Resistance to Endotoxin Shock in Transgenic Mice Overexpressing Endothelial Nitric Oxide Synthase

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Background—Nitric oxide (NO) plays a central role in the pathogenesis of septic shock. However, the role of the NO produced by endothelial NO synthase (eNOS) in septic shock is still unclear. We examined the effect of chronic eNOS overexpression and the role of eNOS-derived NO in lipopolysaccharide (LPS)-induced septic shock using eNOS transgenic (Tg) mice.

Methods and Results—LPS was intraperitoneally injected into Tg and control mice. No differences existed in the peak plasma nitrate and nitrate levels induced by LPS between the 2 genotypes. In LPS-treated control mice, blood pressure progressively declined and reached 60% of basal levels (from 97±3 to 59±3 mm Hg) 24 hours after LPS injection. In contrast, the blood pressure of LPS-treated Tg mice fell only 15% from basal levels (from 84±4 to 71±4 mm Hg) after the first 6 hours and, thereafter, it remained at this level. LPS-induced increases in the expression of the mRNA of both vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 in the lungs were significantly lower in Tg mice than in control mice. LPS-induced pulmonary leukocyte infiltration and increases in lung water content were also significantly attenuated in Tg mice. Histological examination revealed that lung injury after LPS injection was milder in Tg mice. Furthermore, Tg mice exhibited enhanced survival from LPS-induced septic shock compared with control mice.

Conclusions—Chronic eNOS overexpression in the endothelium of mice resulted in resistance to LPS-induced hypotension, lung injury, and death. These effects are associated with the reduced vascular reactivity to NO and the reduced anti-inflammatory effects of NO. (Circulation. 2000;101:931-937.)

Key Words: nitric oxide ■ cell adhesion molecules ■ lung ■ shock

Endotoxin shock is a potentially lethal failure of multiple organs that is initiated by lipopolysaccharides (LPS) derived from the outer membrane of gram-negative bacteria. Endotoxin produces a large amount of nitric oxide (NO) by the activation of inducible nitric oxide synthase (iNOS) in various cell types and tissues.1 Evidence suggests that overproduced NO plays a central role in the pathogenesis of endotoxin-induced systemic hypotension and tissue injury: NOS inhibitors increase systemic blood pressure (BP) in patients with septic shock,2 and iNOS-selective inhibitors yield beneficial effects and improve survival in rodents with endotoxin shock.3 However, several studies have demonstrated the opposite findings. Cobb et al4 reported that NOS inhibitors increased mortality rates in animals injected with LPS. In addition, inconsistent results on resistance to endotoxin shock have been reported in iNOS knockout mice.5,6 In 1 study, no significant difference existed in LPS-induced death between endothelial NOS (eNOS) knockout mice and wild-type mice.7 Thus, the therapeutic benefit of NOS inhibition in septic shock is still controversial and must be clarified.

A low concentration of NO produced by eNOS under physiological conditions is one of the major regulators of arterial BP and regional blood flow. Some investigations have proposed that the maintenance of basal NO synthesis is critical for organ perfusion and survival in endotoxin shock and that the deleterious effects of NOS inhibitors in endotoxin shock might be related to the blockade of eNOS.1 However, little is known about the potential roles of eNOS in endotoxin shock. We generated transgenic (Tg) mice overexpressing the bovine eNOS gene in endothelial cells.8 The purpose of this study was to determine the effects of the chronic overexpression of eNOS in the endothelium and the role of eNOS-derived NO in endotoxin shock. To this end, we examined the process of LPS-induced shock in eNOS-Tg mice.

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Methods

Materials
LPS (Escherichia coli, Serotype 055:B5) and other drugs were purchased from Sigma, and the nitroglycerin (NTG) was obtained from Nippon Kayaku. The LPS was dissolved in 0.9% saline.

Animal Preparation
We generated Tg mice overexpressing the bovine eNOS gene in the endothelium using the preproendothelin-1 promoter. Once they reached 12 to 16 weeks of age, heterozygous Tg mice and their littermate control mice were used in this study. All animal experiments were conducted according to the “Guidelines for Animal Experiments at Kobe University School of Medicine.”

