Selective in vivo inhibition of inducible nitric oxide synthase in a rat model of sepsis

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Scott, J. A., and D. G. McCormack. Selective in vivo inhibition of inducible nitric oxide synthase in a rat model of sepsis. J. Appl. Physiol. 86(5): 1739–1744, 1999.—Elevated production of nitric oxide (NO) by the inducible NO synthase (type II, iNOS) may contribute to the vascular hyporesponsiveness and hemodynamic alterations associated with sepsis. Selective inhibition of this isoenzyme is a possible therapeutic intervention to correct these pathophysiological alterations. Aminoguanidine has been shown to be a selective iNOS inhibitor and to correct the endotoxin-mediated vascular hypocontractility in vitro. However, to date aminoguanidine has not been shown to selectively block iNOS activity in vivo. The in vivo effects of aminoguanidine were assessed in the cecal ligation and perforation model of sepsis in rats. Aminoguanidine (1.75–175 mg/kg) was administered to septic and sham-operated rats for 3 h before euthanasia and harvest of tissues. NO activities were determined in the thoracic aorta and lung from these animals. Aminoguanidine (17.5 mg/kg) did not alter the mean arterial pressure; however, it did inhibit induced iNOS (but not constitutive NOS) activity in the lung and thoracic aorta from septic animals. Only the higher dose of aminoguanidine (175 mg/kg) was able to increase the mean arterial pressure in septic and sham-operated animals. Thus selective inhibition of iNOS in vivo with aminoguanidine is possible, but our data suggest that other mechanisms, in addition to iNOS induction, are responsible for the loss of vascular tone characteristic of sepsis.

aminoguanidine; selective; enzyme inhibition

NITRIC OXIDE (NO) is a potent vasodilator synthesized from the semiessential amino acid L-arginine by a family of enzymes, NO synthases (NOS) (14). These isoenzymes are generally subclassified into constitutive forms (cNOS), which are continuously expressed basally and mediate normal homeostatic regulation in the organism, and an inducible form (iNOS), which is expressed in response to inflammatory stimuli, such as cytokines and bacterial lipopolysaccharide, and contribute to an organism’s response to disease. The inducible isozyme of NOS is expressed in inflammatory diseases such as sepsis and is believed to account, at least in part, for the vascular hypocontractility and loss of vascular tone observed in this syndrome (8). This is supported by investigations using “selective” inhibition of iNOS, which prevents the decrease in blood pressure and the accumulation of the metabolic end products of NO, nitrite and nitrate, in the plasma from septic animals (1, 7, 9, 21, 22). By contrast, the predominant role of NO in the systemic hypotension characteristic of sepsis is not supported by investigations, which have demonstrated sepsis-induced hypotension in knockout mice that do not express the iNOS gene (11). Thus the role of induced production of NO in the hypotension of sepsis remains controversial.

To investigate the role of iNOS in sepsis, it is essential to selectively block this isozyme. The first generation of NOS inhibitors used modified analogs of the natural substrate L-arginine, such as N⁶-nitro-L-arginine methyl ester (L-NAME) and N⁶-monomethyl-L-arginine, to competitively block enzyme activity. These inhibitors are nonselective for the different isozymes of NOS (17). The next generation of NOS inhibitors are more selective for particular isozymes, such as 7-nitroindazole for the neuronal cNOS, N⁶-nitro-L-arginine for the endothelial cNOS, and 5-methylisothiourea (18) and aminoguanidine for the inducible isozyme (17). Rather than acting as a competitive inhibitor of NOS, aminoguanidine has been reported to act as a mechanism-based or suicide inhibitor, which forms an enzyme-activity-dependent covalent attachment to the NOS enzyme (19, 20). Aminoguanidine blocks iNOS activity at concentrations ~100 times less than that required to block the constitutive isozymes in vitro (13, 16). We have demonstrated that aminoguanidine reverses the vascular hypocontractility induced by incubation of vascular rings with lipopolysaccharide in vitro (16).

Although there are many reports documenting selective inhibition of iNOS in vitro (13, 19, 21, 22), there has not yet been conclusive evidence of selective iNOS inhibition in vivo. This is important because the selectivity of these inhibitors for iNOS vs. cNOS may vary in different tissues, and these inhibitors often inhibit cNOS at higher doses. Thus we used a clinically relevant model of sepsis, induced by cecal ligation and perforation (CLP) in rats, to examine the hemodynamic effects of aminoguanidine administration in vivo. Furthermore, we investigated the effect of increasing doses of aminoguanidine on constitutive and iNOS activities in lung and vascular tissue from these animals.

