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Inhibition of Inducible Nitric Oxide Synthase Prevents LPS-Induced Acute Lung Injury in Dogs

Mari Numata,* Shunsuke Suzuki,2* Naoki Miyazawa,* Akira Miyashita,* Yoji Nagashima,† Satoshi Inoue,* Takeshi Kaneko,* and Takao Okubo*

Nitric oxide (NO) is produced by inducible NO synthase (iNOS) after LPS stimulation, and reacts with superoxide to form peroxynitrite. We hypothesize that in LPS-induced lung injury, NO generated by iNOS plays a key role through the formation of peroxynitrite. We developed an acute lung injury dog model by injecting LPS, and examined the effects of selective iNOS inhibitors, aminoguanidine (AG) and S-methylisothiourea sulfate (SMT), on the LPS-induced lung injury. At 24 h after LPS injection, arterial oxygen tension and mean arterial pressure decreased, and shunt ratio and lung wet-to-dry weight ratio increased. On histology, the LPS group had marked neutrophil infiltration and widening of the alveolar septa. On immunohistochemistry, the LPS group had marked neutrophil infiltration and widening of the alveolar septa. On immunohistochemistry, iNOS was injured has been the subject of recent intense investigation.

Nitric oxide (NO) is a highly reactive radical synthesized from the amino acid L-arginine by the action of nitric oxide synthases (NOS) (5). Several isoforms of NOS have been identified and divided into two categories with different regulation and activities (6–8). The constitutive NOS (cNOS) exists in endothelial, neuronal, and various cells, and comprises the low output path on demand in homeostatic processes such as neurotransmission or blood pressure regulation (6, 7). In addition, there are inducible isoforms (iNOS) that may be expressed after exposure to endotoxin and certain cytokines (IL-1, TNF, IFN-γ) in macrophages, neutrophils, mast cells, endothelial cells, and vascular smooth muscle cells (9, 10). Induction of iNOS is a much greater stimulus of NO production than activation of cNOS. Under physiologic states, NO may serve a protective function by scavenging superoxide to protect lung tissues, but the excessive production of NO may contribute to tissue damage in which NO reacts with superoxide to form peroxynitrite, a strong oxidant (11, 12). It is suggested that peroxynitrite is an important oxidant in various diseases (13–15).

Stimulation by LPS induces large amounts of NO and superoxide in alveolar macrophages, lung epithelial, endothelial, and interstitial cells for prolonged periods (6, 8, 11). Overproduction of NO following cytokine- or endotoxin-mediated expression of iNOS can result in shock (16, 17). Endotoxin is reported to trigger the induction of iNOS and form peroxynitrite in the rat aorta (18). A major product from the reaction of peroxynitrite with protein is nitrosyls (11, 12). Recently, nitrosyls were detected in patients and animals with acute lung injury (19, 20).

We hypothesize that NO generated by iNOS plays a key role in LPS-induced acute lung injury by forming peroxynitrite. To test the hypothesis, we developed an animal model of acute lung injury, comparable physiologically and histologically to human ARDS. We examined, with the use of selective iNOS inhibitors, aminoguanidine (AG) (21, 22) and S-methylisothiourea (SMT) (23), whether NO and peroxynitrite contribute to the development of acute lung injury in LPS-injected animals.

Materials and Methods

Animal preparation

Beagles weighing 10.4 ± 1.7 (SD) kg were used for the experiment. Anesthesia was induced with i.v. thiopental sodium (30 mg/kg), and maintained with the use of pentobarbital sodium (2 mg/kg/h). The animals were intubated with an endotracheal tube and spontaneously breathed room air. Anesthesia was maintained to keep the end-tidal CO2 at approximately 40 mm Hg throughout the experiment. A femoral artery was cannulated with a catheter (8 Fr) for monitoring of systemic arterial pressure and for drawing arterial blood for gas analysis. A Swan-Ganz catheter (131H-8F; Baxter Healthcare, Irvine, CA) was inserted into the main pulmonary artery for measurement of pulmonary hemodynamics. Animals were observed for...
24 h on a surgical table using a heating pad and were administered Ringer’s solution throughout the experiments (4 ml/kg/h). Pressures and ventilation were recorded on a six-channel strip-chart recorder (Rectigraph 8K; San-ei NEC, Tokyo, Japan).

