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Increased Microvascular Reactivity and Improved Mortality in Septic Mice Lacking Inducible Nitric Oxide Synthase

Steven M. Hollenberg, Marque Broussard, Jailan Osman, Joseph E. Parrillo

Abstract—Persistent vasodilation characteristic of septic shock may result from overproduction of nitric oxide and can lead to pressor-refractory hypotension and death. To evaluate the significance of cytokine-inducible nitric oxide synthase (iNOS) in the pathogenesis of sepsis, we used a clinically relevant mouse model of sepsis and compared mortality and microvascular reactivity in wild-type (WT) mice and transgenic mice deficient in iNOS. WT C57BL/6 and iNOS-deficient mice were made septic by cecal ligation and puncture. Treated mice were given fluids and antibiotics every 6 hours. Microvascular vasoconstriction in response to topical norepinephrine was measured in cremasteric arterioles (15 to 30 μm) by videomicroscopy. Mortality at 48 hours was significantly lower in treated septic iNOS-deficient mice (45%) than in treated septic WT mice (76%), untreated septic iNOS-deficient mice (87%), or untreated WT mice (100%) (P<0.01). Norepinephrine-induced vasoconstriction was decreased in WT septic mice (EC50 200±56 nmol/L) compared with WT and iNOS-deficient shams (16±4 and 13±6 nmol/L), and vasoconstriction was significantly improved in septic iNOS-deficient mice (35±13 nmol/L, P<0.01). Microvascular catecholamine responsiveness and survival were improved in iNOS-deficient mice in a clinically relevant model of sepsis, suggesting that iNOS plays an important, but not exclusive, role in refractory vasodilation in patients with septic shock. (Circ Res. 2000;86:774-779.)

Key Words: sepsis $\boldsymbol{\text{ vascular reactivity}}$ $\boldsymbol{\text{ nitric oxide}}$ $\boldsymbol{\text{ inducible nitric oxide synthase}}$ $\boldsymbol{\text{ videomicroscopy}}$

The characteristic hemodynamic abnormality in septic shock is persistent vasodilation with a normal or high cardiac output, which results in hypotension, hypoperfusion, and a high mortality. This vasodilation is often refractory to vasopressor therapy with catecholamines. Nitric oxide (NO), a potent endogenous vasodilator, has been implicated in both the vascular relaxation associated with sepsis.$^{1}$ NO is synthesized from l-arginine by the enzyme NO synthase (NOS), which has constitutive and inducible isoforms encoded by distinct genes.$^{2}$ The inducible NOS isoform (iNOS) is present in macrophages, hepatocytes, and vascular smooth muscle cells; is regulated at the transcriptional level; and releases large amounts of NO in a sustained fashion after stimulation with mediators associated with sepsis, such as endotoxin, tumor necrosis factor, interleukin-1, interleukin-2, and interferon-γ.$^{2}$ Overproduction of NO by activation of iNOS has been well documented both in animal models of sepsis and in septic patients.$^{1-6}$ The vasodilatory effects of NO have been implicated in both the vascular relaxation associated with hypotension in sepsis$^{1,7}$ and in refractoriness to vasoressor catecholamines.$^{8,9}$

The major determinant of vascular resistance in the systemic circulation is the tone of resistance arterioles, as the principal pressure drop in the vascular tree occurs at the level of the microvasculature.$^{10}$ Previous studies in our laboratory have used in vivo videomicroscopy to assess responses of microvascular arterioles in vascular smooth muscle of animals made septic by cecal ligation and puncture (CLP), a model of sepsis that reproduces the hemodynamics seen in patients with septic shock.$^{9,11}$ We have documented hyporesponsiveness to the vasoconstrictive actions of norepinephrine in the microvasculature in septic mice and have shown that hyporeactivity can be reversed both by nonselective inhibition of NOS and by selective inhibition of the iNOS isoform.$^{9,12}$

A more direct evaluation of the role of iNOS can be achieved using transgenic mice deficient in iNOS.$^{13}$ In one study of endotoxin infusion in iNOS-deficient mice on a C57BL/6 background, hypotension was markedly attenuated and early death was decreased compared with wild-type mice, although liver damage and overall survival were not affected.$^{13}$ A similar effect was shown with endotoxin infusion in another iNOS knockout mouse strain, also on a C57BL/6 background.$^{14}$ In a third knockout strain, iNOS-deficient transgenic mice showed increased susceptibility to the intracellular parasite Leishmania but had improved survival when challenged with endotoxin; the background of these mice was 129/Sv.$^{15}$ Endotoxin infusion, however, can have important differences from infection induced by CLP and may not reproduce the entire clinical picture of sepsis. The current...
study was designed to evaluate the role of iNOS in sepsis by comparing mortality and microvascular catecholamine reactivity in iNOS-deficient transgenic mice and wild-type mice made septic by CLP; C57BL/6 mice generated by Laubach et al\textsuperscript{14} were used.

