Background: Previous studies have shown that lipopolysaccharide-induced inflammation in the lung results in tyrosine nitration. The objective of this study was to evaluate the contribution of myeloperoxidase and peroxynitrite pathway to the tyrosine nitration in lipopolysaccharide-administered lungs of rats that were otherwise untreated or leukocyte-depleted by cyclophosphamide or received inhaled nitric oxide (NO).

Methods: The authors analyzed the immunoreactivity of inducible nitric oxide synthase (iNOS), nitrotyrosine (a product of the myeloperoxidase or peroxynitrite pathway), and chlorotyrosine (a byproduct of the myeloperoxidase pathway) by use of specific antibodies. The number of neutrophils in bronchoalveolar lavage fluid (BALF) and levels of myeloperoxidase activity in lung homogenates were also measured.

Results: Lipopolysaccharide enhanced the immunoreactivity of iNOS, nitrotyrosine, and chlorotyrosine in alveolar macrophages, alveolar neutrophils, and neutrophils. Leukocyte depletion by cyclophosphamide and inhibition of leukocyte accumulation in the lungs by NO inhalation did not eliminate the increase in iNOS immunoreactivity in alveolar macrophages after lipopolysaccharide treatment, but nitrotyrosine and chlorotyrosine were not produced in these cells. Tyrosine nitration in response to lipopolysaccharide was associated with increases in neutrophil count in BALF and in myeloperoxidase activity in lung homogenates, whereas NO inhalation suppressed the neutrophil count in BALF and reduced tyrosine nitration and chlorination.

Conclusions: These findings suggest that myeloperoxidase pathway has a role in tyrosine nitration in the lungs of lipopolysaccharide-treated rats, and that NO inhalation during early phase of inflammation does not increase but rather decreases tyrosine nitration and chlorination, possibly by reducing neutrophil sequestration.

NITROTYROSINE is a product of the peroxynitrite pathway (fig. 1) that has been used as a probe to detect nitric oxide (NO)-mediated oxidative reactions in vivo and has been found in the lungs of patients with the acute respiratory distress syndrome (ARDS). If nitrotyrosine is produced via the peroxynitrite pathway in the injured lung, inhaled NO will increase the reaction between superoxide and NO to enhance tissue nitration and exacerbate injury. In a previous study of lung injury induced by lipopolysaccharide, however, we found otherwise: inhaled NO (20 ppm) decreased leukocyte accumulation in the lungs and inhibited tyrosine nitration in response to lipopolysaccharide instillation.

Recently, it has been indicated that leukocyte myeloperoxidase may contribute to nitrotyrosine formation during the accumulation and activation of these cells. Neutrophil myeloperoxidase can use nitrite and hydrogen peroxide or hypochlorous acid as substrates to catalyze tyrosine nitration in proteins; this nitration via a myeloperoxidase pathway is enhanced by the addition of NO2− or by flux of NO. During this reaction, a part of tyrosine is chlorinated to become chlorotyrosine as a byproduct of the myeloperoxidase pathway (fig. 1). It has been shown that inhaled NO increases the formation of nitrotyrosine and chlorotyrosine in bronchoalveolar lavage fluid (BALF) of patients with ARDS.

Our aim of this study was to evaluate the contribution of myeloperoxidase and peroxynitrite pathway to the tyrosine nitration in lipopolysaccharide-administered lungs of rats that were otherwise untreated or leukocyte-depleted by cyclophosphamide or received inhaled NO. We analyzed the immunoreactivity of inducible nitric oxide synthase (iNOS), nitrotyrosine, and chlorotyrosine by use of specific antibodies, and the number of neutrophils in BALF and levels of myeloperoxidase activity in lung homogenates were also measured.

Materials and Methods

General Protocol

Animal care was in accordance with the guidelines of the Animal Care Committee of Kitasato University, and the conduct of these studies conformed to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Science. Male (200–250 g) Sprague-Dawley rats (Clea Japan, Tokyo, Japan) were assigned to one of four groups: untreated controls (control group), lipopolysaccharide-treated (LPS group), leukocyte-depleted and lipopolysaccharide-treated (CPA + LPS group), and lipopolysaccharide-treated + NO inhalation (LPS + NO group). There were no deaths associated with lipopolysaccharide treatment, leukocyte depletion, or NO exposure.
**Lipopolysaccharide Administration**

