Inhaled nitric oxide attenuates acute lung injury via inhibition of nuclear factor-κB and inflammation

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Kang, Jihee Lee, Wann Park, In Soon Pack, Hui Su Lee, Mi Jung Kim, Chae-Man Lim, and Younsuck Koh. Inhaled nitric oxide attenuates acute lung injury via inhibition of nuclear factor-κB and inflammation. J Appl Physiol 92: 795–801, 2002; 10.1152/japplphysiol.00202.2001.—The effect of inhaled nitric oxide (NO) on inflammatory process in acute lung injury (ALI) is unclear. The aims of this study were to 1) examine whether inhaled NO affects the biochemical lung injury parameters and cellular inflammatory responses and 2) determine the effect of inhaled NO on the activation of nuclear factor-κB (NF-κB) in lipopolysaccharide (LPS)-induced ALI. Compared with saline controls, rabbits treated intravenously with LPS showed increases in total protein and lactate dehydrogenase in the bronchoalveolar lavage (BAL) fluid, indicating ALI. LPS-treated animals with NO inhalation (LPS-NO) showed significant decreases in these parameters. Neutrophil numbers in the BAL fluid, the activity of reactive oxygen species in BAL cells, and the levels of interleukin (IL)-1α and IL-8 in alveolar macrophages were increased in LPS-treated animals. In contrast, neutrophil numbers and these cellular activities were substantially decreased in LPS-NO animals, compared with LPS-treated animals. NF-κB activation in alveolar macrophages from LPS-treated animals was also markedly increased, whereas this activity was effectively blocked in LPS-NO animals. These results suggest that inhaled NO attenuates LPS-induced ALI and pulmonary inflammation. This attenuation may be associated with the inhibition of NF-κB activation.

lipopolysaccharide; cytokines; oxidants

RECENT EVIDENCE SUGGESTS THAT nitric oxide (NO) is an important endogenous regulatory molecule, implicated in both proinflammatory and anti-inflammatory processes in the lung (24, 25). In experimental models, NO decreased lung parenchymal damage, alveolar macrophage and neutrophil function, and the transendothelial migration of activated neutrophils during acute lung injury (ALI) (4, 20). In contrast, NO and nitrite interacted with neutrophil myeloperoxidase to stimulate oxidative reactions during inflammation (13). Moreover, NO pretreatment potentiated ALI in an isolated rabbit lung model of oleic acid-induced ALI (25). These findings suggest that NO can exert detrimental as well as beneficial effects. The mechanisms involved in the variation in NO effects leading to ALI are not clear. These disparate effects could be related to differences in the timing of administration and/or in the concentration of inhaled NO species or to model differences. NO administration before the establishment of lung injury has been suggested to protect against ALI by causing a decrease in the availability of oxygen radicals, as well as by modulation of the interaction between the vascular endothelium and inflammatory cells (4, 7). Recent data have indicated that reducing the concentration of NO might attenuate its potentially deleterious effects. Inhaled NO at 10–30 parts/million (ppm) attenuates lung injury and does not result in any toxic effects in human and animal models. In patients with acute respiratory distress syndrome (ARDS), the beneficial effect on oxygenation occurred at doses of inhaled NO from 1.25 to 40 ppm in patients with ARDS (12).

Neutrophil infiltration in the lung, followed by the extracellular release of reactive oxygen species (ROS) and proteolytic enzymes, proinflammatory cytokine activity in the lung, and endothelial cell expression of intracellular adhesion molecule-1 have been considered as primary events in the pathogenesis of ALI (4). However, the molecular and cellular mechanisms underlying the effects of inhaled NO on the pathogenesis of ALI in vivo still remain to be clarified.

One of the mechanisms by which NO may modulate lung inflammation is through its interaction with the transcription factor nuclear factor (NF)-κB. NF-κB is a multiprotein complex that regulates a variety of diverse genes, including interleukin (IL)-1, -2, -6, and -8, tumor necrosis factor (TNF)-α, various adhesion mole-

