Nitric oxide synthase inhibition reverses arteriolar hyporesponsiveness to endothelin-1 in septic rats

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Hollenberg, Steven M., Mark J. Piotrowski, and Joseph E. Parrillo. Nitric oxide synthase inhibition reverses arteriolar hyporesponsiveness to endothelin-1 in septic rats. Am. J. Physiol. 272 (Regulatory Integrative Comp. Physiol. 41): R669–R674, 1997.—Persistent vasodilatation refractory to vasopressor agents is the hemodynamic abnormality characteristic of septic shock. Induction of nitric oxide synthase (NOS) by sepsis-induced cytokines has been hypothesized to play a pathogenetic role in this refractory vasodilation. To evaluate the mechanism of vasodilation in sepsis, we used in vivo videomicroscopy to measure responses of resistance arterioles (15–20 μm) to topical suffusion of the potent vasoconstrictor, endothelin-1 (ET-1), in rat cremaster muscle. Rats made septic by cecal ligation and puncture were compared with controls that underwent sham ligation. Responses to topically suffused ET-1 were assessed in septic and control rats before and after superfusion of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA). Sepsis produced a decrease in ET-1-induced vasoconstriction; the ET-1 concentration-response curve was shifted to the right in septic rats (P < 0.05). Contractions at ET-1 concentrations of 1, 10, and 100 nM were 20, 28, and 32%, respectively, of sham controls. Superfusion of the muscle with L-NMMA restored arteriolar responsiveness to ET-1 in the septic rats, significantly increasing arteriolar constriction at 1 and 10 nM. This effect was reversed with superfusion of excess L-arginine (1 mM). This study demonstrates that impaired vasoconstriction in response to ET-1 in resistance arterioles of septic rats in vivo is reversibly NO-dependent. Taken together with previous studies showing sepsis-induced impairment of vasoconstriction with norepinephrine, a vasopressor with a mechanism of action different from ET-1, these findings suggest a generalized abnormality in the responsiveness of resistance arterioles in sepsis. Reversal of hyporesponsiveness to both of these vasopressor agents by NO inhibition suggests an important role for nitric oxide as a mediator of refractory vasodilation in sepsis.

HYPOTENSION IN SEPTIC SHOCK results from persistent vasodilation and impaired arteriolar constriction to vasopressor substances, which results in decreased peripheral vascular resistance (13). The most important determinant of peripheral vascular resistance is the tone of resistance arterioles (10), which is determined by a complex interplay of local vasodilators and vasoconstrictors. The refractory vasodilation seen in septic shock likely results from perturbation of normal vasoregulation and represents an important therapeutically target in the management of patients with septic shock.

In clinical septic shock, peripheral vasodilation occurs despite elevated levels of endogenous catecholamines (6), and decreased vasopressor responsiveness is manifested as a blunted rise in blood pressure in response to administration of catecholamines (6, 8). Whether this decreased responsiveness is specific to catecholamines or whether the abnormality in vascular function in sepsis is generalized has not yet been determined. Modulation of microvascular tone is due not only to actions of vasopressors but also to a complex interplay of local vasodilators and vasoconstrictors. One newly identified endogenous vasoconstrictor is endothelin-1 (ET-1), a 21-amino acid peptide produced by vascular endothelial cells (35). The vasopressor effects of ET-1 are potent and long lasting, persisting up to 60 min after administration in humans, but ET-1 has a serum half-life of only about 2 min and is thought to have predominantly local effects (17). Plasma ET-1 levels are elevated in septic patients (31), but whether the vasoconstrictor effects of ET-1 are altered in sepsis has not been studied.

The mechanisms of sepsis-induced refractory vasodilation have not been precisely defined. Our underlying hypothesis is that hypotension and abnormal distribution of blood flow in sepsis result from derangements in responses to endogenous vasoactive substances at the microvascular level. We have used an open cremaster model (2) to investigate microvascular responsiveness in skeletal muscle in vivo. Using this model, we have documented hyporesponsiveness to topically applied norepinephrine in rats made septic by cecal ligation and puncture (15). The hyporesponsiveness of microvascular resistance arterioles documented using in vivo videomicroscopy in this septic model is analogous to the blunted rise in blood pressure in response to exogenously administered vasopressors seen in septic shock (6, 8, 13). The current study sought to extend these observations by assessing arteriolar responses to ET-1 in an attempt to define the extent to which abnormal vascular reactivity is specific to catecholamines or represents a generalized abnormality in vascular function.