**Materials and Methods**

The study was performed in accordance with NIH guidelines for the use of experimental animals, and the protocol was approved by the institutional Animal Care and Use Committee. Animals were made septic by CLP, and arteriolar responses to topical norepinephrine were quantified using in vivo videomicroscopy.

**Cecal Ligation and Puncture**

Sepsis was induced surgically by CLP as previously described.\textsuperscript{9,16} Wild-type C57BL/6 and iNOS-deficient transgenic C57BL/6 mice\textsuperscript{14} were anesthetized for laparotomy. The cecum was ligated and punctured with an 18-gauge needle. For sham operations, laparotomy was performed but ligation and puncture omitted. Animals were bolused with normal saline 100 mL/kg SC.

**Videomicroscopy**

Mice were prepared for videomicroscopic observations 12 to 15 hours after CLP. The mice were anesthetized with intramuscular ketamine and acepromazine. The carotid artery was cannulated for measurement of systemic blood pressure.

The cremaster muscle was dissected and exteriorized onto an optically clear viewing platform with blood and nerve supplies preserved and suffused with physiological Krebs solution.\textsuperscript{16,17} The preparation was placed on the stage of an upright microscope (Nikon), and the transilluminated microcirculation was viewed through a $\times 40$ objective. The image was projected by video camera onto a monitor and recorded on a VCR. Arteriolar diameter was measured offline by image analysis.

**Experimental Protocol**

After the preparation was in place, 60 minutes were allowed for arteriolar tone to reach steady state. Vasoactive substances were administered topically onto the arteriole of interest by micropipette in a 50-\mu L volume. Vessels selected for study were third-order arterioles\textsuperscript{18} ranging from 15 to 30 \mu m.

Microvascular catecholamine reactivity was assessed by measuring vasoconstrictive responses to norepinephrine applied topically on the arteriole under observation in 50-\mu L volumes in increasing concentrations ($10^{-11}$ to $10^{-4}$ mol/L), with sufficient time allowed between applications for vessels to return to baseline. At the end of the videomicroscopy protocol, the animal was euthanized.

**Mortality Experiments**

Survival was tested in a separate cohort of animals. Mice were made septic by CLP; controls underwent sham ligation. Treated mice were resuscitated with fluids (50 mL/kg saline every 6 hours) and treated with antibiotics (ceftriaxone 30 mg/kg IM and clindamycin 30 mg/kg IM) every 6 hours. Mice were observed continuously for 48 hours. Some mice were euthanized at 12 hours for blood cultures.

**Materials**

Norepinephrine and adenosine were obtained from Sigma Chemical Co. Appropriate dilutions were made with modified Krebs solution. Ceftriaxone (Roche) and clindamycin (Upjohn) were obtained from the hospital pharmacy and reconstituted in saline.

Transgenic mice deficient in inducible NOS were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice have been shown to lack detectable iNOS mRNA and iNOS protein and not to produce NO (as detected by serum nitrate/nitrite levels) after endotoxin stimulation.\textsuperscript{14}

**Data Analysis**

Data are reported as mean±SD, with n indicating the number of animals. In each experimental animal, only 1 vessel was tested. For concentration-response curves, the areas under the curve were calculated, and statistical analyses were performed using 2-tailed unpaired t tests. Kaplan-Meier survival analysis was used for mortality experiments.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

Systemic arterial pressure was stable during the course of the videomicroscopic protocol and was not affected by topical suffusion of test compounds. In wild-type mice, the mean arterial pressure in cecal ligation animals (79±11 mm Hg) was slightly but significantly lower than that in sham-ligated control animals (91±7 mm Hg, \textit{P}<0.05). Mean arterial pressure in iNOS-deficient controls was 94±5 mm Hg, which was not different from that of wild-type controls. Mean arterial pressure in septic iNOS-deficient mice was 85±10 mm Hg, which did not differ significantly from that of either wild-type or iNOS-deficient controls.

Baseline arteriolar diameter was comparable in wild-type and iNOS-deficient sham ligation controls (mean 23.0±3.2 versus 23.9±2.2 \mu m, respectively, \textit{P}=0.58). Baseline diameter was similar in wild-type and iNOS-deficient septic mice as well (mean 23.8±2.7 versus 20.7±4.3 \mu m, respectively, \textit{P}=0.64).