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these hypotheses, this investigation aimed to through a NF-κB-dependent mechanism. To address /H9260 /LPS-induced ALI in vivo, and NO involves the inhibition of NF-κB activation in vivo. We hypothesize that 1) inhaled NO may attenuate LPS-induced ALI in vivo, and 2) this may occur through a NF-κB-dependent mechanism. To address these hypotheses, this investigation aimed to 1) examine whether inhalation of NO (10 ppm), given 10 min after LPS treatment, affects the biochemical lung injury parameters and cellular inflammatory responses, and 2) determine the effect of inhaled NO on the activation of NF-κB in LPS-induced ALI. In this study, we evaluated the degree of ALI by measuring protein levels and lactate dehydrogenase (LDH) activity in the bronchoalveolar lavage (BAL) fluid. The cellular inflammatory response in the lung was assessed by measuring neutrophil numbers in the BAL fluid, the activity of ROS in BAL cells, and the levels of proinflammatory cytokines, such as IL-1β and IL-8, in alveolar macrophages.

METHODS

Experimental animals. New Zealand White rabbits (3.0 ± 0.5 kg) were used for this investigation. Rabbits were fed a controlled diet (Sam-yang Feed, Seoul, Korea) and were housed in individual cages before the experiment. The Animal Care Committee of the Asan Life Science Institute approved the experimental protocol. The rabbits were cared for and handled according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, Bethesda, MD 20892]. The rabbits were placed supine under a radiant warmer to keep rectal temperatures between 38 and 39°C. Ketamine (25 mg/kg; Boehringer Ingelheim, St. Joseph, MO) was administered intramuscularly in the thigh, and then a marginal ear vein was cannulated with a 24-gauge angiocath, which was later used as the route for intravenous anesthesia. The rabbits were tracheostomized, with additional local anesthesia at the neck, induced with 2% lidocaine. After tracheostomy, a 3.5-mm cuffless endotracheal tube was inserted 3–4 cm deep into the trachea and tied firmly to prevent gas or liquid leakage. The carotid artery was cannulated with a 22-gauge angiocath and connected to an Escort II pressure monitor (Medical Data Electronics, Arleta, CA) to record the pulse rate and the arterial pressure, referenced to the midthoracic level. Anesthesia was induced with intravenous thiopental sodium (20 mg/kg), given in two divided doses, and maintained at 3 mg·kg⁻¹·h⁻¹, with intermittent muscle paralysis using intravenous vecuronium (0.1 mg/kg, every 30 min; Haver, New York, NY). The rabbits were given 5% dextrose and normal saline (50:50 vol/vol), intravenously at 7.5 ml·kg⁻¹·h⁻¹ by using an infusion pump. The rabbits were ventilated until the end of the experiment. The Servo 300 mechanical ventilator (Siemens-Elema, Solna, Sweden) was initially set at a tidal volume of 18 ml/kg [taking the compression volume of the circuit into consideration (2.2 ml/cmH₂O), the actual inspired volume was ~9 ml/kg], at a frequency of 24/minute, an inspired O₂ fraction of 1.0, a positive end-expiratory pressure of 0 cmH₂O, and an inspiration-toexpiration ratio of 1:1. With the administration of LPS or saline, the positive end-expiratory pressure was set at 2 cmH₂O, which was maintained to the end of the study. Other ventilation parameters were not changed during the period of study. Normal saline was given as necessary to keep the blood pressure within normal ranges in all rabbits. However, there were no actual decreases in arterial pressure throughout the study period.

Experimental design. Rabbits were divided into four groups: 1) a saline (control) group of rabbits, infused with 20 ml saline, without NO inhalation; 2) a saline-NO group, infused with 20 ml saline, with 10 ppm NO inhalation; 3) a LPS group, infused with 5 mg/kg body wt of LPS (Escherichia coli lipopolysaccharide, 455B5, Sigma Chemical, St. Louis, MO) in 20 ml saline, without NO inhalation; and 4) an LPS-NO group, infused with 5 mg/kg body wt of LPS in 20 ml saline, with 10 ppm NO inhalation. LPS was administered after anesthesia with xylazine and ketamine. Control rabbits received identical anesthesia and surgery. Recent evidence indicates that a sufficient inflammatory response can be induced in the lung 6 h after LPS treatment, demonstrable as neutrophil accumulation in the lung with neutrophil oxidant burst (4) and as proinflammatory cytokine activity in the lung (31). Therefore, we chose to examine the rabbits at a time point 6 h after LPS treatment. In each group of animals, we performed the following: 1) total cell count and a differential cell count in the BAL fluid, 2) measurement of total protein and LDH activity in the BAL fluid, 3) measurement of chemiluminescence generation in the BAL cells, 4) measurement of IL-1β and IL-8 in alveolar macrophages, and 5) an assay for NF-κB in alveolar macrophages. Phorbol 12-myristate 13-acetate (PMA) is a well-known stimulant of inflammatory cells, such as neutrophils and alveolar macrophages (4, 20), whereas zymosan selectively stimulates macrophages (21). Therefore, PMA and zymosan were used to stimulate the cells and to amplify their activities, in an attempt to determine the effects of inhaled NO on the activities of ROS, proinflammatory cytokines, and NF-κB in the cells.