Nitric oxide (NO), a potent endogenous vasodilator, has been implicated in vascular relaxation and hypotension in sepsis (13, 24). NO is formed from L-arginine by the enzyme NO synthase (NOS) and stimulates the soluble guanylyl cyclase in smooth muscle cells to convert GTP to guanosine 3',5'-cyclic monophosphate (cGMP), resulting in vascular relaxation. Under physiological conditions, NO formed by the constitutive NOS in the endothelial cell plays an important role in the regulation of blood pressure and local tissue perfusion. When stimulated by inflammatory mediators such as cytokines, an inducible form of NOS, present in macrophages and vascular smooth muscle cells, is formed;
Although this large amount of NO may represent a useful antimicrobial weapon, the large sustained flux of NO can result in profound vasodilation (24).

Investigations in animal modelos have suggested a role for the NOS pathway in the loss of vascular responsiveness in sepsis addition to mediating hypotension. Decreased responsiveness of isolated rat aortic rings to norepinephrine after intraperitoneal endotoxin was eliminated by the NOS inhibitor NO-monomethyl-L-arginine (L-NMMA) and by methylene blue, an inhibitor of guanylyl cyclase (16); the rise in vascular cGMP levels was also eliminated by these agents. When endotoxemic rats were studied in vivo, NOS inhibitors restored catecholamine responsiveness, an effect that was reversed with l-arginine (11). Late hypotension and the decreased vascular responsiveness in rats given endotoxin can be prevented by pretreatment with dexamethasone, which blocks induction of the inducible form of NOS (25). The effects of dexamethasone on immediate hypotension after endotoxin infusion, which may result from activity of constitutive NOS, have been variable; when given after vascular hyporeactivity is already present, dexamethasone has no effect (25).

Most of these studies of arterial tone were conducted in vitro using large vessels. Because the role of NOS in conduit vessels may differ from that in the microvasculature, we have used videomicroscopy to investigate the effects of NOS inhibition on microvascular catecholamine responsiveness in skeletal muscle in vivo and have shown that superfusion of the muscle with L-NMMA restores the arteriolar responsiveness of septic rats (15). To further define the mechanisms of generalized abnormality in vascular function in sepsis, we also measured the effects of NOS inhibition on microvascular responses to ET-1.

METHODS

Cecal ligation and puncture were performed to induce sepsis as described previously (1, 15). Male Sprague-Dawley rats weighing 125–175 g were anesthetized, and a 1-cm incision was made into the peritoneal cavity, exposing the cecum. A tight ligature was placed around the cecum with a 4–0 suture distal to the insertion of the small bowel, forming an area of devitalized tissue while maintaining bowel continuity. The cecum was punctured on its antimesenteric serosal surface with a 16-gauge needle, and a small amount of cecal contents was expressed through the wound. The cecum was then replaced into the peritoneal cavity, and the anterior peritoneal wall and skin were closed with surgical staples. Each animal was given a bolus of normal saline (15 mL/kg) for hydration and allowed to recover overnight. For sham operations, laparotomy was performed in a similar manner, but the ligation and puncture were omitted. Mortality in the cecal ligation and puncture model has been reported by Alexander et al. (1) to be 90%. Blood pressure is stable from 12–20 h after surgery (1, 15), the time during which arteriolar responses were measured.

Rats were prepared for videomicroscopic observations 12–20 h after cecal ligation and puncture. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), and a constant level of anesthesia was maintained throughout the experiment by injection of supplemental doses of 10 mg/kg. Animals breathed room air through a tracheostomy tube placed to maintain a patent airway. The carotid artery was cannulated for measurement of systemic blood pressure with a Statham P23ID pressure transducer connected to a Gould 2400S chart recorder.