Concentration-dependent vasoconstriction to norepinephrine was seen in cremaster arterioles of both septic and sham-ligated wild-type mice, but the septic mice were less sensitive to norepinephrine (see Figure 1). The \textit{EC}_{50} (the concentration that produces half-maximal response) was 200±56 versus 16±4 nmol/L, and the areas under the 2 curves differed significantly (\textit{n}=12 for septic and control, \textit{P}<0.01; see Table). Norepinephrine responsiveness in iNOS-deficient transgenic control mice did not differ from that in wild-type controls (\textit{EC}_{50} 13±6 nmol/L, \textit{n}=10, \textit{P}=0.85; see Figure 2). In the septic iNOS-deficient mice, responsiveness to norepinephrine was improved compared with wild-type septic mice, but not normalized (\textit{EC}_{50} 35±13 nmol/L, \textit{n}=8;
Arteriolar Responsiveness to Norepinephrine

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Baseline Diameter, µm</th>
<th>AUC (per mol/L)</th>
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<tbody>
<tr>
<td>Wild-type sham control</td>
<td>12</td>
<td>23.0±3.2</td>
<td>0.0071±0.0009</td>
</tr>
<tr>
<td>Wild-type septic</td>
<td>12</td>
<td>23.8±2.7</td>
<td>0.0023±0.0005†</td>
</tr>
<tr>
<td>iNOS-deficient sham</td>
<td>10</td>
<td>23.9±2.2</td>
<td>0.0069±0.0009</td>
</tr>
<tr>
<td>iNOS-deficient septic</td>
<td>8</td>
<td>20.7±4.3</td>
<td>0.0053±0.001‡</td>
</tr>
</tbody>
</table>

AUC indicates area under the vasoconstriction/norepinephrine concentration response curve.

*None of the differences among baseline diameters reached statistical significance.
†P<0.001 vs wild-type sham control mice.
‡P<0.05 vs wild-type septic mice.

P=0.01 versus wild-type septic mice, P=0.29 versus iNOS-deficient control mice (see Table).

Septic mice were bacteremic with Gram-negative enteric organisms (Escherichia coli, Klebsiella, and/or Proteus mirabilis). There were no deaths in either wild-type or iNOS-deficient sham-ligated controls (n=10). Survival in untreated septic wild-type mice (n=30) was 0% and increased to 24% with fluid resuscitation and antibiotic treatment (n=55). In untreated iNOS-deficient septic mice, survival was slightly but not significantly higher (13%, n=30). In iNOS-deficient mice treated with fluids and antibiotics, however, survival was significantly higher (55%, n=60, P<0.01 by Kaplan-Meier analysis; see Figure 4).

Discussion

This study used in vivo videomicroscopy to document improved microvascular reactivity in septic iNOS-deficient mice. This improved reactivity corresponded with an increase in mean arterial pressure and translated into improved survival in a clinically relevant model of sepsis with fluid resuscitation and antibiotic treatment.

It is crucial to take the model of sepsis used into account in interpreting the results of this study and placing them in the context of previous findings. Results of previous studies of mortality in iNOS-deficient mice have been mixed, with 1 group reporting improved mortality in untreated septic wild-type mice (n=30) was 0% and increased to 24% with fluid resuscitation and antibiotic treatment (n=55). In untreated iNOS-deficient septic mice, survival was 13%, slightly but not significantly higher (n=30). In iNOS-deficient mice treated with fluids and antibiotics, however, survival increased to 55% (n=60, P<0.01 by Kaplan-Meier analysis). KO indicates iNOS-deficient knockout mice; Rx, treated with fluids and antibiotics; and NoRx, untreated.

Figure 3. Responses to norepinephrine (NE) in wild-type (WT) and iNOS-deficient septic mice. Vasoconstrictive responses are expressed as percentages of baseline diameter. In the septic iNOS-deficient mice, responsiveness to catecholamines was improved compared with wild-type septic mice but not normalized (P=0.29 vs iNOS-deficient control mouse, P=0.25 vs wild-type control mouse).

Figure 4. Survival in wild-type and iNOS-deficient septic mice. There were no deaths in either wild-type or iNOS-deficient sham-ligated controls (n=10 for both groups). Survival in untreated septic wild-type mice (n=30) was 0% and increased to 24% with fluid resuscitation and antibiotic treatment (n=55). In untreated iNOS-deficient septic mice, survival was 13%, slightly but not significantly higher (n=30). In iNOS-deficient mice treated with fluids and antibiotics, however, survival increased to 55% (n=60, P<0.01 by Kaplan-Meier analysis). KO indicates iNOS-deficient knockout mice; Rx, treated with fluids and antibiotics; and NoRx, untreated.
support in this model of sepsis, iNOS-deficient mice had significantly improved survival.