Study protocol
Experimental groups were as follows: 1) control group (n = 7), animals were injected with 20 ml of saline; 2) LPS group (n = 7), animals were injected with LPS i.v.; 3) AG group (n = 5), AG was administered i.v. throughout the experiment; 4) LPS + AG group (n = 7), AG administration was started before LPS injection; 5) SMT group (n = 5), SMT was injected continuously throughout the experiment; and 6) LPS + SMT group (n = 5), SMT administration was started before LPS injection. LPS (Escherichia coli serotype 0111; B4; Sigma Chemical Co., St. Louis, MO), 20 μg/kg, was dissolved in 20 ml of saline and injected i.v. in 10 min. Intravenous administration of AG or SMT (Sigma Chemical Co.) was started 30 min before the injection of saline or LPS at a rate of 2 mg/kg/h or 1 mg/kg/h throughout the experiment, respectively.

Hemodynamic parameters, pulmonary gas exchange, and pulmonary function were measured at 0, 3, 6, 12, and 24 h after LPS or saline injection. At the end of the experiments, the animals were killed by injection of potassium chloride, and the lungs were excised immediately for measurement of wet-to-dry weight (W/D) ratio and for histologic examination.

Hemodynamics
Mean arterial pressure (MAP) was measured by a catheter placed in the femoral artery connected to a pressure transducer (model 023XL; Spectramed, Stratham, CA). Both mean pulmonary arterial pressure (MPAP) and pulmonary capillary wedge pressure (PCWP) were measured with a Swan-Ganz catheter connected to pressure transducers (model 023XL; Spectramed).

Pulmonary gas exchange
Partial pressures of oxygen (PaO$_2$) and carbon dioxide, and pH of arterial blood were measured with a blood gas analyzer (BGM IL-1312; Instrumentation Laboratory, Milan, Italy). Hemoglobin concentration, and the oxygen saturation of arterial blood (SaO$_2$) and mixed venous blood (SO$_2$) were measured with a CO-Oximeter (IL-482; Instrumentation Laboratory). Mixed venous samples were collected through a Swan-Ganz catheter. Alveolar-arterial oxygen difference (A-aDO$_2$) and intrapulmonary shunt ratio (Qs/QT) were calculated using standard formulae.

Pressure-volume curve
The pressure-volume (P-V) curve of the lung was measured by a previously described method (24). Transpulmonary pressure was monitored as a pressure difference between airway pressure and esophageal pressure with a differential pressure transducer (MP-45; Validyne, Northridge, CA). Total lung capacity (TLC) and functional residual capacity (FRC) were determined as the absolute air volume at a transpulmonary pressure of 30 and 5 cm H$_2$O, respectively. Absolute lung volume at FRC was measured by a gas dilution method using Neon gas. Before the measurement of the P-V curve, the animal was mechanically hyperventilated using a respirator (SN-480; Shinnano, Tokyo, Japan) to suppress spontaneous breathing temporarily. The lung volume was increased and then decreased between FRC and TLC in stepwise volume changes of one-sixth of the volume difference from FRC to TLC.

W/D ratio
Extravascular lung water (EVLW) was measured using a modification of a previously described technique (24, 25). Briefly, after all measurements were finished 24 h after LPS or saline injection, three blocks (1 × 1 × 1 cm) were cut from the upper, middle, and lower lobes and homogenized. Each lung homogenate was dried in a 50°C oven until weights were unchanged on 2 consecutive days (7 to 10 days), and dry weight was measured. Other homogenates were centrifuged and hemoglobin was measured in a spectrophotometer (U-1100; Hitachi, Tokyo, Japan) on the cleared supernatant and the whole blood. Then the weight of the blood in the lungs was calculated and EVLW was obtained as a difference between lung water and blood water. The bloodfree W/D ratio was the ratio of EVLW plus the dry weight to the dry weight. An average value of three sites of the lungs was reported.

Lung histology
The left lower lobe was excised and inflated with 10% formaldehyde solution at a pressure of 25 cm H$_2$O for 24 h. After fixation, the lung tissue was sectioned sagittally every 2 to 5 mm, and 10 blocks were sampled randomly for evaluation of histology. These sections were embedded in paraffin and cut to a thickness of 5 μm. They were then stained with hematoxylin-eosin (H-E).