During halothane anesthesia, 100 μl of endotoxin-free saline (control) or 10 μg lipopolysaccharide (Escherichia coli 0127:B8, phenol extract, L-3129, Sigma, St. Louis, MO) in 100 μl saline was sprayed into the trachea close to the bifurcation using a miniaturized nozzle (Model IA-1b, Penn-Century, Philadelphia, PA) to optimize the dose and assure uniform distribution of the solution throughout the lung. After treatment, the rats were housed in a filtered-air chamber for 6 h. Following anesthesia (intraperitoneal sodium pentobarbital, 50 mg/kg body weight), the abdominal aorta was severed. We chose to study a 6-h period after saline- or lipopolysaccharide-treatment based on a preliminary study showing that after lipopolysaccharide treatment, the leukocyte count in BALF (see below) and myeloperoxidase activity in lung homogenates (see below) had increased over the baseline values (3.00 ± 0.71 × 10^6, mean ± SEM, per half lung for neutrophil count in BALF of five animals, and 0.121 ± 0.015, mean ± SEM, optical density unit/g protein for myeloperoxidase activity in lung homogenate of five animals) at 3 h (7.30 ± 1.71 × 10^6 and 0.535 ± 0.086, respectively, for n = 5), peaked at 6 h (7.96 ± 3.14 × 10^6 and 1.42 ± 0.096, respectively, for n = 5), and decreased by 18 h (4.81 ± 9.85 × 10^6 and 0.61 ± 0.12, respectively, for n = 5). Similarly in the human lung after bronchial endotoxin instillation, a high neutrophil count associated with elevated cytokines and chemokines in BALF was found after 6 h, whereas the pulmonary alveolar macrophage (PAM) count did not increase until between 24 and 48 h.

**Leukocyte Depletion**

Rats received intraperitoneal cyclophosphamide (100 mg/kg, Cytoxan, Bristol-Myers, Syracuse, NY) 6 days before lipopolysaccharide treatment and on the day of the lipopolysaccharide treatment (50 mg/kg).

**Nitric Oxide Inhalation**

Rats inhaled 20 ppm NO for 6 h after exposure to lipopolysaccharide. A recent study has demonstrated that this concentration reduces pulmonary inflammation. NO was supplied from a nitrogen-balanced NO gas cylinder (20,000 ppm ultrahigh-pure grade, Sumitomo Seika Chem. Co., Osaka, Japan) through thermal mass-flow controllers (SEC-4400, ESTEC, Kyoto, Japan), and the NO gas was diluted with filtered air (25°C, relative humidity 50-55%) just before the exposure chamber (20 l) to obtain a concentration of 20 ppm NO. The flow rate in each chamber was set at 10 l/min. Three or four animals were housed per chamber. The NO and NO_2 levels were monitored by electrochemical sensors (TM-100 and TM-1002, respectively, Saan, Osaka, Japan), and the NO_2 level in the chamber was found to always be less than 1.0 ppm. Only filtered 104/min air was introduced into the chamber of the animals not exposed to NO inhalation.

**Tissue Fixation and Immunohistochemistry (iNOS, Nitrotyrosine, and Chlorotyrosine)**

Three rats were studied in each group. A polyclonal antibody was prepared in collaboration with Nippon Biotest Laboratories, Tokyo, Japan, by injecting rabbits with chlorotyrosine hapten using bovine serum albumin (BSA) as a carrier protein and with Freund complete adjuvant. Chlorotyrosine was conjugated with BSA by using N-(6-maleimidocaproyloxy) succinimide (Dojindo, Kumamoto, Japan) as a bridge between amino-residue of chlorotyrosine and the SH-residue of BSA. The antibody titer in the immunized rabbit’s serum was checked as follows. The serum (diluted to 1:1 × 10^2 to 2,048 × HATAISHI ET AL.

