Expression and Preferential Inhibition of Inducible Nitric Oxide Synthase in Aortas of Endotoxemic Rats

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ABSTRACT

Septic shock is associated with high mortality. There is in vitro evidence that the induction of nitric oxide synthase (iNOS) in vascular smooth muscle cells may be an important mediator of the systemic vasodilation and hypotension associated with sepsis. In this study, an in vivo murine model of sepsis was used to further examine this important question. Lipopolysaccharide (LPS), the major wall component of gram-negative bacteria, was administered to rats. By the use of a selective cDNA probe for INOS, mRNA for INOS was demonstrated in the aortas of these rats. The functional significance of the expressed iNOS was then examined with aminoguanidine, a preferential inhibitor of iNOS. Aminoguanidine reversed the blunted phenylephrine-evoked contraction of endothelium-denuded aortic rings from LPS-treated rats or rings exposed to LPS in vitro. Aminoguanidine did not impair the relaxation of aortic rings with endothelium to acetylcholine, a known stimulator of endothelial NOS. The reversal of LPS-induced vascular hyporesponsiveness by aminoguanidine therefore strongly supports the functional importance of iNOS mRNA expression in the aorta of endotoxemic rats. Future clinical trials in treating septic shock should therefore consider the preferential inhibition of iNOS while maintaining the integrity of endothelial NOS.

Key Words: Endothelium, lipopolysaccharide, nitric oxide synthase, vascular smooth muscle

Septic shock is associated with a high mortality that correlates with the degree of systemic vasodilation and hypotension. There is in vitro evidence that endotoxin or lipopolysaccharide (LPS), the major component of gram-negative bacteria, induces nitric oxide synthase (iNOS) in endothelium and vascular smooth muscle and therefore may be of primary importance in septic shock (1–13). However, although mRNA has been detected in cytokine-stimulated endothelial (14) and vascular smooth muscle cells (15–20; [VSMC]) in culture, there are no in vivo studies examining this question. Several recent advances make possible the further study of this important question. First, a specific cDNA probe for VSMC iNOS has been developed (19). Second, in contrast to the widely used l-arginine analogues, aminoguanidine (AG) has been shown to inhibit iNOS preferentially while maintaining the integrity of constitutive endothelial NOS (eNOS) (8,21,22). Thus, to further advance the understanding of this important issue, aortas from LPS-treated rats, an in vivo model of sepsis, were examined for the presence of iNOS mRNA by the use of the selective probe from an iNOS cDNA cloned from VSMC. The functional significance of the expressed iNOS mRNA was then investigated by studying the effect of AG on the phenylephrine-evoked contraction in aortic rings from LPS-treated rats. The preferential effect of AG on iNOS was confirmed with aortic rings with endothelium tested with acetylcholine, a known stimulator of endothelial NOS.

MATERIALS AND METHODS

Molecular Biology

Male Sprague-Dawley rats (250 to 300 g; Sasco, Omaha, NE) were injected with LPS (from Escherichia coli O127:B8), 20 mg/kg, or with only 1 mL of saline (vehicle) ip as previously described (9). Four hours after the animals were anesthetized with pentobarbital (50 mg/kg ip), their descending thoracic aortas were isolated, placed in cold physiologic saline, cleaned of clots, and frozen in liquid nitrogen. In some aortas, the endothelium was mechanically removed and a ring of these aortas was cut to confirm the absence of functional endothelium with acetylcholine (1 μM) after contraction with phenylephrine (1 μM; as described below). After pulverization under liquid nitrogen, total RNA was extracted by the use of a simplified guanidinium thiocyanate protocol (23; RNAzol-B method [Teltest, Inc., Friendswood, TX]). Aortas of two animals were pooled for each extraction; three pairs of aortas from each of the experimental groups were used. Extracted RNA was quantified by absorbance at 260 nm and frozen at –80°C until northern analysis. Twenty micrograms of total RNA was size fractionated by formaldehyde–1.2% agarose gel electrophoresis, electrically transferred to Hybond-N membrane (Amersham, Arlington, Illinois, U.S.A.), and hybridized with radiolabeled iNOS cDNA. Following autoradiography, the relative amount of iNOS mRNA was measured by computer-assisted densitometry using a multiple-lane gel analyzer (BioImage Systems, Boston, MA).
Organ Chamber Studies