METHODS

CLP model of sepsis. This experimental protocol was approved by the Animal Ethics Review Committee at the University of Western Ontario. After a 7- to 14-day acclimatization period in the animal housing facility, Sprague-Dawley rats (300–450 g) were instrumented for fluid and analgesic administration and blood pressure monitoring. Under gen-

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eral anesthesia (2% halothane with O₂), the rat was shaved in the abdominal region and in the dorsal and anterior cervical regions. A 0.3-cm incision was made in the dorsal cervical region for tunneling of the catheters. A 2-cm longitudinal incision was made in the anterior cervical region to expose the right external jugular vein and carotid artery. A Silastic catheter (0.047-in. OD, Silimed, Montreal, Quebec) was inserted into the superior vena cava via the jugular vein for administration of analgesic (fentanyl citrate, 2 µg/ml in 0.9% saline, 10 ml·kg⁻¹·h⁻¹). A PE-50 polyethylene catheter (VWR Canlab, Mississauga, ON) was inserted into the carotid artery for monitoring of blood pressure and sampling of arterial blood. Catheters were tunneled through to the dorsal cervical region and passed through a harness-swivel device (Ealing Scientific, St.-Laurent, Quebec) sutured to the skin with 2-0 silk.

Induction of sepsis was performed by a CLP model as described previously by our laboratory (12). The abdomen was opened with a 3-cm midline incision. The cecum was ligated just distal to the ileocecal valve so that bowel continuity was preserved. Two 18-gauge needle punctures were made through the distal cecum, and fecal contents were allowed to spill into the peritoneum. The midline incision was then closed with 2-0 silk. This model has previously been shown to induce hyperdynamic normotensive sepsis within a 24-h period (12). After surgery, the animals were allowed to recover in their cages with free access to food and water. Sham control animals underwent surgery for the insertion of carotid arterial and jugular venous lines, as above, with an abdominal incision to control for the opening of the peritoneum in the CLP animals. Sham control animals were administered fluids (at the same rate as CLP animals) for 24 h postsurgery until euthanasia and harvesting of tissues.

Starting 20 h after CLP or sham operation, the systemic blood pressure was recorded every 15 min for 4 h, until euthanasia (at t = 24 h postsurgery). From 20 to 24 h postsurgery, arterial blood was drawn on an hourly basis for blood gases, lactate determination, and white blood cell count (0.5-ml aliquots of blood were removed for sampling and replaced with at least 1.0 ml of 0.9% saline). Starting 21 h postsurgery, aminoguanidine was administered as a continuous infusion over 3 h (total dose of 1.75, 17.5, or 175 mg/kg) or saline (note: only the doses of 17.5 and 175 mg/kg aminoguanidine were administered to the Sham animals). At the end of the aminoguanidine administration (i.e., 24 h postsurgery), the rats were euthanized with an overdose of pentobarbital sodium (6.5 mg/kg iv), and the thoracic aorta and lung were harvested for assay of NOS activity. The trachea/heart/lung and thoracic aorta were removed en bloc, and the lung and aorta were perfused with cold Krebs solution (4°C) through the left atria and ventricle, respectively.

NOS assay. NOS activity was quantitated in vitro by the conversion of L-[³H]arginine to L-[³H]citrulline as previously described (16). Rat thoracic aorta and whole lung were homogenized in six volumes (wt/vol) of ice-cold homogenization buffer (pH 7.4; 10 mM HEPES, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.32 M sucrose) by using a Brinkmann Polytron (Brinkmann Instruments, Mississauga, ON). Tissue homogenate (50-µl aliquots) was incubated in quadruplicate for 15 min at 37°C in the presence of incubation buffer [100 µl, pH 7.4; concentrations before addition of homogenate: 50 mM potassium phosphate monobasic; 60 mM L-valine (1 mM bovine serum albumin); 1 mM L-NAME (175 mg/kg); 60 mM L-NAME; 1 mM β-NADPH; 10 µM FAD; 10 µM tetrahydrobiopterin]; 30 µM L-[³H]arginine (200 counts·min⁻¹·µmol⁻¹); and 1.2 mM MgCl₂. When calcium-dependent (cNOS) NOS activity was assayed, 100 nM calmodulin and 2 mM CaCl₂ were added to the incubation buffer. When the calcium-independent (iNOS) activity was assayed, 1.2 mM EDTA and 1 mM EGTA were added to the incubation buffer. The reaction was terminated by the addition of 500 µl of cold stop buffer (4°C; pH 5.5; 100 mM HEPES; 12 mM EDTA). Dowex G250 (200–400, 8% cross-linked, Na⁺ form; 2 ml 1:1 wt/vol with water; ~pH 7.0) was added to the solution to remove any excess L-[³H]arginine. The test tubes were then centrifuged for 20 min at 600 g, and 0.5 ml of the supernatant was added to 4.5 ml of liquid scintillation fluid. The radioactivity was determined by liquid scintillation counting (Beckman Scientific Instruments, Mississauga, ON). The protein concentration of rat lung and aortic homogenates was determined by a microplate modification of the Bradford method, with bovine serum albumin as the standard and homogenization buffer as the blank (2).