The left cremaster muscle was surgically prepared using techniques previously described methods (15, 23); caution was taken to preserve intact blood and nerve supplies and to keep the muscle moist and warm throughout the surgical procedure. During the experiments the muscle was suffused with a modified Krebs solution containing (in mM) 129 NaCl, 4 KCl, 1.96 CaCl2, 1 K2HPO4, 1 MgSO4, and 25 dextrose and was titrated to pH 7.4 with NaHCO3. The temperature of the rat was thermostatically controlled at 37°C with a heating pad (Cole-Parmer, Chicago, IL) placed beneath the animal on the microscope stage, and the temperatures of the superfusion solution and the supporting platform for the muscle were maintained at 34°C during the experiments.

After the cremaster dissection, the preparation was placed on the stage of a Nikon upright microscope and transilluminated using a 0.4 numerical aperture condenser. The microcirculation was viewed through a ×40 objective (Nikon M Plan Fluor 40 ELWD, 0.5 numerical aperture), and the image was displayed via a video camera (Dage CCD-72, Michigan City, IN). Diameters of cremaster microvessels were measured with an image-shearing monitor (IPM-905, Instruments for Physiology and Medicine, San Diego, CA) and stored on videotape for off-line analysis.

After the preparation was in place, 30–45 min were allowed for arteriolar tone to return to baseline. The technical adequacy of the preparation was verified by documenting vasodilative capability measured as dilation with adenosine (100 µM), and smooth muscle responsiveness assessed by appropriate contraction in response to an intermediate concentration of norepinephrine (100 nM). All vasoreactive substances were administered topically onto the arteriole of interest by micropipette in a 50 µl volume without interrupting the flow of superfusion fluid. Vessels selected for study were third-order arterioles (32) ranging from 12 to 25 µm.

The first set of experiments measured the vasoconstrictive effects of ET-1 in eight septic and eight control rats. ET-1 was applied topically on the arteriole under observation in 50 µl volumes in increasing concentrations (0.1 µM to 10 µM), and sufficient time between applications was allowed for vessels to return to baseline.

Further experiments tested the effects of NOS inhibition on the responses to ET-1 in 15 septic and 8 control rats. Topical application of ET-1 at concentrations of 1, 10, and 100 nM was performed with measurement of changes in arteriolar diameter. The superfusion solution was then changed to contain 100 µM l-NMMA, 1 µM l-arginine, or both L-NMMA and l-arginine. After the solution was equilibrated for at least 20 min, the ET-1 concentration-response was measured again. A time-control experiment in which ET-1 was applied twice with Krebs solution superfusion was performed to rule out potentiation of subsequent responses by initial application of ET-1.

An additional set of experiments was performed to compare the effects of norepinephrine and L-NMMA on arteriolar responsiveness to ET-1. In these experiments, responses of septic animals to 1, 10, and 100 nM of ET-1 were measured, and then the superfusion buffer was changed to contain either 10 µM norepinephrine or 100 µM L-NMMA. After the solution was equilibrated for at least 20 min, the ET-1 concentration-response was measured again and the buffer was then changed to contain the other compound. The dose of norepinephrine chosen for this experiment produces a decrease in
baseline vessel diameter (20–25%) equivalent to that produced by the dose of L-NMMA used.

Materials. Adenosine, norepinephrine, and L-arginine were obtained from Sigma Chemical (St. Louis, MO), and ET-1 and L-NMMA were obtained from Calbiochem (San Diego, CA). Appropriate dilutions were made with modified Krebs solution.

Data analysis. Data are reported as means ± SE, with n indicating the number of animals. In each experimental animal only one vessel was tested. Statistical analyses were performed using paired and unpaired t-tests, area under the curve analysis, and a four-parameter curve-fitting program (SigmaPlot, Jandel).

RESULTS

The experimental interventions did not significantly affect systemic arterial pressure, which was stable during the course of the experimental protocol. The mean arterial pressure in cecal ligation animals (115 ± 2 mmHg) was slightly but significantly lower than that in sham-ligated animals (123 ± 3 mmHg, P < 0.05).