NO inhalation. After a 10-min intravenous infusion of saline or LPS, the animals were exposed to 10 ppm NO for 6 h. Because the beneficial effect on oxygenation occurred at doses of inhaled NO from 1.25 to 40 ppm in patients with ARDS (12), we designed the present study to investigate the beneficial effects of inhaled NO at 10 ppm, given 10 min after LPS infusion. NO was delivered from a tank of nitrogen with a flow rate of 400 or 800 ppm, to the high-pressure air port of a Servo Ventilator 300, for 6 h until the end of the experiment. NO was blended with medical air, using a high-pressure blender, before delivery to the ventilator. Inhaled NO and NO₂ concentrations were continuously monitored by electrochemical analysis (TMX-100, Taiyo Toyo Sanso, Osaka, Japan) at the distal portion of inspiratory tube of the mechanical ventilator. The inspired oxygen concentration was also monitored continuously.

Isolation of BAL cells and cell counts. The rabbits were killed 6 h after saline or LPS intravenous infusion. Saline (30 ml per time) was injected slowly into the endotracheal tube and then withdrawn, until the recovered volume reached 150 ml. Recovered BAL fluid was centrifuged (500 g) for 5 min, and the BAL cells were washed and resuspended in HEPES-
buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5.5 mM dextrose, and 1.0 mM CaCl2, pH 7.4). Cell counts and differentials were determined by using an electronic Coulter counter with a cell-sizing analyzer (model ZBI with a channelizer 256, Coulter Electronics, Bedfordshire, UK), as described by Lane and Mehta (18). Red blood cells, lymphocytes, neutrophils, and alveolar macrophages were distinguished by their characteristic cell volumes (6). The recovered cells were 98% viable, as determined by trypan blue dye exclusion.

Measurement of total protein and LDH activity. To assess the permeability of the bronchoalveolar-capillary barrier, total protein was measured according to the method of Hartree (15), using bovine serum albumin as the standard. The LDH activity and total protein were measured in the first aliquots of the acellular BAL fluid. The activity of LDH, a cytosolic enzyme used as a marker for cytotoxicity, was measured with a LDH determination kit (Roche Molecular Biochemicals, Mannheim, Germany). One hundred microliters of BAL supernatant were added to a 100-μl reaction mixture and incubated for 30 min. The absorbance of the samples at 490 nm was measured with an ELISA reader. LDH activity was expressed as units per liter by using an LDH standard.

Measurement of chemiluminescence generation in BAL cells. The activity of BAL cells in producing ROS was determined by measuring cellular chemiluminescence with a luminometer (model LB9505AT, Berthold Instruments, Wildbad, Germany), as previously described (29). Briefly, BAL cells were resuspended in HEPES-buffered medium, at a final concentration of 10⁶ alveolar macrophages/ml. Chemiluminescence was monitored continuously at 37°C for 10 min, in cells at rest and stimulated with either PMA (3 μM) or unopsonized zymosan (2 mg/ml), in the presence of luminol (8 μg/ml). The integral of counts/min vs. time was used to compare the total chemoluminescence between samples.

Measurement of IL-1β and IL-8 in cultured alveolar macrophages. Lavage cells were resuspended in RPMI-1640 medium (Mediatech, Washington, DC), containing 2 mM glutamine, 100 U/ml mycostatin, and 10% fetal bovine serum (28). Aliquots of 1 ml, containing 10⁶ alveolar macrophages, were added to 24-well plates (Costar, Cambridge, MA), and incubated for 30 min at 37°C, in a humidified atmosphere of 5% CO₂ for 2 h. The nonadherent cells were then removed by vigorous washing with two 1-ml aliquots of DMEM. The adherent cells, at rest and stimulated with either PMA (3 μM) or unopsonized zymosan (2 mg/ml), were further incubated in 5 ml DMEM for 1 h. At the end of the incubation, the cells were harvested and resuspended in hypotonic buffer A (100 mM HEPES, pH 7.9, 10 mM KCl, 0.1 M EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice, then vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000 g for 30 s and were resuspended in buffer C (200 mM HEPES, pH 7.9, 20% glycerol, 0.42 M NaCl, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 10,000 g for 2 min, and stored at −70°C.