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**Fig. 1. Myeloperoxidase (MPO) and peroxynitrite pathways to produce nitrotyrosine are shown. The treatment with lipopolysaccharide (LPS) sequesters neutrophils (PMN) in the lungs, thereby activating the MPO or peroxynitrite pathways to produce nitrotyrosine; chlorotyrosine is also produced by myeloperoxidase activity. LPS treatment induces inducible nitric oxide synthase (iNOS) in pulmonary alveolar macrophage (PAM) and type 2 cells, increasing nitric oxide (NO) levels, which combine with superoxide, thereby giving rise to peroxynitrite. LPS treatment also induces iNOS in alveolar macrophages, thereby activating the peroxynitrite pathway, which results in production of nitrotyrosine without chlorotyrosine. The detection of nitrotyrosine together with chlorotyrosine indicates that the myeloperoxidase pathway has a role in the production of the nitrotyrosine, whereas detection of nitrotyrosine mainly contributes to the production of nitrotyrosine.**
TYROSINE NITRATION AND NEUTROPHILS

10^2) was incubated for 60 min at 37°C in micro-enzyme-linked immunosorbent assay (ELISA) plates (Falcon 3912 Assay Plate, Becton Dickinson, Franklin Lakes, NJ) coated with chlorotyrosine. The plates were washed in phosphate-buffered solution (PBS) (× 4), and 50 μl alkaline phosphate-conjugated goat F(ab')2 antirabbit IgG (Biosource Technical Service, Sunnyvale, CA) diluted 1:1,000 in PBS was placed in each well; the wells incubated at 37°C. They were then washed with PBS (× 5) and incubated at 37°C with 200 μl of the phosphatase substrate in PBS (pH 9.5) to develop. Absorbance at 405 nm was measured on a microplate reader (EL307, BioTek Instruments, Winooski, VT). The titer was checked weekly, and after obtaining the plateau titer, all the serum of the immunized animal was obtained and purified through a BSA column (Amersham Pharmacia Biotech, Buckinghamshire, England). No cross-reaction with BSA was observed in the immunoprecipitation method after the purification of the antibody. A polyclonal antibody to iNOS and its blocking peptide and a polyclonal antibody to nitrotyrosine were obtained from commercial sources (Biomol Research Laboratories Inc., Plymouth Meeting, PA, and Upstate Biotechnology, Charlottesville, VA, respectively).

Both lungs were fixed with 4% paraformaldehyde via the trachea at 23 cm H_2O and embedded in paraffin wax. De-paraffinized 4-μm sections were incubated overnight at 4°C with the primary antibody (anti-iNOS, anti-nitrotyrosine, or antichlorotyrosine antibody) diluted to 1:100. The sections were treated with a secondary antibody (biotinylated goat antirabbit IgG) and an enzyme complex (Histostain SP kit, Zymed Laboratories Inc., South San Francisco, CA); reactive sites were visualized with aminoethylcarbazole (producing red reaction product), and the sections were counterstained with hematoxylin.

Although alveolar type 1 and type 2 cells form the alveolar surface, it was not possible to clearly differentiate these two cell types in our 4-μm sections; for this reason, in this study, we refer to them as “alveolar wall cells.”

Quantitative Analysis of the Counts of Immunoreactive Neutrophils and the Degree of Immuno staining in Lung Sections

The numbers of neutrophils and the numbers of neutrophils immunoreactive for nitrotyrosine and chlorotyrosine were counted in three randomly chosen 50-μm square areas of lung sections from three animals in each group (i.e., nine optical fields in each group). A quantitative analysis of the degree of immunostaining was also performed. The immunostaining of nitrotyrosine and chlorotyrosine in three randomly chosen 50-μm square areas of lung sections from three animals in each group (i.e., nine images in each group) was captured and saved as a RGB digital image with 8-bit density resolution in each color with image software (Adobe Photoshop v. 5.5, Adobe Systems Incorporated, San Jose, CA), and the mean density of 334,572-pixel red image of each RGB image was measured using NIH image (Scion Image v. 1.62, Scion Corporation, Frederick, MD) because the reactive sites were visualized with red reaction product.

Validation of Antibody Specificity

The iNOS antibody does not cross-react with neuronal (NOS I) or endothelial NOS (NOS III) by Western analysis (see Manufacturers Data Sheets). Preadsorption experiments confirmed its specificity for the NOS isof orm, i.e., no positive reaction sites were detected after competitive binding of the iNOS antibody with 20 nm of peptide antigen (Biomol Research Laboratories, Inc., Plymouth Meeting, PA).

Negative controls for nitrotyrosine and chlorotyrosine staining were (1) antibody preadsorption with authentic nitrotyrosine or chlorotyrosine (20 μm) and (2) antibody substitution with nonimmune IgG. To exclude the possibility of (artifactual) tyrosine nitration during quenching of endogenous peroxidase activity by H_2O_2 in the presence of intrinsic NO_2^−, tissue sections were flooded with three washes (20 s each) of 1 μM sodium hydrosulphite (adjusted to pH 9.5 with 2 N NaOH), and the sections were quenched (3% H_2O_2) and stained for nitrotyrosine as described above.