Immunofluorescent staining for iNOS and nitrotyrosine
Paraffin-embedded lung tissue was stained with immunofluorescence. The staining was performed as previously described, with minor modifications (20, 26). The sections were dewaxed, dehydrated, and incubated with 10% normal goat serum to block nonspecific protein adsorption. Then the sections were incubated with polyclonal anti-mouse iNOS Ab (diluted 1/500; Affinity Bioreagents, Golden, CO) or anti-nitrotyrosine polyclonal Ab (diluted 1/100; Upstate Biotechnology, Lake Placid, NY) at 4°C overnight. The labeled Ags were visualized after incubation with FITC-conjugated goat anti-rabbit Ig (diluted 1/10; Kirkegaard and Perry, Gaithersburg, MD) at 4°C overnight. Cross-reactivity of anti-mouse iNOS Ab to canine iNOS was confirmed by Western blotting (data not shown). The tissue was then washed with ice-cold PBS to remove unbound Ab, overlaid with a drop of glycerol/PBS (9:1) mounting medium containing 0.01% phenylenediamine to prevent fluorescence breaching, and covered with a coverslip. In addition, we tested the staining with nonspecific IgG. Lung sections were observed with a fluorescent microscope (model BH2-RFC; Olympus, Tokyo, Japan).

Neutrophil chemotaxis
Neutrophils were isolated from peripheral blood of four dogs that were not used for in vivo studies (27). Neutrophil chemotaxis activity was determined by the leading front method using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD), as described elsewhere (27, 28). To examine the effects of iNOS inhibitors on neutrophil chemotaxis, neutrophils (4 × 10$^5$ cells/ml) were preincubated with AG (10$^{-3}$ M), SMT (10$^{-3}$ M), or vehicle for 30 min at 37°C. Neutrophils were then placed in the upper compartment of the chamber and were allowed to migrate through a nitrocellulose filter of 3 μm pore size (Neuroprobe) toward human IL-8 (10$^{-3}$ M) or PAF (10$^{-5}$ M) in the well of the lower compartment for 25 min at 37°C. Human IL-8 has been reported as chemotactic for dog neutrophils (28). The concentrations of IL-8 and PAF were chosen because they caused maximal chemotaxis in our preliminary dose-response studies and in previous studies (28). Chemotactic response was expressed as distance of migration (μm).

Statistical analysis
All results are expressed as mean ± SE. Statistical differences among group means were determined with one-way or two-way ANOVA with repeated measures, followed by a post hoc comparison using Newman-Keuls test. A p value of <0.05 was considered significant. The STATISTICA statistical software package (StatSoft, Tulsa, OK) was used.

Results
All animals survived for 24 h after LPS injection.

Gas exchange
In the LPS group, PaO$_2$ decreased gradually during the experiment (p < 0.01 by ANOVA) (Fig. 1). At 24 h after LPS injection, PaO$_2$ decreased from 103.6 ± 2.6 mm Hg to 76.4 ± 6.1 mm Hg (p < 0.01). Treatments with AG and SMT prevented the decrease in PaO$_2$ in LPS-injected animals, and no changes in PaO$_2$ were observed in the control, AG, and SMT groups. A-ADo$_2$ widened significantly at 12 and 24 h in the LPS group (from 5.3 ± 3.4 mm Hg at baseline to 21.7 ± 7.1 mm Hg at 24 h, p < 0.05 and p < 0.01, respectively). However, treatments with AG and SMT prevented the increase in A-ADo$_2$ by LPS. No change in A-ADo$_2$ was observed in the control, AG, and SMT groups. In the LPS group, Qs/Qt increased gradually from 12 ± 3% at baseline to 48 ± 8% at the end of the experiment (p < 0.01) (Fig. 1). In contrast, treatments with AG and SMT prevented the increase of Qs/Qt. No changes in these parameters were observed in the control, AG, and SMT groups.
Hemodynamics

In the LPS group, the MAP started to decrease 3 h after LPS injection and recovered at 6 h, but finally declined by 15% at 24 h \( (p, 0.01 \text{ by ANOVA}) \), although the control group showed no change in MAP throughout the experiment (Fig. 2). Treatments with AG and SMT prevented the decrease in MAP in LPS-injected animals. The MPAP and PCWP remained unchanged throughout the experiment in all groups.