Electrophoretic mobility shift assay. Binding reaction mixtures (10 μl), containing 5 μg (4 μl) nuclear extract protein, 2 μg poly(dI-dC)-poly(dI-dC) (Sigma Chemical, St. Louis, MO), and 40,000 counts/min 32P-labeled probe in binding buffer (4 mM HEPES, pH 7.9, 1 mM MgCl₂, 0.5 mM dithiothreitol, 2% glycerol, and 20 mM NaCl), were incubated for 30 min at room temperature. The protein-DNA complexes were separated on 5% nondenaturing polyacrylamide gels in 1× Tris-borate-EDTA buffer, and autoradiographed overnight.

The oligonucleotide used as a probe for the electrophoretic mobility shift assay was a double-stranded DNA fragment, containing the NF-κB consensus sequence (5′-CCGTGCTC-CCGGAAATTCTCAGGCCC-3′, binding site underlined), labeled with [α-32P]dATP (Amersham, Buckinghamshire, UK), using DNA polymerase Klenow fragment (Life Technologies, Gaithersburg, MD). Cold competition was performed by adding 100 ng unlabeled double-stranded probe to the reaction mixture.

Statistical analysis. Values are expressed as the median, together with the range. Kruskal-Wallis analysis of variance, with a Mann-Whitney nonparametric test, was employed to detect the differences among the groups, where appropriate. Statistical significance was accepted at P < 0.05.

RESULTS

Protein levels and LDH activity in BAL fluid. BAL protein content and LDH activity (Table 1) were significantly increased in LPS-treated animals compared

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Protein, μg/ml</th>
<th>LDH, U/ml</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td>137.8 (46.2–264.2)†</td>
<td>3.6 (3.1–7.2)</td>
</tr>
<tr>
<td>Saline-NO</td>
<td>137.0 (56.4–183.5)</td>
<td>4.6 (3.6–6.2)</td>
</tr>
<tr>
<td>LPS</td>
<td>525.7 (365.9–663.8)§</td>
<td>82.9 (14.7–141.5)§</td>
</tr>
<tr>
<td>LPS-NO</td>
<td>249.7 (173.3–258.3)¶</td>
<td>7.1 (1.8–29.1)¶</td>
</tr>
</tbody>
</table>

Values are medians with range in parentheses; n = 5 rabbits in each group. Groups represent rabbits treated as follows: saline, intravenous saline; saline-NO, intravenous saline and nitric oxide inhibition; LPS, intravenous lipopolysaccharide; LPS-NO, intravenous LPS and NO inhibition. †Significant difference compared with saline, P < 0.05. §Significant difference compared with LPS group, P < 0.05.
with saline controls, indicating that treatment of the rabbits with LPS induced ALI. In contrast, there were significant decreases in these parameters in LPS-NO animals ($P < 0.05$ vs. LPS animals). There were no significant changes in saline-NO animals in these parameters, compared with saline controls.

**Neutrophil transmigration.** Differential analysis of BAL cells showed that neutrophils accounted for 16.8% (11.7–25.9%) of the total lung lavage cells [54 (40–58) x 10^6] in LPS animals, indicating significant transmigration into the alveolar spaces (Fig. 1; $P < 0.05$ vs. saline controls). The percentage of BAL neutrophils significantly decreased to 7.8% (4.1–9.4%) of the total lavage cells [25 (14–26) x 10^6] in LPS-NO animals ($P < 0.05$ vs. LPS animals).

**ROS production in BAL cells.** Table 2 shows the results of chemiluminescence assays performed to determine the ROS-secreting activity of cells obtained from the BAL fluid. We measured ROS activity at rest and after the stimulation of BAL cells (1.5 x 10^6 alveolar macrophages/0.75 ml HEPES) with either PMA (3 µM) or zymosan (2 mg/ml). Chemiluminescence was significantly increased in the resting, and PMA- and zymosan-stimulated cells of LPS-treated animals, compared with saline controls ($P < 0.05$). The ROS response of cells to the stimulants among LPS animals was greater than for the saline controls (PMA vs. resting, 1.9 vs. 4.5; zymosan vs. resting, 13.4 vs. 24.8). However, the ROS activity in BAL cells of LPS-NO animals, at rest and when stimulated with PMA or zymosan, was significantly decreased compared with the values in LPS-treated animals ($P < 0.05$).

