flow (82). However, the significance of hepatic iNOS expression has been questioned (83) because after 18 h of porcine endotoxemia, only minor iNOS expression was found in liver tissue biopsies. Moreover, the NO donor 3-morpholino-sydnonimine restored the ileal microcirculatory oxygenation and the ileal mucosal-arterial Pco_2 gap in a recent study of swine with endotoxic shock (84).

Our group (50) has conducted a series of investigations using a long-term, normotensive, and hyperdynamic model of porcine endotoxemia. Nonselective NO inhibition with L-NMMA restored preshock systemic blood pressure but failed to improve any of the endotoxin-induced changes in intestinal macro- and microcirculation, oxygenation, and metabolism. Booke et al. (55) reported similar findings in a sheep model, where L-NMMA did not affect superior mesenteric blood flow during bacterial sepsis. L-NMMA did not affect hepatic macro- and microcirculation, oxygenation, or metabolism in porcine endotoxemia either (49). Other authors (85) have also emphasized the importance of NOS isoform selectivity. In endotoxic pigs, the nonselective NOS inhibitor L-NAME further reduced liver blood flow and increased hepatocellular enzyme activities beyond the levels of saline controls, although there were no differences in histopathological organ damage. By contrast, aminoethyl-isothiourea restored liver blood flow to baseline levels and prevented the increase of hepatic enzyme activities.

In our porcine model, 1400W stabilized systemic hemodynamics without affecting hepatosplanchnic macrocirculation, oxygen exchange, or regional venous NOx concentrations. Nevertheless, 1400W prevented the progressive increase in the ileal mucosal-arterial Pco_2 gap and attenuated the endotoxin-induced regional venous acidosis, as well as the progressive increase in lactate/pyruvate ratios, and improved liver lactate uptake (56). The administration of other iNOS inhibitors, such as SMT in endotoxin-challenged swine (51) and ONO1714 in dogs (86), also beneficially influenced intestinal mucosal acidosis. In addition, the latter attenuated the increase of hepatic enzymes and bilirubin, suggesting less hepatocellular damage. Finally, pretreatment with aminoethyl-isothiourea, an iNOS inhibitor with relatively low selectivity, attenuated the decrease in hepatic arterial blood flow during porcine streptococcal septic shock; however, it did not change liver eNOS and iNOS activity (87).

The question of whether a beneficial effect of iNOS inhibition on gut mucosal acidosis in these models was primarily caused by improved microvascular perfusion and oxygen availability or to a direct cellular effect on mitochondrial respiration was addressed in subsequent experiments. We did not find any influence of 1400W on the number or heterogeneity of perfused villi or microvascular perfusion and oxygenation assessed with combined laser Doppler flow and remission spectrophotometry, suggesting that blunted gut mucosal acidosis during 1400W was caused by an effect of redistribution of microvascular perfusion within the gut wall and to an effect on cellular respiration (57). Interestingly, Spronk et al. (84) confirmed this beneficial effect of 1400W on the ileal mucosal-arterial Pco_2 gradient during short-term, volume-resuscitated porcine endotoxemia, but in their study, this effect coincided with restoration of both mucosal and serosal microvascular Po_2. Finally, Matejovic et al. (58) reported that in a porcine model of long-term *Pseudomonas* bacteremia, an even more selective iNOS-inhibitor, L-NIL, not only stabilized macrocirculatory hemodynamics, but also prevented a live bacteria-induced increase of arterial NOx concentration, attenuated the sepsis-induced impairment of the intestinal mucosal microcirculation (assessed with laser Doppler flowmetry), and prevented the decrease in mesenteric venous pH values and the progressive increase of lactate/pyruvate ratios. Ileal mucosal perfusion was only partially restored however, whereas the ileal mucosal-arterial Pco_2 gap was completely normalized so that the authors concluded that improved mitochondrial function at least partially contributed to the beneficial effect of L-NIL. This conclusion was further supported by the reduced hepatocellular injury (assessed by alanine-aminotransferase concentrations) and the attenuation of the bacteremia-induced decrease in hepatic metabolic activity, as assessed by liver lactate clearance and hepatic venous lactate/pyruvate and ketone body ratios. Interestingly, these beneficial effects were accompanied by a decrease in blood 8-isoprostane levels, which are currently regarded as the best available *in vivo* marker of oxidative stress. This latter finding underscores the important contribution of oxidative stress to the toxicity of excess NO release caused by the formation of $ONOO^-$ (39,88,89).