Three groups of male Sprague-Dawley rats (250 to 300 g) were used: rats treated with ip LPS (20 mg/kg; 4 h); rats treated with its vehicle (ip saline); and untreated rats. They were anesthetized with pentobarbital (50 mg/kg ip). In each group, the thoracic aortas were isolated and placed in a cold physiologic salt solution with the following millimolar composition: NaCl, 116.3; KCl, 5.4; MgSO4, 0.83; NaH2PO4, 1.04; CaCl2(H2O)2, 1.8; NaHCO3, 19.0; glucose, 5.5; phenol red sodium sulfate was present at 0.011 g/l as a pH indicator (control solution). The aortas were cleaned of surrounding tissues and cut into four to six rings (2 to 3 mm in length). The endothelium was removed by gentle mechanical abrasion. The rings were suspended between two stainless-steel stirrups in 10 mL of control solution (37°C) and gassed with 74% N2, 21% O2, and 5% CO2 (pH 7.4). One of the stirrups was nipped in 10 mL of control solution (37°C) and gassed with 74% N2, 21% O2, and 5% CO2 (pH 7.4). One of the stirrups was anchored, and the other was connected to a strain gauge (Grass FT03; Grass Instruments, Quincy, MA) to record changes in isometric force. After equilibration and stepwise increases in resting tension to 2 g in 30 min, two sequential contractions were evoked with phenylephrine hydrochloride (1 μM) and the tissues were tested for the presence of the endothelium with acetylcholine hydrochloride (1 μM). All rings were washed with control solution and treated with indomethacin (10 μM) prepared with equimolar sodium carbonate and sonicated until dissolved to inhibit the production of endogenous prostanooids. The rings from the three groups of rats were incubated for 30 min with the preferential inhibitor of iNOS, AG hemisulfate (300 μM (8)), with equimolar sodium sulfate (150 μM) or with vehicle (distilled water, 50 μL control rings). Then, concentration-response curves to phenylephrine (1 nM to 10 μM) were performed. Additional rings from control rats were incubated for 6 h in control solution in the presence or absence of LPS (1 μg/mL) before contraction with phenylephrine. Some of the latter rings, exposed and unexposed to LPS, were incubated with AG (300 μM) during the last 30 min. The rings were then removed from the organ chambers, air dried for 48 h, and weighed. The following parameters (expressed as means ± SE) were compared: the maximal contraction to phenylephrine (Emax; in grams of force per milligram of dried tissue) and the logarithm of the effective molar concentration of phenylephrine causing 50% of maximal contraction (log EC50). A two-tailed unpaired t test was used to compare the above parameters between control rings of LPS- and saline-treated rats; the effect of AG between rings of the same rat, either LPS or saline treated, was compared by a two-tailed paired t test. The effect of control solution, LPS, and/or AG in rings incubated for 6 h was evaluated by analysis of variance (Scheffe’s test). Differences between treatments were considered significant when P < 0.05.

In other experiments, aortic rings with a functional endothelium obtained from control rats and not subjected to denudation were incubated with and without AG (300 μM; 30 min) and contracted with 1 μM phenylephrine. Once a plateau was reached, a concentration-response curve to acetylcholine (1 nM to 10 μM) was performed to test if this concentration of AG affected endothelium-dependent relaxation. Results are expressed as percentage of phenylephrine-evoked contraction ± SE. Maximal relaxation to acetylcholine and the logarithm of the efferent molar concentration of acetylcholine causing 50% of maximal relaxation (log EC50) were compared by a paired t test. All experiments were performed in parallel with a single concentration-response curve per ring, and N is the number of rings from different rats. All reagents and drugs used in molecular biology and physiologic studies were purchased from Sigma (St. Louis, MO) unless otherwise stated.

