Decreased exhaled nitric oxide as a marker of postinsult immune paralysis

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Attalah, Habiba L., Stéphanie Honoré, Saadia Eddahibi, Elisabeth Marcos, Claude-James Soussy, Serge Adnot, and Christophe Delclaux. Decreased exhaled nitric oxide as a marker of postinsult immune paralysis. J Appl Physiol 97: 1188–1194, 2004. First published May 7, 2004; 10.1152/japplphysiol.00840.2003.—Nitric oxide (NO) regulates neutrophil migration and alveolar macrophage functions such as cytokine synthesis and bacterial killing, both of which are impaired in immune paralysis associated with critical illness. The aim of this study was to determine whether NO is involved in immune paralysis and whether exhaled NO measurement could help to monitor pulmonary defenses. NO production (protein expression, enzyme activity, end products, and exhaled NO measurements) was assessed in rats after cecal ligation and puncture to induce a mild peritonitis (leading to ∼20% mortality rate). An early and sustained decrease in exhaled NO was found after peritonitis (from 1 to 72 h) compared with healthy rats [median (25th–75th percentile), 1.5 parts per billion (ppb) (1.2–1.7) vs. 4.0 ppb (3.6–4.3), P < 0.05], despite increased NO synthase-2 and unchanged NO synthase-3 protein expression in lung tissue. NO synthase-2 activity was decreased in lung tissue. Nitrites and nitrates in supernatants of isolated alveolar macrophages decreased after peritonitis compared with healthy rats, and an inhibitory experiment suggested arginase overactivity in alveolar macrophages bypassing the NO substrate. Administration of the NO synthase-2 inhibitor aminoguanidine to healthy animals reproduced the decrease in exhaled NO compared with L-arginine administration [4.8 (3.9–5.7) vs. 1.6 (1.3–1.7) ppb, respectively, P < 0.05]. In conclusion, the decrease in exhaled NO observed after mild peritonitis could serve as a marker for lung immunodepression.

ALTERATIONS IN IMMUNE DEFENSES associated with critical illness, although long described, have only recently been individualized by the term “immune paralysis” (25). Immune paralysis includes functional impairments of monocytes and macrophages and neutrophils (11, 30), the two major phagocytic cells contributing to innate immune defenses. This suggests that immune paralysis may be a major risk factor for nosocomial infections. In keeping with this possibility, we previously described an association between impaired intracellular killing of Staphylococcus aureus by neutrophils and subsequent nosocomial infection (30).

Nitric oxide (NO) regulates neutrophil migration and alveolar macrophage functions such as cytokine synthesis and bacterial killing (15, 36, 19, 34, 23), which are impaired in critically ill patients. One may hypothesize that lung NO production could be affected in these patients because pneumonia is the most common nosocomial infection. Along this line, L-arginine, which is a semessential amino acid, is the substrate for NO synthesis, and arginine blood concentration is often decreased in critically ill patients (3), a fact that could explain the beneficial effect of formulas with high arginine content in terms of reduction of infectious complications (16).

The respiratory tract is accessible by noninvasive investigation, because NO is present and can be measured in exhaled gas (10). Few studies have evaluated the concentration of exhaled NO in ventilated critically ill patients, and they have shown a decrease in exhaled NO after surgery (18, 9). It could be hypothesized that this unexpected decrease (inasmuch as proinflammatory mediators are known to upregulate inducible NO synthase) in exhaled NO could be a marker of immune paralysis in critically ill patients.

To this end, we used a rat model of peritonitis, in which we measured pulmonary NO production and evaluated potential effects of NO on inflammatory cell phenotype modifications, i.e., abnormal neutrophil migration and alveolar macrophage cytokine synthesis, which are components of immune paralysis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (IFFA CREDO Laboratories, L’Arbresle, France) weighing 225–250 g were used in all experiments. The rats were housed in air-filtered, temperature-controlled units and were allowed free access to food and water. The animals were housed as recommended by the European Convention for the Protection of Experimental Animals (Decree no. 2001-131, February 6, 2001). Our local animal subcommittee approved the animal protocols.