In this context, one might evaluate the implications of the difference in outcome of iNOS-deficient mice in this model. Untreated iNOS-deficient mice had slightly prolonged survival compared with untreated wild-type mice, but the difference in survival was not significant. This suggests that although blood pressure may be somewhat improved, this improvement by itself is not sufficient to translate into improved survival. It is possible that in the absence of fluid resuscitation, profound hypotension and decreased cardiac output cause irreversible organ system failure. When the septic animals are resuscitated with fluids and treated with antibiotics, however, improved hemodynamics may allow time for the antibiotics to take effect.23

Induction of NOS by cytokines released by activated macrophages and other cells in sepsis has been shown to play an important role in the pathological vasodilatation and diminished response to α-adrenergic agonists seen in patients with septic shock.7 Overproduction of NO has been well documented in sepsis,3,4 and expression of mRNA for iNOS and increased NO production has been demonstrated in small arteries of septic patients.22 In animal models of sepsis, selective inhibition of iNOS has been shown to improve microvascular reactivity,12 and some studies have shown a mortality benefit as well.23,24 NO causes vasodilation in sepsis in part by activation of guanylyl cyclase, with ultimate reduction of intracellular calcium in vascular smooth muscle,25 but other mechanisms, such as S-nitrosylation of proteins,26 interaction with superoxide to form peroxynitrite,27 and activation of poly (ADP-ribose) synthetase (PARS),28 may contribute as well.

The current model also allows for evaluation of the role of iNOS in microvascular catecholamine reactivity in sepsis without potential nonspecific effects of NOS inhibitors. Vascular reactivity was tested 12 to 24 hours after the onset of sepsis, a time at which humans with sepsis clinically show decreased systemic vascular resistance and decreased vasopressor responsiveness.29 Responsiveness to norepinephrine was tested in third-order arterioles of septic animals, the site of the principal pressure drop in the vascular tree30,31 and thus the major determinant of systemic vascular resistance. Diminished responsiveness of these resistance arterioles results in the failure of endogenous and exogenous vaspressors to induce appropriate increases in blood pressure in patients with septic shock. We have previously documented hyporeactivity of these resistance vessels to the vasoconstrictive effects of norepinephrine.12 The fact that reactivity was improved in iNOS-deficient septic mice but was not entirely normalized in the current study suggests that NO plays an important, but not an exclusive, role in producing microvascular vasopressor hyporesponsiveness in sepsis.

Although NOS inhibition reproducibly increases systemic vascular resistance in animal models of sepsis8,31 and in small clinical trials,32,33 the value of nonselective NOS inhibition is coming into question. A recent randomized clinical trial of administration of the nonselective NOS inhibitor Nω-monomethyl-l-arginine (L-NMMA) to patients with septic shock was stopped because of adverse effects of treatment; the mechanisms of this adverse effect remain to be elucidated.34 Production of NO by constitutive NOS in endothelial cells is an important modulator of both vascular permeability and leukocyte adherence.35,36 The effects of selective and nonselective NOS inhibition have been compared in several recent animal studies. Liaudet et al23 compared the effects of selective iNOS inhibition (with l-canavanine) with those of nonselective NOS inhibition (with Nω-nitro-l-arginine methyl ester [L-NAME]) in mice challenged with endotoxin and found that L-NAME enhanced liver damage and tended to accelerate mortality, whereas l-canavanine reduced mortality significantly, with no measurable adverse effects on organ function. Aranow et al23 compared L-NAME and selective iNOS inhibition with S-methyl-isothiourea in a rat model of bacterial peritonitis and demonstrated that selective iNOS inhibition prolonged survival, whereas nonselective NOS inhibition accelerated early mortality and worsened overall survival. The current study confirmed these findings by using iNOS-deficient transgenic mice, thus avoiding the nonspecific effects of NOS inhibitors, and extended them by documenting that microvascular catecholamine reactivity is improved in iNOS-deficient mice.

Clinical Implications. In this study, iNOS-deficient septic mice had improved microvascular catecholamine responsiveness and improved survival when treated with fluid resuscitation and antibiotics in a clinically relevant model of sepsis. This suggests that activation of iNOS plays an important, but not an exclusive, role in the pathophysiology of hypotension and decreased vasopressor responsiveness in sepsis. Inhibition of iNOS can increase catecholamine reactivity in the microvasculature but may prove most useful in sepsis as an adjunctive measure to support hemodynamics in hypotensive patients along with other therapies.

Acknowledgments
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References