P-V curve

In the LPS group, the P-V curve shifted downward 12 and 24 h after LPS injection \( (p < 0.01 \text{ by ANOVA}) \), and TLC decreased to 86.1 \( \pm \) 4.4% of baseline values at 12 h and to 80.1 \( \pm \) 4.7% at 24 h \( (p < 0.01) \). AG and SMT prevented the downward shift of the P-V curve by LPS. Neither the control, nor AG, nor SMT group caused changes in the P-V curve.

W/D ratio of the lung

The W/D ratio, a parameter of pulmonary edema, was increased in the LPS group \( (p < 0.01 \text{ by ANOVA}) \) (Fig. 4). Treatments with AG and SMT prevented the increase in the W/D ratio \( (p < 0.05 \text{ and } p < 0.01, \text{ respectively}) \). Neither the control, nor AG, nor SMT group showed an increase in the W/D ratio.

Histology

At 24 h after LPS injection, there was a marked inflammatory cell infiltration in the interstitium and airspaces of the lung, predominantly composed of neutrophils (Fig. 5B). Interstitial edema and vascular congestion were also observed. Treatments with AG and SMT markedly attenuated the neutrophil infiltration and lung injury (Fig. 5, D and F). No inflammatory change was observed in the control, AG, and SMT groups (Fig. 5, A, C, and E).
iNOS immunoreactivity in the lung

Paraffin-embedded sections from the LPS group exhibited significant immunostaining with the polyclonal Ab to iNOS (Fig. 6). In the alveolar walls and capillaries of the LPS group, immunostaining of iNOS was demonstrated (Fig. 6B). Patchy staining of neutrophils and alveolar macrophages was also observed. In both the LPS + AG group and LPS + SMT groups, however, immunostaining of iNOS was markedly attenuated, and only weak staining of the alveolar walls was observed (Figs. 6D). No significant staining was detected in the control, AG, and SMT groups (Fig. 6, A, C, E, and F). Minimal background staining was observed in all six groups stained with nonspecific IgG (Fig. 6G).

Immunoreactivity of nitrotyrosine

Fluorescent images of the lung specimens labeled with polyclonal Ab to nitrotyrosine are shown in Figure 7. In the lung specimens of the LPS group, immunohistochemical staining of protein nitrotyrosine residues was observed throughout the lung (Fig. 7B). The lung interstitium, alveolar epithelium, alveolar exudates, and capillary wall were strongly stained. Alveolar macrophage and intra-alveolar neutrophils exhibited significantly strong staining. With treatments of AG and SMT, the alveolar septa and alveolar macrophages were only weakly stained (Fig. 7, D and F). Scant staining of the alveolar septa was observed in the lung tissues of the control, AG, and SMT groups (Fig. 7, A, C, and E). Minimal background staining was observed in all six groups that were stained with nonspecific IgG (Fig. 7G).

Neutrophil chemotaxis

Neutrophils showed chemotaxis to IL-8 and PAF (Table I). Pretreatment with either iNOS inhibitor did not affect random migration (data not shown). AG did not affect neutrophil chemotaxis in response to either IL-8 or PAF. SMT slightly attenuated neutrophil chemotaxis in response to IL-8, but it was not statistically significant.

Discussion

We have shown that LPS injection in dogs causes severe hypoxemia and increases shunt ratio. On histology, interstitial edema and marked neutrophil infiltration in the lung were observed. Intense immunofluorescent staining of iNOS and nitrotyrosine, a specific marker for the presence of peroxynitrite, was observed in capillary wall and alveolar wall. Treatments with AG and SMT almost completely attenuated these physio-logic and histologic changes and the production of peroxynitrite. The localization of iNOS and peroxynitrite suggests that NO may be generated by the induction of iNOS, and peroxynitrite may be responsible in part for the microvascular damage in acute lung injury induced by LPS.