RESULTS

Molecular Evidence for iNOS Expression in Aortas From LPS-Treated Rats

Northern analysis of total RNA extracted from aortas with and without functional endothelium obtained from rats treated with LPS (20 mg/kg ip; 4 h) indicated that iNOS mRNA was expressed in these blood vessels; however, total RNA obtained from aortas with or without endothelium from control rats did not express iNOS mRNA (Figure 1). By comparison with 28S rRNA and an RNA ladder, the calculated size of the expressed iNOS is approximately 4.4 kilobase pairs.

Functional Evidence for iNOS Activity in Aortas From LPS-Treated Rats

Endothelium-denuded aortic rings from LPS-treated rats (20 mg/kg ip; 4 h) showed a marked reduction in reactivity and contractility to phenylephrine (Table 1 and Figure 2b) when compared with rings obtained from saline-treated rats (Table 1 and Figure 2a). The incubation of rings from LPS-treated rats with AG (300 μM; 30 min) significantly enhanced both reactivity and contractility to phenylephrine (Table 1 and Figure 2b). However, AG did not affect either the reactivity or the contractility to phenylephrine in rings obtained from saline-treated rats (Table 1 and Figure 2a). Sodium sulfate (150 μM) had no effect on the phenylephrine-evoked contractions in rings from LPS- or saline-treated rats (N = 6; data not shown).

The in vitro incubation of aortic rings without endothelium from untreated rats with LPS caused a shift to the right in the concentration-response curve to phenylephrine relative to rings incubated in the control solution (log EC50 control versus LPS: −7.5 ± 0.1 versus −7.1 ± 0.1; P < 0.05; N = 7) and a nonsignif-
significant decrease in maximal contraction \( E_{\text{max}} \) control versus LPS: 3.0 ± 0.2 versus 2.4 ± 0.2 g/mg). In other endothelium-denuded aortic rings incubated with LPS, the addition of AG caused a significant enhancement in vascular reactivity \( \log EC_{50} \) LPS + AG versus LPS: \(-7.7 \pm 0.1; P < 0.01; N = 7\) and contractility \( E_{\text{max}} \) LPS + AG: 3.6 ± 0.2 g/mg; \( P < 0.01; \) [Figure 2c]). AG did not significantly affect the vascular reactivity or contractility of aortic rings without endothelium incubated for 6 h in control solution (\( N = 7\); data not shown).

**TABLE 1.*** Phenylephrine-evoked contraction of aortic rings without endothelium from saline- and LPS-treated (Rx) rats in the presence and absence of AG (300 µM; 30 min)

<table>
<thead>
<tr>
<th>Group</th>
<th>log EC(_{50})</th>
<th>( E_{\text{max}} )</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-Rx</td>
<td>Control</td>
<td>(-7.9 \pm 0.1)</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Saline-Rx</td>
<td>AG</td>
<td>(-7.8 \pm 0.1)</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>LPS-Rx</td>
<td>Control</td>
<td>(-6.7 \pm 0.2^a)</td>
<td>1.0 ± 0.2(^a)</td>
</tr>
<tr>
<td>LPS-Rx</td>
<td>AG</td>
<td>(-7.3 \pm 0.2^a)</td>
<td>2.8 ± 0.2(^a)</td>
</tr>
</tbody>
</table>

\(^a\) \( P < 0.0001 \) when compared with control from saline-treated rats.

\(^b\) \( P < 0.001 \) when compared with control from LPS-treated rats.