cNOS activity was calculated as the difference between the calcium/calmodulin sample (cNOS + iNOS activities) and the EDTA-EGTA sample (iNOS activity only). Nonspecific radioactivity and metabolism of L-arginine was accounted for by incubating homogenization buffer or tissue homogenate with L-NAME (1 mM) in the incubation buffer containing EDTA and EGTA. iNOS activity was calculated as the L-NAME-inhibitable portion of the activity in the samples with EDTA and EGTA. Resultant enzyme activities were expressed as Units (1 Unit = 1 pmol L-citrulline evolved·min⁻¹·mg protein⁻¹). Preliminary investigations determined that total inhibition of NOS activities is possible with aminoguanidine in vitro (data not shown).

Chemicals. Potassium phosphate monobasic was purchased from Fisher Scientific (Mississauga, ON). Cytoscint Environmentally Safe scintillation fluid, 1,4 dithiothreitol, phenylmethylsulfonyl fluoride, and Coomassie brilliant blue G250 were purchased from ICN Biomedical (Mississauga, ON). L-[³H]arginine was purchased from Amersham (Oakville, ON). All other reagents were purchased from Sigma Chemical (Sigma-Aldrich Canada, Mississauga, ON).

Statistical analysis. All data are expressed as means ± SE, and multiple groups were compared by using factorial ANOVA with Fisher’s post hoc analysis. P values < 0.05 were considered significant. All statistical tests were calculated by using the StatView-4.5 program on a Macintosh computer.

RESULTS

Effect of CLP and aminoguanidine on mean arterial pressure (MAP). The induction of sepsis by CLP significantly decreased MAP at 21 h postsurgery compared with that in Sham animals (P < 0.0001) (Table 1). Aminoguanidine (1.75 and 17.5 mg/kg) did not alter MAP in either Sham or CLP animals (not significant (NS) compared with either animals not receiving aminoguanidine or to the pretreatment values). Aminoguanidine (175 mg/kg) significantly increased MAP in both Sham and CLP animals (P < 0.05 for each group of animals compared with their preaminoguanidine baseline).

Effect of CLP and aminoguanidine on arterial blood gases, lactate, and leukocytes. Blood gases, arterial lactates, and white blood counts, at 24 h postsurgery, in samples from Sham and CLP animals administered aminoguanidine (0–175 mg/kg) are shown in Table 2. As we have previously reported with this model (4, 5, 12, 23), sepsis resulted in a significant increase in arterial lactate concentration and decrease in leuko-
cyte count. Neither these values nor the blood gases were affected in any way by aminoguanidine treatment (NS compared with baseline samples). None of these parameters was altered in Sham animals administered aminoguanidine (17.5 and 175 mg/kg; data not shown).

Effect of CLP and aminoguanidine on NOS activities. Thoracic aortas from rats made septic by CLP had increased iNOS activity (Fig. 1A) and decreased cNOS activity (Fig. 1B) compared with aortas from Sham rats (P < 0.05 for both iNOS and cNOS activities). A low dose of aminoguanidine (1.75 mg/kg, n = 8) had no effect on cNOS and iNOS activities in CLP rats. By contrast, higher doses of aminoguanidine (17.5 and 175 mg/kg, n = 9 and 8, respectively) inhibited the CLP-induced increase in iNOS activity (P < 0.05 compared with CLP), whereas cNOS activities were unchanged (NS compared with CLP).

As described above, sepsis resulted in a reduction in cNOS activity in thoracic aorta. In this scenario, it would be difficult to measure further inhibition of cNOS activity by aminoguanidine, indicative of nonselectivity.