In sepsis, toxic products activate systemic host defenses including neutrophils, macrophages, monocytes, endothelial cells, and the complement system (3). The activated cells produce toxic host mediators such as cytokines, kinins, eicosanoids, NO, and superoxides (2, 3, 29). Neutrophils have been implicated specifically in the pathogenesis of most cases of human sepsis (1, 30, 31). Consistent with these reports, our model demonstrates that neutrophils accumulated markedly in the interstitium and airspaces of the lung. In our study, treatment with iNOS inhibitors attenuated neutrophil sequestration in the lung. In several animal models of inflammation, a selective inhibitor of iNOS, N-iminoethyl-l-lysine, suppressed the infiltration of inflammatory cells (32, 33). However, the mechanisms by which iNOS inhibitors attenuate infiltration of inflammatory cells are unclear. N\textsuperscript{\textgreek{g}}-monomethyl-l-arginine (l-NMMA), an inhibitor of both isoforms of NOS, inhibits chemotaxis in neutrophils (34). It is also reported that nonspecific NOS inhibitors
attenuated chemotaxis of peripheral blood monocyte, but an iNOS inhibitor did not (35). In the present study, both AG and SMT did not affect neutrophil chemotaxis, suggesting that iNOS inhibitors have no direct action on unstimulated neutrophil chemotaxis. Exogenous NO elicits chemotaxis of neutrophils (36). Therefore, attenuation of neutrophil infiltration by iNOS inhibitors may be caused by inhibition of NO production. Thus, treatment with NOS inhibitors attenuates neutrophil accumulation into the lung in LPS-injected animals.

Large amounts of NO produced by iNOS induction interact with oxygen free radicals derived from neutrophils and macrophages to form peroxynitrite at diffusion-limited reaction (11, 12). Peroxynitrite causes extensive tyrosine nitration and, as a result, forms nitrotyrosine (29). Thus, peroxynitrite is a potent and versatile oxidant that can attack many types of biologic molecules and has strong oxidizing and cytotoxic properties (29, 37). Excess production of NO is reported to contribute to the lung injury induced by immune complexes (13) or paraquat (38), and peroxynitrite is reported to be an important tissue-damaging product (13, 38). Recently, it has been reported that, in lung ischemia-reperfusion, nitrotyrosine is increased, and peroxynitrite is thought to be a causative factor of oxidative lung injury (39).
Several studies have shown that nitrotyrosine levels in the lung increased in patients with ARDS and in lung injury models (19, 20, 40), although these studies did not provide direct evidence that peroxynitrite contributed to lung injury. In our study, iNOS inhibitor treatment in LPS-injected animals prevented the development of pulmonary edema. In immunohistochemistry studies of the LPS group, staining of iNOS was markedly increased, and immunofluorescent staining of nitrotyrosine residue was strongly observed in the interstitium, alveolar epithelium, alveolar exudate, and capillary wall. This localization of iNOS and nitrotyrosine indicates that LPS causes the induction of iNOS and produces excess amounts of NO, resulting in the formation of peroxynitrite in the vascular endothelium and/or blood-borne elements. Thus, iNOS inhibitors prevent the generation of peroxynitrite and the development of pulmonary edema. It has been suggested previously that peroxynitrite may be involved in vascular injury in the acute inflammatory response (13, 38). In our study, therefore, peroxynitrite may have injured the endothelium of the microvasculature, causing increased permeability and edema.

The inducible isofrom of NOS is responsible for the excess production of NO in sepsis of animals, leading to the development of shock (16, 17, 41). In our study, MPAP did not change, although MAP was slightly decreased by LPS. This may indicate that lung injury of our dog model was mild. AG and SMT prevented the decrease in MAP by LPS. Both AG and SMT are selective inhibitors of iNOS and much less effective inhibitors of cNOS (21, 22).

Peroxynitrite contributed to lung injury. In our study, iNOS inhibitors, prevent pulmonary edema and histologic changes induced by LPS. Inhibitors of iNOS, such as AG and SMT, may have potential in the treatment of sepsis-induced ARDS.

### Table I. Effects of iNOS inhibitors on neutrophil chemotaxis

<table>
<thead>
<tr>
<th>Chemottractant</th>
<th>Control</th>
<th>AG (10⁻⁴ M)</th>
<th>SMT (10⁻⁴ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAF (10⁻⁶ M)</td>
<td>68 ± 8</td>
<td>67 ± 10</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>IL-8 (10⁻⁸ M)</td>
<td>59 ± 12</td>
<td>62 ± 12</td>
<td>106 ± 4</td>
</tr>
</tbody>
</table>

* Data are given as mean ± SE of four dogs.
* Migration (μm).
* Migration of vehicle-treated neutrophils in response to IL-8 or PAF.

* Percent of control migration.

### References