Baseline arteriolar diameter was comparable in sham-ligated and septic animals (sham mean, 16.4 ± 1.2 μm, n = 10; septic mean, 16.5 ± 1.5 μm, n = 15; P = 0.89) and decreased slightly more in septic animals than in sham animals (septic, 13.0 ± 1.7 μm (23%); sham, 14.8 ± 1.5 μM (10%); n = 8, P = 0.09) during topical application of L-NMMA. Addition of excess L-arginine to L-NMMA in septic rats restored arteriolar diameter toward baseline (16.0 ± 0.5 μM).

Topical application of ET-1 resulted in a biphasic response, with rapid but brief vasodilation followed by sustained vasoconstriction. When the maximal vasoconstrictive responses were analyzed, cremaster arterioles of septic rats were less sensitive to ET-1; the concentration-response curve was shifted to the right in septic rats compared with sham-ligated rats. The concentration that produces half-maximal response (EC50) was 224 ± 32 vs. 12 ± 7 nM (n = 8 for septic and control, see Fig. 1). The areas under the two curves differed significantly (P = 0.01, see Table 1).

In the sham-ligated rats, superfusion of the cremaster with 100 μM L-NMMA increased arteriolar responsiveness to ET-1 slightly (see Fig. 2), but the areas under the curves were not significantly different (P = 0.35, Table 1). In septic rats, superfusion with 100 μM L-NMMA increased arteriolar responsiveness more profoundly (see Fig. 2) and the areas under the two curves differed significantly (P = 0.01). Arteriolar responsiveness to ET-1 in septic rats after superfusion with L-NMMA did not differ from responsiveness of control rats either at baseline or after L-NMMA superfusion (Fig. 2). Repeat applications of ET-1 at concentrations of 1, 10, and 100 nM caused initial and subsequent vasoconstrictive responses of 18.8 ± 4.2 vs. 10.0 ± 6.0%, 36.1 ± 12.7 vs. 38.8 ± 12.2%, and 92.2 ± 5.5 vs. 93.1 ± 4.6%, respectively (n = 6, P = 0.89), ruling out potentiation of subsequent responses by initial application of ET-1.

When excess (1 mM) arginine, the substrate for NOS, was added to the superfusion buffer along with L-NMMA, arteriolar responsiveness to ET-1 was decreased to the original values (see Fig. 2). L-NMMA also attenuated the transient vasodilatory responses to ET-1, decreasing vasodilation from 10.6 ± 4.6 to 5.1 ± 3.1% of baseline diameter in control rats and from 18.1 ± 3.9 to 8.9 ± 4.1% in septic rats at ET-1 concentration of 1 nM (n = 6, P = 0.05).

In a separate set of experiments, the effects of the addition of norepinephrine and L-NMMA on ET-1-induced arteriolar contractions in nine septic animals were compared. Topical superfusion of norepinephrine and L-NMMA decreased vessel diameters to a similar extent (norepinephrine, 25.3 ± 6.6%; L-NMMA, 25.5 ± 5.0%; P = not significant). L-NMMA increased responsiveness of septic rats to ET-1 (see Fig. 3). In contrast, norepinephrine had no significant incremental vasoconstrictive effect when added to ET-1 (see Fig. 3).

DISCUSSION

This study demonstrates that sepsis produces impaired vasoconstriction in response to ET-1 in resistance arterioles in vivo. This hyporesponsiveness to ET-1 was reversed by the NOS inhibitor L-NMMA. Impaired vasoconstriction was again seen when the cremaster was superfused with both L-NMMA and excess L-arginine. To rule out a nonspecific vasoconstrictive effect of NOS inhibition, we compared the effects of addition of L-NMMA and norepinephrine, both superfused at concentrations that produce similar baseline vasoconstriction (~25%), on vasoconstrictive responses of ET-1. L-NMMA increased responsiveness of septic rats to ET-1 significantly, whereas norepinephrine had no significant incremental vasoconstrictor effect.