To provide positive controls for antinitrotyrosine staining, sections were quenched (3% H_2O_2), washed in PBS (× 3), and incubated in 1 mM peroxynitrite (Upstate Biotechnology, Charlottesville, VA) for 2 h at 37°C; for antichlorotyrosine staining, sections were incubated in PBS with 150 mM chloride ion and then in 500 μM HOCI for 1 h to generate chlorotyrosine.

To exclude cross-reactivity between chlorotyrosine and the antinitrotyrosine antibody and between nitrotyrosine and the antichlorotyrosine antibody, the antinitrotyrosine antibody was incubated with 20 μM authentic chlorotyrosine and used on the nitrotyrosine-rich tissue treated with peroxynitrite; the antichlorotyrosine antibody was incubated with 20 μM authentic nitrotyrosine and used in the chlorotyrosine-rich tissue treated with HOCI.

Bronchoalveolar Lavage

Ten animals were studied in each group. The left main bronchus was ligated, and the right lung lavaged (× 5) with endotoxin-free saline (Otsuka Pharm, Naruto, Japan). Each wash volume was 0.0175 (ml/g) multiplied by body weight (g). The recovered amount was always more than 90%. A nucleated cell count and differential cell count were performed, and the nitrite-nitrate content was measured by the Griess method using an automated analyzer (TCI-NOx 1000 and S-3200, Tokyo Kasei Kogyo, Tokyo, Japan).

Anesthesiology, V 97, No 4, Oct 2002
**Table 1. Peripheral Blood Findings**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leukocytes (×10⁴/mm³)</th>
<th>Erythrocytes (×10¹²/mm³)</th>
<th>Hemoglobin (g/dl)</th>
<th>Hematocrit (%)</th>
<th>Neutrophils (% of total cells)</th>
<th>Eosinophils (% of total cells)</th>
<th>Basophils (% of total cells)</th>
<th>Lymphocytes (% of total cells)</th>
<th>Monoocytes (% of total cells)</th>
<th>Platelets (×10³/mm³)</th>
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<tbody>
<tr>
<td>Control</td>
<td>Mean 7,243</td>
<td>697</td>
<td>14.3</td>
<td>52.6</td>
<td>1,540</td>
<td>32</td>
<td>177</td>
<td>5,196</td>
<td>298</td>
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<tr>
<td></td>
<td>SEM 814</td>
<td>16.4</td>
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<td>0.8</td>
<td>102</td>
<td>14</td>
<td>55</td>
<td>853</td>
<td>61</td>
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<tr>
<td>Lipopolysaccharide</td>
<td>Mean 9,610</td>
<td>724</td>
<td>15.3*</td>
<td>57.2*</td>
<td>4,691</td>
<td>337</td>
<td>375</td>
<td>3,782</td>
<td>425</td>
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<td></td>
<td>SEM 1,028</td>
<td>8.5</td>
<td>0.1</td>
<td>0.3</td>
<td>605</td>
<td>135</td>
<td>79</td>
<td>690</td>
<td>125</td>
<td>85.2</td>
</tr>
<tr>
<td>Cyclophosphamide + lipopolysaccharide</td>
<td>Mean 572#</td>
<td>692</td>
<td>14.0‡</td>
<td>51.1‡</td>
<td>79#</td>
<td>68</td>
<td>12§</td>
<td>293†</td>
<td>120</td>
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<td></td>
<td>SEM 89</td>
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<td>0.9</td>
<td>34</td>
<td>34</td>
<td>4</td>
<td>33</td>
<td>34</td>
<td>5.9</td>
</tr>
<tr>
<td>Lipopolysaccharide + NO</td>
<td>Mean 8,490</td>
<td>682</td>
<td>14.4**</td>
<td>53.7</td>
<td>3,099**</td>
<td>39**</td>
<td>494</td>
<td>4,276</td>
<td>582</td>
<td>112.5</td>
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<td>SEM 706</td>
<td>15.5</td>
<td>0.3</td>
<td>1.5</td>
<td>260</td>
<td>13</td>
<td>80</td>
<td>819</td>
<td>99</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*P < 0.05 and †P < 0.001 comparing control and lipopolysaccharide. §P < 0.005, and #P < 0.001 comparing lipopolysaccharide and cyclophosphamide + lipopolysaccharide. **P < 0.05 comparing lipopolysaccharide and lipopolysaccharide + nitric oxide (NO).