In conclusion, whereas nonselective NOS inhibition, at best, was neither beneficial nor detrimental for the hepatosplanchnic region, selective iNOS inhibition significantly attenuated the sepsis-induced effects on the gut and the liver.

Kidney

The dual characteristics of NO as friend and foe are present in sepsis-induced renal failure. Whereas cNOS activity in the kidney contributes to vasodilatation

under normal conditions to maintain renal blood flow, there is experimental evidence that sepsis-induced NO overproduction caused by iNOS activation downregulates cNOS, and thus, in addition to NO-induced systemic hypotension, it contributes to renal vasoconstriction, ultimately resulting in acute renal failure. However, the increased local NO concentration may also counteract renal vasoconstriction. Moreover, locally produced NO may inhibit platelet aggregation and thereby prevent the intraglomerular formation of thrombi. Finally, excess NO- and ROS-originated $ONOO^-$ causes direct tubular injury (90).

In swine, endotoxemia increased renal medullary NO content and renal blood flow favoring medullary perfusion, and both L-NAME and SMT decreased renal blood flow. L-NAME also reduced the glomerular filtration rate and increased renal sodium excretion and oxygen extraction. The authors concluded that NO is beneficial for kidney function during endotoxemia and that at least the tested NOS inhibitors are likely to be detrimental (91). By contrast, nonselective NOS inhibition with L-NMMA did not affect renal blood in healthy or septic sheep (55).

Selective iNOS inhibition with ONO1714 prevented the endotoxin-induced decrease in creatinine clearance and increased urinary output in endotoxemic dogs (86). Furthermore, in endotoxemic ewes, BBS-2 maintained urinary output and thereby prevented acute renal failure but also attenuated the increase in microvascular permeability in virtually every organ (60). Finally, Matejovic et al. (58) recently observed that L-NIL maintained renal function in bacteremic swine, as documented by the prevention of the otherwise progressive increase in serum creatinine concentration. However, based on the available studies, it remains unclear whether any beneficial effect, if present, is related to a direct specific renal effect of iNOS inhibition, to the maintained macrocirculatory perfusion and thus prevention of sepsis-induced organ hypoperfusion, or whether it is related to the attenuation of microvascular permeability.

Central Nervous System (CNS)

Various isoforms of NOS are present in the CNS. NO is a neurotransmitter, and in addition, it seems to play an important role in the sepsis-induced pathological changes in the CNS. Cobb et al. (48) reported that L-NAA administered in large doses (10 mg/kg) to healthy awake dogs resulted in neurotoxic side effects, such as neuromuscular rigidity and seizure-like activity. However, in bacteremic sheep, L-NMMA did not alter cerebral blood flow. Given its systemic vasoconstrictor effect, NOS inhibition even redistributed blood flow to the brain, as measured by colored microspheres (92). Interestingly, whereas L-NMMA had no effect, L-NAME significantly reduced carotid artery blood flow to less than preendotoxemic values, as assessed with flow probes (93).

There is increasing evidence now that central autonomic nervous system dysfunction contributes to hemodynamic failure in septic shock. Human postmortem neuropathology findings have demonstrated significantly higher vascular iNOS expression in the brains of patients who died of septic shock than in the brains of nonseptic patients, and this effect was directly related to more pronounced apoptosis of autonomic center neurons. The authors concluded that septic shock induces diffuse cerebral damage, and specific neuronal apoptosis may be associated with iNOS activity and NO production (94,95).