To induce septic shock in mice, the animals received an intraperitoneal injection of 80 mg/kg LPS. This dose was chosen because preliminary tests showed that it led to a mortality rate of >90% in wild-type C57BL/6 mice. For survival studies, 18 sex- and age-matched mice of both genotypes were used. To examine the effect of NOS inhibition on LPS-induced death, LPS was given to another 8 mice from both genotypes 30 minutes after the intraperitoneal injection of N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester (L-NAME; 300 mg/kg). Survival was monitored every 12 hours for the first 2 days and daily thereafter.

Studies of Vascular Reactivity Ex Vivo
Isometric tension was recorded as previously described. Briefly, 3-mm-wide aortic rings were suspended in organ baths containing Krebs’ solution and equilibrated at 37°C with a 95% O\textsubscript{2}/5% CO\textsubscript{2} gas mixture. The rings were mounted on stainless steel hooks attached to force transducers to measure isometric tensions. After precontracting with prostaglandin F\textsubscript{2}\textalpha, NTG was added in a cumulative manner.

Measurement of BP in Mice
Under anesthesia with 80 mg/kg pentobarbital sodium (Abbott Laboratories), BP was measured by femoral artery catheterization, as previously reported. Then, NTG was injected through the vein cannula, and BP was continuously monitored using a Macintosh computer with MacLab systems.

For the continuous measurement of BP under conscious and unrestrained conditions, the femoral catheter was connected to a transducer using a free-moving cannulation system (Tsumura), and the signals were continuously monitored. At least 6 hours after recovery from anesthesia, BP was monitored for 24 hours after LPS injection.

Northern Blot Analysis
Total RNA was extracted with ISOGEN (Nippon Gene) from the organs of the mice. The RNA was subjected to electrophoresis on 1% agarose gels containing formaldehyde and transferred to nylon membranes. The membranes were hybridized with a \textsuperscript{32}P-labeled cDNA probe and then washed and analyzed with a BAS2000 Autoimage Analyzer (Fuji Photo Film). To detect iNOS mRNA, we used a mouse iNOS cDNA probe, as previously described. To detect the mRNA of both vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), we used mouse VCAM-1 and rat ICAM-1 cDNA probes, as previously reported.

Measurement of Plasma Nitrite and Nitrate Levels
Cyclic guanosine monophosphate (cGMP) levels were measured using an enzyme immunoassay kit (Amersham Life Science), as previously described.

Lung Myeloperoxidase Activity
The left lung was surgically removed, externally rinsed with saline, blotted dry, and weighted. The lung tissue was homogenized and sonicated in 2 mL of a 50 mmol/L potassium phosphate–buffered solution (PBS) (pH 7.4) containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was centrifuged at 25000g for 15 minutes at 4°C; the supernatant was decanted for myeloperoxidase (MPO) measurements, and the pellet was resuspended in 1 mL of hexadecyltrimethylammonium bromide–PBS. This extraction procedure was repeated 3 times. The supernatants were mixed 1:30 (vol/vol) with 50 mmol/L PBS (pH 6.0) containing 0.167 mg/mL o-dianisidine and 0.0005% hydrogen peroxide; the absorbance change was then measured at 460 nm for 5 minutes. MPO activity, as based on wet lung weight, was calculated as a change in absorbance over time.

Lung Wet-to-Dry Weight Ratio
A lung wet-to-dry weight ratio (W/D ratio) was used as a parameter of lung water accumulation after LPS injection. LPS-treated mice were studied 4 and 24 hours after LPS injection. Lung wet weight was determined immediately after removal of the right lung. Lung dry weight was determined after the lung had been dried in an oven at 50°C for 24 hours, and the W/D ratio was calculated by dividing the wet weight by the dry weight.

Bronchoalveolar Lavage
The mice were anesthetized with pentobarbital sodium, their tracheas were cannulated, and bronchoalveolar lavage (BAL) was performed 3 times using 0.4 mL of PBS. We counted the total number of cells using the hemocytometer method. BAL fluid was centrifuged using a cytopsin, and BAL cells were stained with Diff Quik. Differential cell counts were determined by counting 300 cells per slide.