Table 1. Mean arterial pressure of Sham and CLP animals before and after administration of aminoguanidine

<table>
<thead>
<tr>
<th>Group/Aminoguanidine Dose (mg/kg)</th>
<th>n</th>
<th>Pre-AG (mmHg)</th>
<th>Post-AG (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>117 ± 2</td>
<td>114 ± 4†</td>
</tr>
<tr>
<td>17.5</td>
<td>6</td>
<td>119 ± 4†</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>8</td>
<td>125 ± 1†</td>
<td></td>
</tr>
<tr>
<td>CLP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>101 ± 1*</td>
<td>94 ± 5*</td>
</tr>
<tr>
<td>1.75</td>
<td>8</td>
<td>100 ± 6*</td>
<td></td>
</tr>
<tr>
<td>17.5</td>
<td>9</td>
<td>102 ± 2*</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>8</td>
<td>111 ± 4†</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE, n, No. of rats; AG, aminoguanidine; Pre, before; Post, after; Sham, sham-operated rats; CLP, cecal ligation and perforation. Mean arterial pressure at baseline (t = 21 h postsurgery, pre-AG) and during the 3rd hour postinfusion of AG or saline in Sham and CLP rats (post-AG) are shown. *P < 0.05 compared with untreated Sham, †P < 0.05 compared with untreated CLP, #P < 0.05 compared with pre-AG (factorial ANOVA).

Table 2. Arterial blood data for Sham and CLP animals at 24 h

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (n = 8)</th>
<th>CLP (n = 8)</th>
<th>CLP + 1.75 AG (n = 8)</th>
<th>CLP + 17.5 AG (n = 9)</th>
<th>CLP + 175 AG (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate, mM</td>
<td>0.5 ± 0.0</td>
<td>0.9 ± 0.1*</td>
<td>1.2 ± 0.1*</td>
<td>0.8 ± 0.1*</td>
<td>1.0 ± 0.1*</td>
</tr>
<tr>
<td>Leukocytes, ×10⁹/l</td>
<td>11.2 ± 1.5</td>
<td>3.4 ± 0.3*</td>
<td>3.8 ± 0.5*</td>
<td>3.8 ± 0.6*</td>
<td>4.1 ± 0.6*</td>
</tr>
<tr>
<td>pH</td>
<td>7.49 ± 0.01</td>
<td>7.46 ± 0.01</td>
<td>7.46 ± 0.02</td>
<td>7.46 ± 0.02</td>
<td>7.44 ± 0.01*</td>
</tr>
<tr>
<td>Pco₂, Torr</td>
<td>36.5 ± 2.0</td>
<td>36.8 ± 1.2</td>
<td>38.1 ± 1.4</td>
<td>36.2 ± 1.9</td>
<td>36.9 ± 3.8</td>
</tr>
<tr>
<td>Po₂, Torr</td>
<td>83.9 ± 3.7</td>
<td>84.3 ± 5.1</td>
<td>89.7 ± 2.5</td>
<td>94.4 ± 8.1</td>
<td>82.6 ± 6.0</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.33 ± 0.01</td>
<td>0.29 ± 0.01*</td>
<td>0.33 ± 0.01</td>
<td>0.29 ± 0.02*</td>
<td>0.30 ± 0.1</td>
</tr>
<tr>
<td>Platelets, ×10⁹/l</td>
<td>596 ± 17</td>
<td>286 ± 33*</td>
<td>288 ± 37*</td>
<td>331 ± 40*</td>
<td>291 ± 41*</td>
</tr>
<tr>
<td>Weight, g</td>
<td>398 ± 15</td>
<td>368 ± 14</td>
<td>390 ± 9</td>
<td>339 ± 7*</td>
<td>347 ± 11*</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

Values are means ± SE, with AG in mg/kg, n, No. of rats. Twenty-four-hour arterial blood-gas values, plasma lactate concentration, leukocyte count, and hematocrit from Sham animals and CLP animals administered aminoguanidine (0–175 mg/kg) are shown. *P < 0.05 compared with Sham, (factorial ANOVA).
of the NOS inhibition. Thus, to confirm that aminoguanidine did not inhibit cNOS activities, aminoguanidine (17.5–175 mg/kg) was administered to Sham animals. Thoracic aortas from these rats showed no significant effect of aminoguanidine (17.5 mg/kg) compared with the Sham control (NS compared with Sham; Fig. 1B). However, Sham rats administered a higher dose of aminoguanidine (175 mg/kg) exhibited significantly less cNOS activity compared with Sham control animals (P < 0.001 compared with Sham; Fig. 1B).

Similar to the thoracic aortas, lungs from septic rats were found to have an increase in iNOS activity (Fig. 2A) and a decrease in cNOS activity (Fig. 2B) compared with lungs from Sham rats (P < 0.05 for both iNOS and cNOS activities). The aminoguanidine (1.75–175 mg/kg) dose-dependently inhibited iNOS activity in the lungs from septic animals (Fig. 2A). By contrast, aminoguanidine did not have any effect on cNOS activity in lungs from these animals (Fig. 2B). Lungs from sham-operated rats administered aminoguanidine (17.5–175 mg/kg) showed no significant effect of aminoguanidine on cNOS activity compared with the Sham control rats (NS compared with Sham untreated).