Control = aerosolized with saline and placed in room air for 6 h; Lipopolysaccharide = exposed (aerosolized) to lipopolysaccharide and placed in room air for 6 h; Cyclophosphamide + lipopolysaccharide = after intraperitoneal injection of cyclophosphamide, aerosolized with lipopolysaccharide and placed in room air for 6 h; Lipopolysaccharide + NO = exposed to lipopolysaccharide and placed in 20 ppm nitric oxide for 6 h.

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**Myeloperoxidase Activity in the Lung**

Ten animals were studied in each group. Myeloperoxidase activity in left lung homogenates was assayed as described by Hirano14 at an optical density of 460 nm (OD460). Myeloperoxidase activity was then normalized to protein content, which was measured using a bichinonic acid protein kit according to the manufacturer’s instruction (Pierce, Rockford, IL).

**Statistical Analysis**

A one-way analysis of variance (ANOVA) with Scheffé post hoc test was used to detect the difference between groups. P values less than 0.05 were considered significant.

**Results**

**Neutrophil Number in Blood**

After lipopolysaccharide treatment, the number of neutrophils circulating in peripheral blood was significantly increased above the control value (P < 0.001), and NO inhalation did not alter this response. Leukocyte depletion decreased the number of neutrophils circulating in response to lipopolysaccharide (compared with lipopolysaccharide treated group, P < 0.001; table 1).

**Validation of Antibody Specificity**

The negative controls for nitrotyrosine and chlorotyrosine staining, i.e., (1) antibody preadsorption with authentic nitrotyrosine or chlorotyrosine (20 μM; fig 2) and (2) antibody substitution by nonimmune IgG, did not stain. There was no (artifactual) tyrosine nitration during quenching of endogenous peroxidase activity by H₂O₂ in the presence of intrinsic nitrite.

**Positive Controls for Nitrotyrosine and Chlorotyrosine Staining**

No cross-reactivity between chlorotyrosine and the anti-chlorotyrosine antibody or between nitrotyrosine and the anti-chlorotyrosine antibody was observed as a result of nitrotyrosine staining after preadsorption of antinitrotyrosine antibody with authentic chlorotyrosine or as a result of chlorotyrosine staining after preadsorption of antichlorotyrosine antibody with authentic nitrotyrosine (fig. 2).

**iNOS, Nitrotyrosine, and Chlorotyrosine in Lung Cells**

In the lungs of rats in the untreated control group, iNOS protein was weakly expressed by some alveolar wall cells but not by PAMs or by the rare neutrophils present. Lipopolysaccharide treatment enhanced the iNOS immunoreactivity of alveolar wall cells, PAMs, and neutrophils. Most of the large numbers of neutrophils present were immunopositive. Leukocyte depletion and NO inhalation decreased neutrophil accumulation in the lungs of lipopolysaccharide-treated rats. In these animals, iNOS immunoreactivity was present in PAMs and alveolar wall cells (figs. 2 and 3).

In the control group, no nitrotyrosine-positive cells were detected. In the lungs of lipopolysaccharide-treated rats, however, many positive alveolar wall cells, PAMs, and neutrophils were present. After leukocyte depletion and NO inhalation, no nitrotyrosine-positive cells were detected (figs. 2 and 3).

Chlorotyrosine immunoreactivity was similar to that of nitrotyrosine. In control rats, no chlorotyrosine-positive cells were detected, and in lipopolysaccharide-treated rats, many chlorotyrosine-positive alveolar wall cells, PAMs, and neutrophils were present. After leukocyte depletion and after NO inhalation, no chlorotyrosine-positive cells were detected in the lungs of rats treated with lipopolysaccharide (figs. 2 and 3).

Quantitative analysis of tissue sections also showed that lipopolysaccharide exposure resulted in neutrophil accumulation with nitrotyrosine-positive and chlorotyrosine-positive neutrophils and that the photometric red color density of immunostained images of lung sections had increased, whereas leukocyte depletion and NO...
inhalation had decreased neutrophil accumulation and red-color density in the lungs of lipopolysaccharide-exposed rats (table 2).