Histopathological Examination
Mouse lungs were excised at various times after LPS injection and inflated at a pressure of 20 cm H\textsubscript{2}O with 4% paraformaldehyde/PBS. Livers and kidneys were perfusion-fixed (30 cm H\textsubscript{2}O) in situ with 4% paraformaldehyde/PBS. Organs were fixed overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistics
Data are presented as mean ± SEM. An unpaired Student’s t test was used to detect significant differences when 2 groups were compared. Statistical differences among group means were determined by 1-way ANOVA with repeated measures; this was followed by a post hoc comparison. Time-dependent changes in mean arterial BP in different groups were compared by ANOVA with a Bonferroni correction. Survival data were analyzed using the Kaplan-Meier test. P<0.05 was considered statistically significant.

Results
eNOS-Tg Mice Exhibited Reduced NO-Mediated Vasorelaxation
In accordance with our previous report, relaxation with NTG was significantly reduced in the aortas from Tg mice compared with those from control mice, as measured by shifts in ED50 (61.5±9.5 versus 20.0±2.7 mmol/L; P<0.01) and by maximal relaxation (64.3±2.7% versus 89.7±1.6%; P<0.01) (Figure 1A). Reductions in arterial BP induced by NTG (100 mg/kg) injection were monitored to investigate the in vivo effects of NTG on hemodynamics. As depicted in Figure 1B, NTG injection caused a profound and sustained decrease in
BP in control mice. In contrast, in Tg mice, NTG injection induced only a slight and transient decrease in BP compared with control mice (changes in mean BP, 217 ± 7 versus 247 ± 3 mm Hg; P < 0.05). The same observation applied to the response to sodium nitroprusside (data not shown). These findings likely show that Tg mice exhibit reduced NO-mediated vasorelaxation in vivo in resistant vessels, as well as ex vivo in aortas.

iNOS mRNA and Plasma NOx Levels
We did not detect iNOS mRNA in organs at baseline in either rat genotype (data not shown). LPS induced marked increases in iNOS mRNA and plasma NOx levels in both control and Tg mice (Figure 2). No significant differences existed between the 2 groups in LPS-induced iNOS mRNA levels (Figure 2A) and iNOS activity (data not shown) in the organs. Moreover, no significant differences existed in peak plasma NOx levels between the 2 genotypes, although plasma NOx levels in Tg mice at baseline and 3 hours after LPS injection were significantly higher than those in control mice (Figure 2B).

cGMP Levels in the Aorta
Basal cGMP levels were significantly higher in the aortas from Tg mice than those from control mice (0.91 ± 0.10 versus 0.59 ± 0.09 pmol/mg; P < 0.05). Aortic cGMP levels increased after LPS injection in both genotypes. However, increases in aortic cGMP levels 12 hours after LPS injection were significantly attenuated in Tg mice compared with control mice (1.96 ± 0.21 versus 2.87 ± 0.34 pmol/mg; P < 0.05).

Changes in BP After LPS Injection
As was the case with our previous study, baseline BP was significantly lower in Tg mice than in control mice (mean BP, 84 ± 4 versus 97 ± 3 mm Hg; P < 0.01). Control mice exhibited a progressive and severe drop in BP after LPS injection (Figure 3A). By 24 hours, the BP of control mice fell to ~60 mm Hg (a 40% reduction) (Figure 3B). In contrast, the BP of Tg mice only fell ~10 mm Hg (a 15% reduction) after the first 6 hours; thereafter, it remained at this level (Figure 3). Thus, Tg mice were more resistant to LPS-induced hypotension than control mice.

LPS-Induced Organ Damage
LPS induces pulmonary leukostasis, edema, and toxicity. We assessed pulmonary granulocyte infiltration by measuring lung MPO activity. At basal levels, only low levels of lung MPO activity were detected, and no differences existed in the levels between the 2 genotypes (Figure 4A). The MPO
activity 4 hours after LPS injection increased 16-fold in control mice but only 10-fold in Tg mice ($P$, 0.05). Moreover, MPO activity 24 hours after LPS injection was significantly lower in Tg mice (Figure 4A). BAL revealed that the numbers of neutrophils and macrophages migrating into the alveolar space significantly increased after LPS injection in both genotypes. However, the migration of both cell types was significantly reduced in Tg mice (Table). The W/D ratio, a parameter of pulmonary edema, increased in both Tg and control mice after LPS injection (Figure 4B). LPS-challenged lungs of control mice had a significant increase in the W/D ratio 4 hours after LPS injection compared with that of Tg mice ($P$, 0.01). However, the differences in W/D ratio between the 2 groups 24 hours after LPS injection were negligible (Figure 4B).