Animal Model of Peritonitis

Sepsis was produced in the rats by cecal ligation and puncture (CLP), as described elsewhere (2). After halothane anesthesia, a 3-cm midline abdominal incision was made to expose the cecum, which was ligated with 3-0 silk (Ethicon) suture, just distal to the ileocecal valve to avoid intestinal obstruction. The cecum was punctured twice with a 19-gauge needle. The cecum was then replaced in the peritoneal cavity, and the incision was closed with a continuous 3-0 silk suture. The control (sham-operated) rats underwent the same procedures except that the cecum was neither ligated nor punctured. All rats were given 1 ml of physiological saline subcutaneously for fluid resuscitation during the postoperative period. By using this low number of punctures, a mild localized sepsis was obtained as previously described (2), given a low spontaneous mortality rate (∼20%). This

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design was chosen inasmuch as we previously showed that a postaggressive inhibition of neutrophil migration occurs in this setting that further allows studying almost all rats.

At designated time points after CLP (1, 4, 24, 48, and 72 h), the rats were anesthetized by intraperitoneal injection of pentobarbital, and tracheotomy was performed for mechanical ventilation with a rodent ventilator (tidal volume of 7 ml/kg; respiratory rate of 60/min).

**Measurement of Exhaled NO Concentration**

Exhaled NO was measured with a chemiluminescence analyzer (EVA 4000, Sèrè, Aix en Provence, France), with a lower limit of detection of 1 part per billion (ppb) and a NO sampling rate of 30 l/h. Calibration was performed with a zero gas and standard 100 and 800 ppb NO gas (AGA).

All rats were ventilated with NO-free air. Exhaled breath from each rat was collected in a 6-liter polyethylene bag that did not react with NO gas (AGA). Calibration was performed with a zero gas and standard 100 and 800 ppb NO gas (AGA).

**Plasma Collection**

After exhaled gas collection, rats were killed by exsanguination. Blood was obtained, and plasma was separated and stored at −80°C.

**Wet-to-Dry Weight Ratio**

One lobe of the freshly harvested left lung was separated, weighed, dried at 40°C for 72 h, and weighed again. The wet-to-dry lung weight ratio was calculated by dividing the initial weight by the dry weight. The remaining lobe was frozen in liquid nitrogen and stored at −80°C until use.

**Bronchoalveolar Lavage**

Bronchoalveolar lavage (BAL) was performed as previously described (2). The left bronchus was clamped for BAL of the right lung by using the tracheotomy tube. Five 4-ml aliquots of 37°C, sterile, pyrogen-free, 0.9% saline were flushed through the tracheotomy tube. The five fractions were recovered and pooled. The total number of cells was counted using a standard hemocytometer. The BAL fluid was then centrifuged at 300 g for 7 min. The supernatant was collected and stored at −80°C for further analysis.

**Alveolar Macrophage Isolation**

After BAL centrifugation, the cell pellet (>97% macrophages) was suspended in DMEM (Life Technologies, Cergy-Pontoise, France) containing antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 100 mg/ml gentamicin). The cells, at a concentration of 1×10^6/ml, were incubated for 4 h at 37°C in 5% CO2 with and without stimulation by lipopolysaccharide (LPS, *Escherichia coli* endotoxin 055:B5 Sigma, 1 μg/ml). Cell supernatants were collected and stored at −80°C for further analysis.

**Measurement of NO End Products (Nitrite/Nitrate)**

NO is stable in the presence of oxygen and is rapidly converted into NO2− and NO3− (NO end products: NOx) in liquid media. For measurements of NO production in biological samples (plasma, BAL fluid, and alveolar supernatants), both NO2− and NO3− were converted into NO by using the reducing agent vanadium (III) chloride in acidic solution [hydrochloric acid (HCl) 6N] at 90°C (Nitroflux NT system, Sèrè), as described elsewhere (37). NO gas was carried into the NO analyzer (EVA 4000 analyzer) with a constant flow of N2 gas and was quantified with reference to sodium nitrite standards. Before plasma NOx measurement, the frozen plasma was thawed and mixed with methanol (1:1 vol/vol) to precipitate the proteins and then centrifuged at 12,000 g for 20 min at 4°C.