Neutrophil and PAM Number and Nitrite–Nitrate Levels in BALF

Lipopolysaccharide treatment increased the neutrophil count \((P < 0.001)\) in BALF, whereas leukocyte depletion prevented this increase and NO inhalation suppressed it \((P < 0.05)\). The number of PAMs did not change after lipopolysaccharide treatment, leukocyte depletion, or NO inhalation. Nitrite–nitrate levels increased after lipopolysaccharide treatment \((P < 0.05)\), were unchanged by leukocyte depletion, and were further increased by NO inhalation (fig. 4).

**Myeloperoxidase Activity in Lung Homogenate**

Lipopolysaccharide treatment increased myeloperoxidase activity in lung homogenates \((P < 0.001)\). This activity was decreased by leukocyte depletion \((P < 0.001)\) and suppressed by NO inhalation \((P < 0.05;\) fig. 5).

**Discussion**

The present study shows that instillation of lipopolysaccharide via a nozzle enhances iNOS immunoreactivity and the production of nitrotyrosine and chlorotyrosine in lung cells and is associated with high numbers of neutrophils in blood and BALF and an increase in myeloperoxidase activity in lung tissue. It further demonstrates that neither leukocyte depletion nor NO inhalation influences iNOS immunoreactivity in PAMs and alveolar wall cells in response to lipopolysaccharide treatment, but that each eliminates nitrotyrosine and chlorotyrosine production in lung cells and suppresses the influx of neutrophils into BALF and lung myelo-
oxidase activity. These data indicate that neutrophil-derived myeloperoxidase contributes to tissue nitration in lipopolysaccharide-induced lung injury, and that during the early phase of inflammation, when neutrophils sequester and infiltrate the lung, inhaled NO has an inhibitory effect on the nitration response.

Lipopolysaccharide-induced Tissue Nitration

Clearly, a reduced number of sequestrated neutrophils in the lung results in reduced levels of iNOS and myeloperoxidase generation. Although neutrophil depletion reduces levels of tissue nitration and chlorination in response to lipopolysaccharide via reduced neutrophil-derived myeloperoxidase activity and iNOS expression (fig. 6), our finding of the continued expression of lipopolysaccharide-induced iNOS in PAMs suggests that, combined with superoxide production, this could be a source of peroxynitrite generation. While cyclophosphamide treatment has been shown to stimulate superoxide production in rat lungs in vivo,15 in our study, the

Table 2. Quantitative Analysis of Immunoreactive Neutrophils and Immunostaining in the Lung Sections

<table>
<thead>
<tr>
<th>Treatment (n = 9 in each group)</th>
<th>Neutrophil Count</th>
<th>Red Image Mean Density (bit/pixel)</th>
<th>Chlorotyrosine</th>
<th>Neutrophil Count</th>
<th>Red Image Mean Density (bit/pixel)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean Density</td>
<td></td>
<td></td>
<td>Mean Density</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Positive</td>
<td></td>
<td>Total</td>
<td>Positive</td>
</tr>
<tr>
<td>Control</td>
<td>1.4</td>
<td>0.0</td>
<td>33.0</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>SEM</td>
<td>0.2</td>
<td>0.0</td>
<td>0.4</td>
<td>0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>Mean 22.6*</td>
<td>11.6*</td>
<td>48.2*</td>
<td>Mean 21.4*</td>
<td>17.8*</td>
</tr>
<tr>
<td>SEM</td>
<td>1.5</td>
<td>1.4</td>
<td>0.6</td>
<td>1.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Cyclophosphamide + lipopolysaccharide</td>
<td>Mean 1.2†</td>
<td>0.0†</td>
<td>33.9†</td>
<td>Mean 1.9†</td>
<td>0.0†</td>
</tr>
<tr>
<td>SEM</td>
<td>0.3</td>
<td>0.0</td>
<td>1.3</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Lipopolysaccharide + NO</td>
<td>Mean 5.3‡</td>
<td>0.4‡</td>
<td>35.5‡</td>
<td>Mean 6.1‡</td>
<td>0.3‡</td>
</tr>
<tr>
<td>SEM</td>
<td>1.6</td>
<td>0.2</td>
<td>1.1</td>
<td>2.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* P < 0.0001 comparing control and lipopolysaccharide. † P < 0.0001 comparing lipopolysaccharide and cyclophosphamide + lipopolysaccharide. ‡ P < 0.0001 comparing lipopolysaccharide and lipopolysaccharide + nitric oxide (NO).