Light microscopic examination of lungs taken from LPS-treated control mice 24 hours after LPS injection demonstrated the infiltration of numerous polymorphonuclear leukocytes and macrophages in the interstitial spaces and marked swelling of alveolar walls (Figure 5C). These changes were attenuated in LPS-treated Tg mice (Figure 5D). We also examined the expression of VCAM-1 and ICAM-1 mRNA levels in the lungs. Although LPS treatment increased the expressions of lung VCAM-1 and ICAM-1 mRNA levels in both genotypes, the expression levels were attenuated in LPS-treated Tg mice compared with LPS-treated control mice (Figure 6).

LPS treatment did not induce notable liver damage in our model. We observed only slight hepatocellular vacuolation in the liver, and histopathological examination yielded findings that were indistinguishable between control and Tg mice (data not shown). LPS-treated mice had glomerular and tubular abnormalities in their kidneys, which were characterized by increases in glomerular cells, an increased mesangial matrix, and adjacent connective tissue with inflammatory

### Results of BAL After LPS Injection in Mice

<table>
<thead>
<tr>
<th></th>
<th>Total No. of Cells, $\times 10^4$ cells/mL</th>
<th>Neutrophils, $\times 10^4$ cells/mL</th>
<th>Macrophages, $\times 10^4$ cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mice (6)</td>
<td>11.7±1.5</td>
<td>0.07±0.03</td>
<td>11.2±1.4</td>
</tr>
<tr>
<td>Tg mice (6)</td>
<td>12.2±1.2</td>
<td>0.06±0.03</td>
<td>11.5±1.1</td>
</tr>
<tr>
<td>LPS-treated control mice (8)</td>
<td>30.9±5.9†</td>
<td>4.46±2.13†</td>
<td>23.5±3.2‡</td>
</tr>
<tr>
<td>LPS-treated Tg mice (8)</td>
<td>20.4±1.7†</td>
<td>0.60±0.15†</td>
<td>18.1±1.6‡</td>
</tr>
</tbody>
</table>

Migration of inflammatory cells into the air space was detected by BAL in mice. BAL was examined in un-treated mice and LPS-treated mice 8 hours after LPS injection. The number of independent experiments are shown in parentheses. Results are shown as mean±SEM.

* $P$, 0.05 versus LPS-treated control mice; † $P$, 0.05 and ‡ $P$, 0.01 relative to baseline values.
infiltrates. The histopathological changes were equivalent in the 2 genotypes (data not shown).

**LPS-Induced Death**

LPS-treated mice of both genotypes showed signs of sepsis such as apathy, fur ruffling, conjunctivitis, and diarrhea, which were more prominent in control mice. Figure 7 shows the survival curves for both Tg and control mice after LPS injection. Within 24 hours after LPS injection, 28% of LPS-treated control mice died; no deaths occurred in LPS-treated Tg mice at this time point. By 72 hours after LPS injection, 94% of control mice died, in contrast to 56% of Tg mice. Therefore, eNOS overexpression significantly increased the survival from LPS-induced septic shock ($P < 0.05$). Moreover, L-NAME treatment dramatically increased the mortality rate in both genotypes and canceled the beneficial effect of eNOS overexpression.

**Discussion**

The present study determined that eNOS-Tg mice were resistant to LPS-induced hypotension (Figure 3). Furthermore, Tg mice showed a reduced hypotensive response to the NO donor NTG (Figure 1B). Previous in vitro studies showed reduced NO-mediated vasorelaxation in isolated aortas from Tg mice. The mechanisms of the reduced vascular reactivity have not been completely clarified. Some evidence suggests that long-term exposure of vessels to NO donors results in an alteration of vascular reactivity due to desensitization of soluble guanylate cyclase. In our model, the sodium nitroprusside–induced increase in cGMP levels via the activation of soluble guanylate cyclase was markedly attenuated in the aortas of Tg mice compared with those from control mice. In the present study, LPS stimulated iNOS expression and produced a large amount of NO in both genotypes (Figure 2). Although iNOS-mediated NO production did not differ between the 2 groups, the LPS-induced increase in aortic cGMP levels and the extent of the LPS-induced reduction in BP were attenuated in Tg mice (Figure 3). We speculate that the desensitization of soluble guanylate cyclase is responsible, at least in part, for the resistance to LPS-induced hypotension in Tg mice.