Cellular Energy Metabolism

In patients with septic shock, increased NO production was not only associated with increased norepinephrine requirements and higher Scale for the Assessment of Positive Symptoms II scores (32,96), but also with decreased complex I activity (29). Furthermore, NOx concentrations were larger and adenosine triphosphate (ATP) levels were smaller in nonsurvivors than in survivors and controls in this study. Finally, exposure of human endothelial cells to the serum of patients with septic shock was associated with depressed mitochondrial oxygen use and reduced ATP concentrations, and the addition of the NOS inhibitor AG and the poly-(ADP-ribose) polymerase-blocker 3-aminobenzamide completely reversed this effect to the levels found after exposure to serum from healthy volunteers (97). These data suggest that mitochondrial dysfunction as a cause of bioenergetic failure is an important underlying mechanism in sepsis and multiple organ failure and that NO plays a major role in this context as a known inhibitor of complexes I and IV. This conclusion is supported by the results of Matejovic et al. (58) in bacteremic pigs, in which selective iNOS blockade with L-NIL blunted the otherwise progressive decrease in hepatic venous ketone body ratio, demonstrating a retained mitochondrial energy charge level. Finally, NO is involved in the regulation of apoptosis/necrosis in sepsis by its effect on mitochondria (98), but data are inconsistent regarding its exact role as well as the type of NOS isoenzyme involved in the process (94,99,100)

Immunological Effects

NO is an important element of host defense, a central component of innate immunity, and an effective antimicrobial agent (101,102). Excess NO release resulting from iNOS activation, in addition to being regarded as the final common mediator of sepsis, is also essential for the upregulation of inflammatory response (103). The possible mechanisms of direct microbicidal effects include DNA strand breaks leading to inhibited DNA replication and inhibition of mitochondrial function (103). Both mechanisms can, of course, be detrimental to the host. Several studies, mostly in rodents, addressed the role of NO on NOS activity or its inhibition in host defense and especially on bacterial clearance or on phagocytosis and respiratory burst (reviewed in (104)). The few studies available on large animals did not show any deleterious effects on host defense against bacteria using nonspecific NO scavenging with stroma free hemoglobin in dogs (105) or L-NMMA in sheep (93).

Based on the available data, the contribution of excess NO formation to tissue damage and cell death during sepsis seems to be well established. However, NO is also essential for the regulation of macro- and microcirculatory perfusion, as well as for the host defense under these conditions. Consequently, iNOS activation has to be regarded as part of the normal stress response, whereas excessive NO production probably reflects an out-of-control system.

Several factors may explain why therapies aimed at inhibiting NO synthesis have met with limited success. Although often referred to as a final mediator of sepsis (10), NO may not be the only player in the "death opera," and other pathogenic mechanisms may even be responsible for tissue damage (106,107). Consequently, selective targeting of one molecule among a myriad may only reverse some aspects of this cell death cycle and consequently may not be sufficient to prevent organ dysfunction and death. In addition, the surrounding milieu, or conditions of pronounced oxidative stress (e.g., the presence and degree of tissue hypoxia (108), the availability of a local substrate [i.e., L-arginine] for NO synthesis (109), and the simultaneously enhanced formation of free oxygen radicals (5,88)) determines whether excess NO release exerts protective or deleterious effects. Furthermore, the timing of NOS inhibition[1] and other interventions, such as surgery, antibiotic therapy, and inotropes, is not uniform and therefore can influence the results of trials. Finally, the question of the appropriate dose of a NOS inhibitor required to achieve adequate tissue levels remains unresolved, even for molecules that are highly selective for iNOS or nNOS. This will require a robust methodology for the estimation of NO production rate at an organ-specific level. Until these issues are better understood, NOS inhibition will remain a research tool, and applying it clinically must to be cautioned.

[1] Poeze M, Bruins MJ, Vriens IJ, et al. Selective iNOS inhibition decreases *in vivo* NO production, but increases mortality during porcine endotoxemia. Intensive Care Med 2001;27:S243.

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