Results from experimental studies of sepsis must be interpreted in light of the choice of animal model. The current study utilizes animals 12–24 h after the onset of sepsis, a time at which humans with sepsis clinically show decreased systemic vascular resistance and decreased vasopressor responsiveness (13). The choice of vessels for study is also important, especially for studies of the NOS pathway (20); the rat cremaster model...
elucidates responses of resistance arterioles in vivo, surmounting some of the difficulties in extrapolating results from in vitro models that use conductance vessels. The present study shows decreased responsiveness to ET-1 in 15- to 20-μm arterioles of septic animals. Arteriolar tone is the major determinant of systemic vascular resistance, as the principal pressure drop in the vascular tree occurs in the microvasculature (5, 10). Diminished responsiveness of these resistance arterioles results in the failure of endogenous and exogenous vasopressors to induce appropriate increases in blood pressure in some patients with septic shock.

Using this model, we previously showed impaired vasoconstrictive effects of norepinephrine in septic rats (15). Together with the current results, the decreased responsiveness to two vasopressors with different mechanisms of action suggests a generalized abnormality in the responsiveness of resistance arterioles in sepsis. Although ET-1 and norepinephrine act at different membrane receptors, their intracellular actions involve activation of several of the same second messengers, among them inositol trisphosphate and protein kinase C, ultimately leading to increased intracellular calcium. It is possible that abnormalities in one or more of these intracellular pathways might explain decreased responsiveness to different vasopressors in sepsis.

Impaired vasoconstriction in response to ET-1 in the septic rats in vivo was reversed by NOS inhibition; only a minimal incremental effect was seen after addition of a dose of norepinephrine with vasoconstrictive effect similar to ET-1, suggesting that reversal is specifically mediated by decreased NO production. In our previous studies the impaired response to norepinephrine was also reversed by NOS inhibition. The reversal of the hyporesponsiveness to both of these vasopressor agents by NOS inhibition suggests that NO plays a crucial role in the refractory vasodilation seen in septic patients. This suggestion is supported by numerous previous reports in the animal literature, albeit primarily using conductance vessels. Decreased contractile responses

![Fig. 2. Effects of nitric oxide synthase (NOS) inhibition on arteriolar responsiveness to ET-1 in septic rats. Results expressed as percentages of maximal vasoconstriction. Rats were made septic by cecal ligation and puncture (n = 15). In the septic group, cremaster was superfused with buffer, and 1, 10, and 100 nM of ET-1 were applied topically to arteriole of interest. In sequence, superfusion buffer was then changed to contain 100 pM NG-monomethyl-L-arginine (L-NMMA) and then 100 pM L-NMMA + 1 mM L-arginine; after stabilization, topical application of ET-1 was repeated with both of these superfusion solutions. Application of 100 μM L-NMMA was performed in shank-operated controls that underwent laparotomy alone (n = 8). Response of septic animals to ET-1 (open bars) was decreased compared with sham controls (solid bars, *P < 0.05). Topical suffusion with L-NMMA (crosshatched bars) restored vascular responsiveness to ET-1 to control levels. L-NMMA suffusion in control rats (shaded bars) increased contractility slightly from control baseline and from L-NMMA suffusion in septic animals. Superfusion with excess L-arginine (hatched bars), as expected, reversed effects of L-NMMA in septic animals, and responsiveness to ET-1 was decreased compared with sham controls (*P < 0.05).

![Fig. 3. Effects of norepinephrine (NE) and L-NMMA on arteriolar responsiveness to ET-1. Results expressed as percentages of maximal vasoconstriction. Responses of septic animals to 1, 10, and 100 nM of ET-1 were measured (solid bars), and then superfusion buffer was changed to contain either 10 pM NE or 100 pM L-NMMA. After equilibration for at least 20 min, ET-1 concentration-response was measured again, and buffer was then changed to contain the other compound. Decreases in baseline vessel diameter were similar for NE (25.3 ± 6.6%) and L-NMMA (25.5 ± 5.0%). L-NMMA (hatched bars) increased responsiveness of septic rats to ET-1 significantly (*P < 0.05), whereas NE (open bars) had no significant incremental vasoconstrictor effect.](http://ajpregu.physiology.org/)
to norepinephrine have been shown in aortic rings of septic rats (22), and NOS inhibition restores catecholamine responsiveness in explanted aortas of endotoxic rats (9, 16). Decreased responsiveness to intravenous vasopressors in anesthetized rats after infusion of endothelin can be reversed by NOS inhibition with intravenous L-NMMA (11).