It has been reported that LPS administration in mice induces lung neutrophil infiltration and the development of lung injury. In the present study, we demonstrated that MPO activity was lower and leukocyte infiltration into airspaces was less in LPS-treated Tg mice than in LPS-treated control mice (Figure 4A, Table). Adhesion molecules expressed on vascular endothelial cells are essential for the regulation of the trafficking of leukocytes across the vascular endothelial barrier; they are critically involved in the inflam-
respectively; \( P<0.05 \)). This result may also relate to the reduced lung injury in Tg mice.

Caterina et al\(^7\) reported that NO reduced the cytokine-induced endothelial expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin. Moreover, it has been reported that NOS inhibition worsens LPS-induced lung injury\(^18\) and that the injection of L-arginine, a precursor of NO, prevents endotoxin-induced lung neutrophil accumulation.\(^19\) In this study, we showed that the extent of VCAM-1 and ICAM-1 expression was less in LPS-treated Tg mice than in LPS-treated control mice (Figure 6). We noticed that eNOS was prominently overexpressed in the pulmonary small vessels in Tg mice\(^8\) and that lung eNOS activity in Tg mice was \( \sim 20 \) times as much as that of control mice at basal conditions (507.2 versus 27.6 fmol of citrulline \( \cdot \) mg\(^{-1} \cdot \) min\(^{-1} \); \( P<0.01 \)). Moreover, we found that plasma NOx levels were higher in the early phase after LPS injection in Tg mice than in control mice (Figure 2B). This finding agrees with the report of Szabó et al.\(^20\) who showed that LPS produced NO by activating eNOS. For these reasons, eNOS-derived NO is likely to be overproduced in the pulmonary vessels of Tg mice. Thus, although NO inhibits the inflammatory process apart from its effects on cell adhesion, we propose that the inhibition of adhesion molecule expression by eNOS-derived NO is at least partly responsible for the reduced lung tissue injury. This finding implies that eNOS-derived NO has anti-inflammatory actions in vivo.

We also showed that LPS induced severe renal damage, the severity of which was not different between control and Tg mice. A recent article demonstrated that eNOS-derived NO plays an important role in the prevention of renal injury by maintaining organ perfusion, whereas iNOS-derived NO inhibited glomerular eNOS activity and worsened renal injury.\(^21\) In the present study, eNOS overexpression did not affect LPS-induced renal injury, likely because only weak overexpression of eNOS occurred in the kidneys of Tg mice, as we have previously reported.\(^8\) Otherwise, a large amount of NO from iNOS might overcome the beneficial effect of eNOS overexpression on LPS-induced renal injury.

We demonstrated that chronic eNOS overexpression prevented LPS-induced death (Figure 7). One of the mechanisms of the reduced mortality was the attenuated lung injury in Tg mice. Another was the secondary prevention of organ damage due to the maintenance of BP and organ perfusion after LPS injection. The inhibition of thrombus formation in microvessels by overproduced NO from the endothelium might also serve to maintain tissue perfusion, although we did not determine this in the present study. Added to these, the reduction in TNF-\( \alpha \) release could also attribute to reduced mortality. Our results are not consistent with those from the previous study by Shesely et al.\(^7\) which demonstrated that eNOS knockout mice were as susceptible to LPS-induced death as wild-type mice. However, survival 1 day after LPS treatment seemed to be lower in the eNOS knockout mice than in the wild-type mice in their study, although they did not mention it.\(^7\) This may agree with the fact that eNOS-derived NO serves to inhibit LPS-induced tissue injury, as noted in our study.
In summary, chronic overexpression of eNOS in the endothelium causes reduced vascular reactivity to NO, which is associated with resistance to LPS-induced hypotension. In addition, eNOS overexpression prevents LPS-induced lung injury, which is likely related to the anti-inflammatory effects of NO. These effects of overproduced NO derived from overexpressed eNOS result in the resistance to LPS-induced death. The present study provides evidence supporting the fact that nonselective NOS inhibitors are not always effective in therapy for septic shock. The development of selective inhibitors for iNOS would serve to improve the outcome of septic shock.

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