DISCUSSION

Our results have demonstrated that administration of aminoguanidine in vivo improved MAP in a rat model of sepsis, although only at doses higher than that which selectively inhibits iNOS. We have also shown that sepsis results in a depression of cNOS activity compared with sham-operated animals in all tissues studied. Furthermore, at appropriate doses, aminoguanidine can selectively inhibit the sepsis-induced elevation of iNOS activity in both the thoracic aorta and the lung, with no significant effect on cNOS activities in these tissues.

The finding that selective inhibition of iNOS is possible in vivo with aminoguanidine is supported by previous investigations using models of sepsis induced by endotoxin. With these models, investigators have reported a decrease in plasma nitrate and nitrite levels after administration of doses of aminoguanidine similar to those used in the present study (15, 18, 22) and have demonstrated selective iNOS inhibition in vitro by using different tissues (18). Furthermore, Alden et al. (1) have recently demonstrated that aminoguanidine decreased plasma concentration of NO metabolites in a rat model of peritoneal sepsis. However, our work is the first to measure both cNOS and iNOS activities in tissues from septic and control animals after administration of aminoguanidine. These data indicate that aminoguanidine selectively inhibits iNOS in the thoracic aorta and lung to varying degrees. The reason for this variability may be related to regional heterogeneity in the distribution, uptake, and/or metabolism of the drug.

In septic animals, we found decreased cNOS activity in all tissues studied. This depression of cNOS activity in sepsis is supported by previous work by us (16) and others (10), in which endotoxin produced an upregulation of iNOS activity and mRNA, with a concomitant downregulation of cNOS activity and mRNA. This is also supported by the previous demonstration that cytokines, important in the induction of iNOS, also produce an instability of cNOS mRNA (24). Previous work using short-term (i.e., 5-h in vitro incubation) endotoxin stimulation of rat thoracic aorta has suggested that the resulting depression of cNOS activity is due to reversible inhibition of the constitutive isozymes by the elevated production of NO by iNOS (16). This has been supported by evidence indicating that cNOS isozymes are more susceptible than is the iNOS isozyme to NO “self-inhibition” by NO binding to the heme-iron core of the enzyme (3, 6). In contrast, investigations using the endotoxin model in vivo have shown that the elevation in iNOS expression is associated with a concomitant reduction in cNOS mRNA (10), hence a depression of cNOS activity that is not restorable through pharmacological means (i.e., through iNOS inhibition or by increasing substrate availability). Thus the finding in the present study, that 24 h of
sepsis resulted in increased iNOS activity and that the depressed cNOS activity was not restored after selective inhibition of iNOS, is consistent with the previous in vivo observation (10) that there is a downregulation of cNOS mRNA and, consequently, of functional protein in sepsis. Thus it appears that the chronic (i.e., >12 h) in vivo models of sepsis produce downregulation of cNOS, whereas the acute in vitro model results in a reversible depression of iNOS activities.

Septic animals exhibited a decreased MAP compared with sham-operated animals. Similar to other investigators (1), a moderate dose of aminoguanidine [17.5 mg/kg in the present study; 25 mg/kg in the case of Alden et al. (1)] did not alter MAP in either septic or control rats. An improvement of MAP in septic animals was only observed after administration of a high dose (175 mg/kg) of aminoguanidine, much higher than the dose found to selectively inhibit iNOS in the thoracic aorta and lung. The lack of an effect on blood pressure of the moderate dose of aminoguanidine, which selectively inhibited iNOS activity in thoracic aortas and lung in vitro, would indicate either 1) that the amino-
guanidine is not affecting the smaller resistance vessels that are responsible for the regulation of systemic blood pressure; 2) that the drug is not reaching or effective in the vascular beds of significance in the sepsis-induced hypotension; or, more likely, 3) that factors in addition to NO, such as vasodilator prostanooids or other vasoac-
tive mediators, contribute to the vascular hypoco
tractility and resultant hypotension of sepsis. This latter hypothesis is supported by studies using iNOS knock-
out mice, which have provided compelling evidence that mechanisms in addition to iNOS induction contrib-
ute to the hypotension in sepsis (11).

In summary, our data show that aminoguanidine can selectively inhibit iNOS activity in vivo. The degree of this inhibition may be variable among tissues investi-
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