**IL-1β and IL-8 production in alveolar macrophages.** IL-1β and IL-8 were chosen in our experiments as representative proinflammatory cytokines because of their prominent roles in the acute inflammatory response and because the expression of their genes is NF-κB-dependent (2). IL-1β and IL-8 concentrations in the supernatants obtained from 24-h cultured alveolar macrophages are given in Table 3 and Table 4, respectively. There was a significant increase ($P < 0.05$) in the ability of alveolar macrophages to produce IL-1β at rest in LPS-treated animals compared with the values in saline controls. Furthermore, IL-1β production by cells stimulated with either PMA or zymosan was also significantly increased in LPS-treated animals ($P < 0.05$ vs. saline controls). Decreases in IL-1β production by cells at rest and when stimulated with either PMA ($P < 0.05$ vs. LPS animals) or zymosan were observed in LPS-NO animals. IL-1β production in alveolar macrophages showed no significant changes in saline-NO animals compared with saline controls.

As with IL-1β production, IL-8 production by alveolar macrophages at rest and when stimulated with either PMA or zymosan was significantly increased in LPS-treated animals ($P < 0.05$ vs. saline controls). In contrast, there were complete decreases in LPS-NO animals compared with the LPS-treated animals ($P < 0.05$). IL-8 production by alveolar macrophages did not change significantly in the saline-NO animals compared with saline controls.

Table 3. Production of IL-1β in alveolar macrophages at rest and stimulated with either PMA or zymosan

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Saline</th>
<th>Saline-NO</th>
<th>LPS</th>
<th>LPS-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>3.4 (3.4–4.2)</td>
<td>3.6 (2.8–4.7)</td>
<td>9.6 (5.2–12.9)*</td>
<td>5.0 (3.6–6.0)†</td>
</tr>
<tr>
<td>PMA</td>
<td>6.9 (4.1–11.9)</td>
<td>9.0 (4.8–9.8)</td>
<td>33.7 (19.7–79.5)*</td>
<td>13.6 (8.0–14.7)†</td>
</tr>
<tr>
<td>Zymosan</td>
<td>55.6 (23.2–77.5)</td>
<td>49.7 (21.6–75.0)</td>
<td>235.0 (162.9–297.0)*</td>
<td>421 (25.3–93.1)†</td>
</tr>
</tbody>
</table>

Values are medians with range in parentheses expressed in pg/ml; $n = 4$ rabbits in each group except LPS ($n = 6$). IL-1β, interleukin-1β. *$P < 0.05$ vs. saline group. †$P < 0.05$ vs. LPS group.

Table 2. Chemiluminescence generation in bronchoalveolar lavage cells at rest and stimulated with either PMA or zymosan

<table>
<thead>
<tr>
<th>Stimulant</th>
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<th>Saline-NO</th>
<th>LPS</th>
<th>LPS-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>3.4 (3.4–4.2)</td>
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<td>421 (25.3–93.1)†</td>
</tr>
</tbody>
</table>

Values are medians with range in parentheses expressed in counts/min; $n = 5$ rabbits in each group. PMA, phorbol 12-myristate 13-acetate. *$P < 0.05$ vs. saline group. †$P < 0.05$ vs. LPS group.
Table 4. Production of IL-8 in alveolar macrophages at rest and stimulated with either PMA or zymosan

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Saline</th>
<th>Saline-NO</th>
<th>LPS</th>
<th>LPS-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>108(90–111)</td>
<td>99(77–111)</td>
<td>131(121–153)&lt;sup&gt;±&lt;/sup&gt;</td>
<td>90(0–133)</td>
</tr>
<tr>
<td>PMA</td>
<td>111(108–129)</td>
<td>99(77–116)</td>
<td>148(130–166)&lt;sup&gt;±&lt;/sup&gt;</td>
<td>53(0–132)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zymosan</td>
<td>115(109–120)</td>
<td>92(53–120)</td>
<td>132(121–147)&lt;sup&gt;±&lt;/sup&gt;</td>
<td>94(0–98)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are medians with range in parentheses; n = 4 rabbits in each group except LPS (n = 6). IL-8, interleukin-8. *P < 0.05 between LPS and saline group. †P < 0.05 between LPS-NO and saline group.