A specific interaction between NO and ET-1 at the level of vascular smooth muscle is supported by studies showing enhanced contractile effects of ET-1 after endothelial removal and attenuation of ET-1-induced contraction with stimulation of endothelium-derived NO (19). ET-1 acts on vascular ETA receptors to produce vasoconstriction and on ETB receptors on endothelial cells to produce vasodilation via release of NO (34). It appears that this action of ET-1 to release NO is involved in maintaining an appropriate balance of local vasodilator and vasoconstrictor tone. In this context, it seems plausible that overabundant production of NO in sepsis could disrupt this balance by attenuating the vasopressor actions of ET-1.

Induction of NOS by cytokines released from activated macrophages in sepsis has also been shown to play an important role in vascular hypersponsiveness (4, 14, 21). Tumor necrosis factor (TNF) reduced microvascular reactivity after a 2-h suffusion (30), and this hyporeactivity was at least partially reversed by NOS inhibition (3), suggesting that induction of NOS was involved. Pretreatment with anti-tumor necrosis factor antibodies attenuated both hyporeactivity to norepinephrine and induction of NOS after endotoxin infusion in rats (29). Dexamethasone pretreatment also prevented vascular hyporesponsiveness to catecholamines after endotoxin infusion in rats (25).

NOS inhibition with L-NMMA has been shown to attenuate endotoxin-induced hypotension in a rat model; catecholamine therapy with phenylephrine was much less effective (28). Increases in systemic vascular resistance and vasopressor responsiveness have been seen with NOS inhibition after endotoxin infusion in whole animal models (7). NOS inhibitors given concomitantly with catecholamines have been shown to increase systemic vascular resistance and blood pressure in two small trials of hypotensive septic patients (18, 26). Not all studies have shown complete reversal of vascular hypersponsiveness with NOS inhibition. In rats given intravenous endotoxin, decreased norepinephrine responsiveness was completely reversed by the guanylyl cyclase inhibitors methylene blue and LY-83583 but only partially reversed by NOS inhibition (33). Similarly, acute arteriolar vasodilation to TNF was partially reversed by NOS inhibition and completely reversed only with simultaneous NOS and cyclooxygenase inhibition (3). It is also relevant that L-NMMA has been shown to have nonspecific actions, among them inhibition of cyclooxygenases (27), which may potentially explain the increased vasopressor responsiveness seen in the current study.

We have shown that impaired microvascular vasoconstriction in response to ET-1 in septic rats is reversed by NOS inhibition. Taken together with previous studies showing impaired vasoconstriction using norepinephrine, a vasopressor with a different mechanism of action, these findings suggest a generalized abnormality in the responsiveness of resistance arterioles in sepsis. The reversal of the hyporesponsiveness to both of these vasopressor agents by NOS inhibition suggests that NO may play a pivotal role in the refractory vasodilation seen in septic patients.

**Perspectives**

The finding that NOS inhibitors can reverse a number of the vascular abnormalities induced by both cytokine administration and experimental sepsis has led to optimism that they will prove useful in clinical sepsis. That they would be expected to affect most prominently those vascular beds with the most abundant overproduction of NO gives them theoretical advantages over other vasopressor agents. Despite these theoretical advantages, other considerations underscore the potential for detrimental effects of NOS inhibitors in sepsis. NOS inhibition has been shown to increase arterial pressure and systemic vascular resistance, both in animal models of sepsis (7) and in small studies of septic patients (18, 26); however, cardiac output was decreased (7, 18, 26). In one canine study of endotoxin infusion, mortality was increased with NOS inhibition (7). NO is an important modulator of both vascular permeability and leukocyte adherence, and NOS inhibition increases microvascular leakage in both normal rats and rats given endotoxin (12). Clinical studies conducted to date have used only relatively nonselective NOS inhibitors that block both the constitutive and induced forms of the enzyme. Inhibitors selective for the induced isoform might prove more beneficial in sepsis than nonselective inhibitors. In addition, the degree of NOS inhibition may be important. NOS inhibitors, when used in moderate doses, may eventually prove most useful in septic patients as adjunctive agents to improve vasopressor responsiveness in septic shock.

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