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**Figure 1.** Northern blots of total RNA (20 µg) extracted from aortas of LPS (LP; 20 mg/kg ip; 4 h)- or normal saline-treated rats (CT). Left pair, aortas without endothelium; right pair, aortas with endothelium. The RNA was electrically transferred to a Hybond-N membrane and hybridized with an 830-base-pair \(^32\text{P}\)-labeled probe obtained from the 5'-end of the VSMC iNOS. The positions of 18S and 28S rRNA are depicted on the right side of the blot. A picture of the 18S and 28S RNA bands on the formaldehyde/agarose gel is depicted to demonstrate equal loading of RNA in both lanes.

**Figure 2.** Phenylephrine-evoked contraction of rat aortic rings without endothelium from: (a) saline-treated rats (1 mL ip; 4 h) in the presence (squares) or absence (circles) of AG (300 µM; 30 min; \( N = 12\)); (b) LPS-treated rats (20 mg/kg ip; 4 h) in the presence (squares) or absence (circles) of AG (300 µM; 30 min; \( N = 8\)); (c) aortic rings without endothelium from control rats treated in vitro with LPS (1 µg/mL; for 6 h) in the presence (squares) or absence (circles) of AG (300 µM; 30 min; \( N = 7\)). Data are shown as means ± SE.
Absence of Effect of AG Either on Phenylephrine-Evoked Contraction or on Acetylcholine-Evoked Relaxation in Aortas With Endothelium From Control Rats

In aortic rings with endothelium from saline-treated rats, AG (300 μM) did not affect the contraction evoked by 1 μM phenylephrine (control versus AG: 1.3 ± 0.2 versus 1.5 ± 0.2 g/mg). The endothelium-dependent relaxation to acetylcholine (1 nM to 10 μM) was not affected by AG either (Figure 3). The log EC50 values for control versus AG were −7.0 ± 0.1 versus −7.1 ± 0.1; the maximal relaxations were 86 ± 5 versus 86 ± 4% (14 ± 5 versus 14 ± 4% of phenylephrine-evoked contraction [N = 5]).

DISCUSSION

The increased production of nitric oxide has been suggested to be important in septic shock. Biochemical and pharmacologic data suggest that LPS and cytokines (e.g., interleukin-1β and tumor necrosis factor-α) increase nitric oxide production in isolated blood vessels (1,2,4–6,8–10,12,13,22) and in both endothelial (1,24) and VSMC in culture (7,11,25). Julou-Schaeffer et al. (9) demonstrated that the hyperresponsiveness of aortas from endotoxemic rats was reversed by L-arginine analogues, which nonselectively inhibit NOS activity. Using a similar murine model of sepsis, we have extended these pharmacologic data suggesting that LPS and cytokines (approximately 4.4 kilobases; Figure 1).

The VSMC iNOS has been cloned and shows 99% homology with the isoform found in macrophages but only 50% homology with eNOS (19). The 830-base-pair cDNA fragment corresponding to the 5'-end of the VSMC iNOS used to generate our probe therefore shares minimal sequence similarity with eNOS. If this probe was not specific for iNOS, we should have found hybridization with eNOS in the RNA extracted from aortas with endothelium of control animals; however, the iNOS message was only detected in aortic total RNA extracted from rats exposed to LPS. Interleukin 1β-activated rat aortic endothelial cells can also express iNOS (14) in addition to constitutive eNOS. In this study, the persistence of iNOS expression in aortas without functional endothelium suggests that VSMC can express iNOS in vivo; the results, however, do not exclude that resident or invading macrophages or other cells may express iNOS in the blood vessels of LPS-treated rats.

There are studies that suggest that LPS may stimulate iNOS in organs other than blood vessels. For example, rats treated with Propionibacterium acnes and LPS expressed mRNA for the hepatic (Ca2+-dependent) iNOS isoform and Ca2+-dependent NOS activity in the liver, lung, spleen, and colon; blood vessels were not evaluated (26). Moreover, the NOS activity induced by LPS in blood vessels is Ca2+-independent (4). An immunohistochemical study using a polyclonal antibody for hepatic iNOS isoform reported positive staining of the aortic endothelial cells from LPS-stimulated rats (27), but detection was equivocal in VSMC (27). LPS treatment in vivo resulted in the expression of iNOS mRNA in lung, liver, spleen, skeletal muscle, and kidney, but again, blood vessels were not reported (28). These results are therefore the first to demonstrate the expression of iNOS mRNA in the vascular tissues of endotoxemic animals.