NF-κB activation in alveolar macrophages. The DNA-binding activity of NF-κB in alveolar macrophages from LPS-treated animals was enhanced three-fold at rest (Fig. 2, lane 7), compared with saline controls (Fig. 2, lane 1). Furthermore, NF-κB activation was also enhanced in cells stimulated with PMA (lane 8 vs. lane 2) or zymosan (lane 9 vs. lane 3). To ensure the band on the autoradiogram was specific for NF-κB binding, we also tested samples obtained from LPS-treated animals in the presence of cold competitor. The addition of cold competitor eliminated the specific bands (lanes 13–15). LPS-NO animals showed complete inhibition of NF-κB activation, at rest and with stimulation (lanes 10–12). There were no marked differences in NF-κB activation in alveolar macrophages, when at rest and stimulated with PMA or zymosan, between saline-NO animals (lanes 4–6) and saline controls.

DISCUSSION

The present study was undertaken to characterize the cellular inflammatory response in ALI after intravenous infusion of LPS into rabbits and to determine the activation of NF-κB in alveolar macrophages in this process. Furthermore, the effects of inhaled NO on the LPS-induced cellular inflammatory responses and NF-κB activation were assessed. The results of the present study indicate that LPS causes significant increases in 1) the levels of total protein and LDH in the BAL fluid; 2) neutrophil transmigration, ROS activity in BAL cells, and proinflammatory cytokines (IL-1β and IL-8) in alveolar macrophages at rest and stimulated with either PMA or zymosan; and 3) DNA-binding activity of NF-κB in alveolar macrophages at rest or stimulated with either PMA or zymosan. In addition, inhaled NO (10 ppm) given 10 min after LPS treatment attenuates these increases in inflammatory response and NF-κB activation, which are associated with parameters representing lung injury, such as the levels of total protein and LDH in the BAL fluid.

The timing of administration and the concentration of inhaled NO remain issues of importance. Beneficial effects of inhaled NO have been reported when NO is administered before ALI. Lung injury is attenuated and alveolar-capillary membrane integrity is preserved with the administration of NO right at the beginning of reperfusion (22), 20 min before the start of *Pseudomonas aeruginosa* infusion (4), simultaneously with 100% O₂ (14), or just after LPS instillation (16). Furthermore, delayed NO administration given 15 min after the beginning of reperfusion (22), or at 30 or 60 min after the start of *P. aeruginosa* infusion (5), also reduced lung damage. Conversely, inhaled NO administered posttreatment in the presence of an established injury is ineffective in reversing ischemia-reperfusion-induced microvascular leakage (7) or LPS-induced lung injury (17). The concentration of inhaled NO may also explain the discrepancies in the effects of NO. It is difficult with heterogeneity and variable response of species or experimental models to establish an optimal dose for inhaled NO to minimize the risk of potential toxicity. However, our results suggest the possibility that the beneficial effects of inhaled NO may be related to the timing of its administration, before the estab-
lishment of lung injury, and reducing the concentration of NO.

Similar inhibitory effects of inhaled NO on neutrophil transmigration and ROS activity have been reported in neutrophils from the BAL fluid of ARDS patients (8) and in an LPS-induced ALI animal model (4).

Recently, it has been proposed that not only neutrophil activation but also macrophage function during LPS-induced ALI contributes to lung inflammation and parenchymal damage during this process (20). In the present study, we examined the effects of inhaled NO on the activity of ROS in BAL cells, including $1.5 \times 10^6$ alveolar macrophages. However, the chemiluminescence in response to zymosan is considered to arise only in alveolar macrophages, because unopsonized zymosan selectively stimulates macrophages. Therefore, our data support the hypothesis that macrophages contribute to lung inflammation and parenchymal damage during this process. These data also suggest that the beneficial effects of NO during ALI may be partly attributable to decreased ROS activity in alveolar macrophages.