**Western Blotting of NO Synthase-2 and NO Synthase-3 Proteins**

Immunoblotting for NO synthase (NOS)-2 and NOS-3 proteins was performed as described elsewhere (26). The frozen tissues were homogenized on ice by using a Polytron homogenizer (Kinetamica, Steinholhalde, Switzerland) in 0.1 mM PBS (pH 7.4) containing protease inhibitors (1 μm leupeptin, 1 μM pepstatin A). The homogenates were centrifuged at 490 g for 15 min at 4°C. Protein concentrations of the supernatants were measured by the Bradford method (Pierce, Rockford, IL), and supernatants (150 μg of protein loaded per lane) were then examined by SDS-PAGE. The proteins were transferred to nitrocellulose membranes by electroblotting in a transblotBio-Rad transfer apparatus (Bio-Rad Laboratories, Richmond, CA). The nitrocellulose membranes were soaked in electroblotting buffer (25 mM Tris-HCl, 193 mM glycine, 20% methanol, pH 8.0) for 15 min before being transferred. After protein transfer, which was carried out for 12 h at 4°C, the membrane was blocked with 5% Tween Tris-buffered saline (TBS-T, 0.15 M NaCl, 10 mM Tris-HCl, 0.05% Tween 20, 5% BSA, pH 8.0) for 1 h at room temperature. The membranes were incubated with primary antibody, the endothelial NOS-3 protein (Transduction Laboratories, Lexington, KY) or the inducible NOS-2 protein (Transduction Laboratories) 1:1,000 for 1 h. The membranes were washed three times in 1% TBS-T. Specific protein was detected by use of horseradish peroxidase (Amersham), and the blots were developed by the Amersham enhanced chemiluminescence detection system. The bands for NOS-2 and/or NOS-3 were visualized and quantified by use of densitometry (personal densitometer SI; Molecular Dynamics).

**Measurement of NOS Activity**

NOS activity was measured in the lung homogenates on the basis of transformation of L-[H3]arginine to L-[H3]citrulline as described elsewhere (26). The tissue (100 mg) was homogenized on ice with a homogenizer, in four volumes of buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.125 mM EGTA, 1% mercaptoethanol, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM PMSF (buffer A). The homogenate was centrifuged at 10,000 g for 30 min at 5°C. The supernatant fraction was used to evaluate NOS-2 activity (cytosolic activity). The pellet (membrane fraction) was resuspended in homogenization buffer containing the detergent CHAPS (20 mM), 1 M KCl, and glyceral (10% vol/vol) and was then allowed to stand on ice for 30 min before centrifugation at 100,000 g for 30 min at 5°C. Activity of NOS-3 in the resultant supernatant fraction (membrane fraction) was determined by measuring Ca2+-dependent conversion of L-[H3]-arginine to L-[H3]-citrulline. The specific activity of L-[H3]-arginine was 320 mCi/mM. The reaction mixture contained the supernatant (<10% in volume), 0.2 μM L-[H3]-arginine, 50 μM L-arginine, 100 μM NADPH, 25 μM tetrahydrobipterin, 2 μM FAD, and 2 μM flavin mononucleotide. This mixture was incubated for 45 min at 37°C under a calcium-dependent condition, with 0.6 mM Ca2+, corresponding mainly to NOS-3 activity. After the reaction was stopped by the addition of 0.5 M perchloric acid, L-[H3]-citrulline and L-[H3]-arginine were separated on a cationic ion-exchange column (Shim-pack ISC-05/S0504; Bio-Rad) in a HPLC system (Waters Associates, Milford, MA). The citrulline fraction was collected by a fraction collector (model 201; Gilson, Villiers-le-Bel, France). After addition of a scintillation cocktail, the isotope was counted for 3 min. Specific transformation of L-[H3]-citrulline was calculated as follows: the isotope counts at the citrulline fraction eluted from the column of the incubation mixture with the enzymes were subtracted from the isotope counts eluted from the incubation without enzymes, and the
remaining counts were then divided by the specific activity (320 mCi/mM). Next, the specific activity of NOS was expressed as picomoles of citrulline formation per milligram protein per 45 min.