Next, in this study, AG, a preferential inhibitor of iNOS (8,21,22), was used to test the functional significance of iNOS mRNA expression in the rat aorta. AG enhanced the phenylephrine-evoked response in endothelium-denuded aortic rings of LPS-treated rats. This pharmacologic finding with AG extends previous experiments using nonselective L-arginine analogues (2,4,6,7,9,10,12,13,25), which inhibit both iNOS and eNOS. These results of the reversal by AG of the hyperresponsiveness observed in aortic rings of untreated rats incubated in vitro with LPS are consistent with recently published studies that compared AG with L-arginine analogues (22). Those authors found AG equipotent to the L-arginine analogues in endothelium-denuded rings exposed to LPS but detected no
action on control rings with endothelium. Equimolar L-arginine prevented the action of AG. In this study, AG also did not affect the responsiveness of control rings without endothelium from saline-treated rats. Previous observations have also shown that the hyporesponsiveness of the pulmonary arteries of endotoxemic rats is reversed by AG (8). Additionally, in this study, AG did not affect endothelium-dependent relaxation to acetylcholine in aortic rings. A similar finding was observed in the pulmonary arteries of control rats (8) and in the rat aorta with a lower concentration of AG (22). Other observations have shown the preferential inhibition of iNOS relative to the constitutive neuronal and endothelial NO by AG using purified enzyme preparations (21). These results from septic rats demonstrating the preferential functional inhibition of iNOS has potential clinical implications. Specifically, AG’s preferential inhibition of iNOS is appealing as compared with the nonselective effects of L-arginine analogues because the regulated release of NO by endothelial cells is maintained while the production of nitric oxide by iNOS is blocked. Despite some reports suggesting that LPS or cytokines may impair endothelium-dependent relaxation (13, 29, 30) or cause increased degradation of eNOS mRNA (17, 31), the microvasculature of endotoxemic rats shows normal acetylcholine relaxation (5). This suggests that the ability of local blood flow regulation by the endothelium is preserved in these animals. AG’s preferential action on iNOS, while maintaining eNOS integrity, may therefore be advantageous in the therapy of sepsis and other states associated with the overproduction of inflammatory cytokines. For example, in spite of the data supporting the marked increase of nitric oxide production in animal models of sepsis and in septic patients, both the biologic significance of this hyperproduction and the desirability in intervening in that pathway with nonselective inhibitors, i.e., L-arginine analogues, are controversial. The unexpected increased mortality observed in septic dogs treated with L-arginine analogues (32), after encouraging initial reports (3), is particularly disturbing. The nonselective effects of L-arginine analogues to impair eNOS integrity could counterbalance and obscure a beneficial effect of blocking iNOS. Therefore, survival studies and systemic and regional hemodynamic evaluations are necessary to further evaluate the usefulness of AG in these states. This more selective approach would block iNOS while preserving the endothelial regulation of the microvasculature by the constitutive eNOS.

In summary, we have demonstrated in aortas of LPS-treated animals both the in vitro expression of iNOS mRNA and the reversal of LPS-induced hyporesponsiveness to phenylephrine by AG, a preferential inhibitor of iNOS. These data extend previous biochemical and pharmacologic evidence of the expression of iNOS activity by LPS. In addition, this model may be an excellent positive control to evaluate the expression of iNOS in other diseases thought to be associated with induction of iNOS in vivo, e.g., hepatic cirrhosis. Finally, the results confirm the usefulness of AG in the study of diseases where iNOS is expressed. Further studies will be necessary to examine the mechanisms involved in the LPS-mediated iNOS expression in vivo and whether iNOS mRNA abundance increases by transcriptional activation or by reduced mRNA degradation.

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