Consistent with our in vivo results that indicate a decrease in LPS-induced proinflammatory cytokine production with inhaled NO, Meldrum et al. (20) have reported that L-arginine decreases the production of alveolar macrophage proinflammatory cytokines (IL-1β and TNF-α) during LPS-induced ALI, by an NO synthase-dependent mechanism. Furthermore, Walley et al. (31) reported that $N^\omega$-nitro-L-arginine methyl ester, a NO synthase inhibitor, increases TNF-α and IL-6 protein and mRNA expression in lung homogenate samples obtained from mice after intratracheal injection with endotoxin. These authors also reported that, in addition to the in vivo model, exogenous or endogenous NO downregulates inflammatory cytokine production in lung macrophages in vitro.

The mechanism by which NO decreases cytokine production has not been elucidated. Recent experimental evidence suggests that NO may inhibit proinflammatory cytokine production through its interaction with NF-κB, which is activated by diverse inflammatory stimuli. Indeed, NF-κB activation has been reported in lung tissue during LPS-induced alveolitis, in bronchoalveolar cells obtained from silica-induced lung inflammation in animal models (3), and in alveolar macrophages from patients with ARDS (26). In the present study, we have demonstrated increases in the DNA-binding activity of NF-κB in alveolar macrophages at rest and when stimulated with either PMA or zymosan. In contrast, inhaled NO markedly inhibited this NF-κB activation, suggesting NO may prevent the production of proinflammatory cytokines by alveolar macrophages, by inhibiting NF-κB. This is the first demonstration of an inhibitory role for NO in the activities of NF-κB and the proinflammatory cytokines in alveolar macrophages, in a model of LPS-induced ALI. Furthermore, these results suggest a correlation between the inhibition of NF-κB activation and the suppression of the inflammatory response. This conclusion is supported by the results of in vitro models. DNA-binding of NF-κB in LPS-stimulated mouse peritoneal macrophages was further increased by $N^\omega$-nitro-L-arginine methyl ester and was decreased by nitroprusside (30). Furthermore, Thomassen et al. (28) reported that NO downregulates inflammatory cytokine production by human alveolar macrophages in vitro and that LPS-induced NF-κB activation is decreased by NO.

The molecular mechanisms by which NO downregulates LPS-induced NF-κB activation have not been resolved. ROS are involved in the signal transduction associated with NF-κB activation. In our study, inhaled NO decreased the activities of ROS, as well as of NF-κB. This result suggests that inhaled NO may affect a still undetermined membrane component required for the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which results in the oxidative burst in macrophages or neutrophils (9) and simultaneously blocks the signal pathways that are initiated by ROS. NO may also inhibit NF-κB directly, through S-nitrosylation of the p50 subunit (11, 23). Peng et al. (23) reported that NO inhibits LPS-induced NF-κB activation in a murine macrophage cell line, via its effect on the transcription and stability of the NF-κB inhibitory protein IκBα. This effect is independent of guanylate cyclase. This independence of guanylate cyclase has also been demonstrated in the inhibition of NF-κB activation by NO (31) and in the reduction in endothelial cell expression of vascular cell adhesion molecule 1 by NO (10). Alternatively, NO may interact directly in the intracellular signaling pathways leading to NF-κB activation, even though NO target molecules have not been identified.

In this study, the degree of increase in IL-1β production after LPS treatment was greater than that of IL-8 (5.9-, 7.5-, and 12.6-fold vs. 1.3-, 1.3-, and 1.2-fold for resting and PMA- and zymosan-stimulated cells, respectively). It is uncertain whether NF-κB has a significant role in the differential production of NF-κB-dependent cytokines, although NF-κB appears to play a critical role in regulation of the gene expression of these cytokines in response to inflammatory stimuli. The relative amounts of IL-1β and IL-8 produced by LPS treatment are probably functions of the interactions between NF-κB and other transcription factors, as well as factors independent of NF-κB.

The present data suggest that the beneficial effects of inhaled NO on ALI may be partly due to inhibition of the inflammatory cellular activity of ROS, proinflammatory cytokines, and NF-κB activity. ROS (30) and proinflammatory cytokines (1) have been reported to strongly induce the DNA-binding activity of NF-κB. Therefore, inhaled NO may block NF-κB-mediated uncontrolled inflammation and lung injury in a positive-feedback fashion. Our findings suggest that NO inhalation might be considered as a treatment in the early stages of ARDS to attenuate the inflammatory cascade closely associated with NF-κB activation.

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REFERENCES