Total = neutrophil number in alveoli in a 50-μm² area; Positive = immunopositive neutrophil number in the alveoli in a 50-μm² area; Mean Density = mean density of red color image of each RGB image of 8-bit resolution in each color; Control = aerosolized with saline and placed in room air for 6 h; Lipopolysaccharide = exposed (aerosolized) to lipopolysaccharide and placed in room air for 6 h; Cyclophosphamide + lipopolysaccharide = after intraperitoneal injection of cyclophosphamide aerosolized with lipopolysaccharide and placed in room air for 6 h; Lipopolysaccharide + NO = exposed to lipopolysaccharide and placed in 20 ppm nitric oxide for 6 h.

Fig. 4. Lung cells and nitrite–nitrate levels in bronchoalveolar lavage fluid (BALF) per half lung are shown. (A) Total number of cells and of pulmonary alveolar macrophages and polymorphonuclear leukocytes (mean ± SEM; n = 10 in each group). Lipopolysaccharide (LPS) treatment increased the number of polymorphonuclear leukocytes, whereas leukocyte depletion prevented and NO inhalation suppressed this increase. The number of alveolar macrophages was unchanged by LPS, leukocyte depletion, or NO inhalation. PAMs = pulmonary alveolar macrophages; PMNs = polymorphonuclear leukocytes. (B) Nitrite–nitrate levels (mean ± SEM; n = 10 in each group). LPS treatment increased nitrite–nitrate level (compared with control: *P < 0.05), and LPS with NO inhalation increased the level more than LPS treatment alone did (¶P < 0.0001 LPS + NO vs. LPS). For abbreviations (LPS, CPA) see also table 1.
lack of nitrotyrosine production in PAMs in neutrophil-depleted rats indicated that the contribution of these cells to this pathway and to tissue nitration was minor. Cyclophosphamide is known to decrease NO production by PAMs and nitrotyrosine formation in mice 72 h after infection with *Mycoplasma pulmonis,* but in our study, the nitrite–nitrate levels in BALF increased 6 h after lipopolysaccharide treatment and were unchanged after leukocyte depletion by cyclophosphamide, suggesting that NO production by lung cells was not significantly influenced by cyclophosphamide treatment in our study. However, in this study, the possibility that the cytotoxicity of cyclophosphamide may also have modified enzyme function, resulting in reduced production of nitrotyrosine and chlorotyrosine, cannot be ruled out.

Similar to cyclophosphamide, inhaled NO inhibits leukocyte sequestration and reduces lung damage in animals administered lipopolysaccharide either intravenously or by inhalation, enhancing leukocyte deformability and inhibiting the expression of adhesion molecules by pulmonary vascular endothelium and leukocytes. An increase in NO levels in tissue can be expected in response to NO inhalation and is indicated in the present study by our finding of an increased content of nitrite–nitrate in BALF. Despite any enhancement by inhaled NO on the reaction between NO and superoxide that may have occurred in the lipopolysaccharide-injured lung, we found that it did not result in nitrotyrosine production. It appears that the inhibitory effect of NO on neutrophil sequestration in the lung supervened its effects in enhancing tissue nitration *via* PAMs and the peroxynitrite pathway (fig. 6).

**Myeloperoxidase Pathway in Tyrosine Nitration**

A number of peroxidases, such as lactoperoxidase in airway cells and eosinophil peroxidase, are found in the lung, in addition to myeloperoxidase. Loss of nitrotyrosine and chlorotyrosine production in lung cells after leukocyte depletion suggests, however, that among the possible peroxidases, the contribution of myeloperoxidase in lipopolysaccharide-induced lung injury is significant. Our finding of nitrotyrosine and chlorotyrosine generation at the intracellular level demonstrates that the neutrophils and the myeloperoxidase pathway play a role in tissue nitration. We were able to evaluate the contribution of this pathway by the use of a specific antibody to chlorotyrosine, which was raised for this study. To our knowledge, this is the first report of the use of such an antibody to dissect the contribution of the myeloperoxidase pathway to tissue nitration.

Although analysis of lung homogenates demonstrated an increase in myeloperoxidase activity, ideally our data for

**MYELOPEROXIDASE PATHWAY**

**PEROXYNITRITE PATHWAY**

PMN: polymorphonuclear leukocyte; PAM: pulmonary alveolar macrophage; MPO: myeloperoxidase; LPS: lipopolysaccharide; iNOS: inducible nitric oxide synthase; NO: nitric oxide
chlorotyrosine in lung cells would be supported by quantita-
tive measurement of chlorotyrosine levels by high-per-
formance liquid chromatography (HPLC) or by gas chro-
matography combined with mass spectrometry.

The cellular pathways of nitrotyrosine production via the myeloperoxidase pathway will be further clarified by administering a selective myeloperoxidase inhibitor or by using genetically manipulated mice. However, be-
cause any inhibitor has nonspecific effects and it is likely that other radical systems, such as the peroxyanitrite pathway, would be activated to compensate for loss of the myeloperoxidase system in genetically manipulated animals, these studies await the availability of condi-
tional knockout mice to disclose the relative role of myeloperoxidase in tyrosine nitration in the injured lung.

Increased expression of iNOS in PAMs in response to lipopolysaccharide indicates a further cellular source for nitrotyrosine production, i.e., NO for peroxyanitrite pathway and nitrite for myeloperoxidase pathway. Although this expression persisted after leukocyte depletion and NO inhalation, nitrotyrosine and chlorotyrosine production in PAMs was suppressed. It is possible that in response to lipopolysaccharide, nitrotyrosine and chlorotyrosine were generated directly via the myeloperoxidase pathway in these cells and that they contained myeloperoxidase released by neutrophils undergoing phagocytosis. Alter-
natively, myeloperoxidase derived from neutrophils in the tissue could have indirectly induced nitrotyrosine and chlorotyrosine production in these cells. We cannot ex-
clude the possibility that cyclophosphamide induced a change in the population or function of type 2 cells, but lipopolysaccharide treatment induced iNOS in alveolar macrophages in the presence of cyclophosphamide or inhaled NO.

Clinical Relevance of NO Inhalation

Clinically, inhaled NO can be expected to decrease the number of sequestered leukocytes in the lung and to reduce the myeloperoxidase activity, thereby reducing tyrosine nitration and chlorination. This beneficial effect can offset tyrosine nitration and chlorination in the in-
jured lung that can alter protein function and affect the activity of the enzymes that have tyrosine at their active site, resulting in enzyme malfunction.

Nitric oxide inhalation, however, likely has contradic-
tory effects in lung, one being to increase tissue nitration as a result of increased amounts of NO and nitrite, thereby enhancing the production of peroxyanitrite and the reaction via peroxyanitrite as a source of nitrite, and the other being to decrease nitration by inhibiting leu-
kokocyte accumulation in the lungs, thereby reducing the production of NO and superoxide (i.e., peroxyanitrite) and the amount of myeloperoxidase.

In a previous study in which we measured nitroty-
rosine quantitatively by HPLC, we confirmed that in-
haled NO decreases the production of nitrotyrosine. In this study, we further confirmed that inhaled NO eliminated nitrotyrosine together with chlorotyrosine in the lungs of lipopolysaccharide-treated rats, indicating that the myeloperoxidase pathway mainly contributed to the production of nitrotyrosine, and, because the inhibitory effect of inhaled NO on leukocyte accumulation in the lung supervened the effect of NO delivery to the lung tissue, that NO inhalation did not enhance the reaction of myeloperoxidase-dependent tyrosine nitration.

Installation of lipopolysaccharide was used as a model of clinical lung injury caused by severe gram-negative bacterial pneumonia or by inhalation of massive amounts of environmental endotoxin; however, the lung injury in clinical settings is not so simple as this animal model, and the possible adverse effect of inhaled NO during the later phase of inflammation remains to be evaluated. Never-
theless, our data indicate that inhaled NO has an inhibi-
atory effect on nitration and chlorination of cellular com-
ponents during the early phase of inflammation caused by intrapulmonary lipopolysaccharide, possibly by redu-
ducing neutrophil sequestration in the lungs.

The authors thank Tomoyuki Tomita, M.D., Ph.D. (Professor Emeritus, Depart-
ment of Medicine, Kitasato University School of Medicine, Sagamihara, Kanag-
awa, Japan) for his advice and encouragement in their study. The authors also thank Hisatsugu Mitsutani, M.D., Ph.D., Kinya Honda, M.D., Ph.D., and Masami Tanaka, Technician, (Department of Medicine, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan) for their excellent technical support.

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