Arginase Inhibition Assay

In macrophages, NOs and arginases compete for the same substrate, L-arginine, to produce NO and urea, respectively (6). Because L-arginine availability is a major determinant for NO synthesis in activated macrophages, we hypothesized that arginase might reduce NO production. To test this hypothesis, we investigated the effect of the specific arginase inhibitor N-hydroxy-L-arginine, diacetate salt (NOHA (33)) (Calbiochem) on NO production. Isolated alveolar macrophages from both CLP rats and healthy control rats were suspended in presence of endotoxin stimulation (LPS, 1 μg/ml) for 4 h in the presence or absence of 10 or 100 μM NOHA. NOx concentrations in cell supernatants were measured as described previously.

Macrophage Inflammatory Protein-2 Concentration Measurement in Isolated Alveolar Macrophage Supernatant

Macrophage inflammatory protein-2 (MIP-2) was assayed by use of a specific rat ELISA kit as recommended by the manufacturer (BioSource). The detection limit was 1 pg/ml.

Alveolar Instillation of Endotoxin

As a functional parameter characterizing immune paralysis, neutrophil influx into the lungs was assessed by endotoxin instillation, as previously described (20). Briefly, the rats were anesthetized by halothane anesthesia, the trachea was exposed, and 0.5 ml/kg body wt of LPS (200 μg/kg) dissolved in sterile physiological saline was instilled via a 25-gauge needle. The skin incision was closed with surgical staples, and the rats were allowed to awaken. Four hours later, the rats were anesthetized by intraperitoneal pentobarbital administration to allow biological sample collection, as described previously.

Treatment with the NOS Inhibitor Aminoguanidine

Healthy rats were injected subcutaneously with aminoguanidine (30 mg/kg), and 4 h later exhaled NO was measured to verify NO inhibition. In a separate set of experiments, 4 h after aminoguanidine injection, the rats were instilled with endotoxin (see above) and then killed 4 h later for BAL to assess whether in vivo NO inhibition was associated with modifications in alveolar neutrophil influx.

Table 1. Time course of exhaled NO and NOx levels in BAL, alveolar macrophages, and plasma after CLP or sham surgery in rats, as compared with healthy rats

<table>
<thead>
<tr>
<th></th>
<th>Healthy Rats</th>
<th>H4 (n = 4)</th>
<th>H24 (n = 4)</th>
<th>H1 (n = 4)</th>
<th>H4 (n = 6)</th>
<th>H24 (n = 6)</th>
<th>H48 (n = 6)</th>
<th>H72 (n = 5)</th>
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<tbody>
<tr>
<td>Total BAL fluid cell count, 10⁶</td>
<td>89 (74–100)</td>
<td>121 (82–176)</td>
<td>198 (144–252)†</td>
<td>63 (45–76)</td>
<td>160 (116–180)</td>
<td>180 (106–181)</td>
<td>130 (65–192)</td>
<td>126 (90–156)</td>
</tr>
<tr>
<td>Wet-to-dry lung ratio</td>
<td>3.9 (3.5–4.0)</td>
<td>ND</td>
<td>4.1 (4.0–4.4)</td>
<td>4.4 (3.6–4.5)</td>
<td>4.0 (3.9–4.1)</td>
<td>4.3 (4.1–4.4)</td>
<td>4.4 (4.3–4.6)*</td>
<td>4.4 (4.1–4.5)*</td>
</tr>
<tr>
<td>Exhaled NO, ppb</td>
<td>4.0 (3.6–4.3)</td>
<td>2.6 (2.5–3.5)</td>
<td>9.1 (5.9–9.3)‡</td>
<td>2.7 (1.8–3.4)</td>
<td>2.9 (2.2–3.7)</td>
<td>1.5 (1.2–1.7)†</td>
<td>1.2 (1.0–1.4)†</td>
<td>2.0 (1.2–2.2)*</td>
</tr>
<tr>
<td>NOx, μM</td>
<td>0.8 (0.3–2.1)</td>
<td>0.7 (0.4–1.1)</td>
<td>1.4 (1.0–2.6)</td>
<td>1.0 (0.6–1.2)</td>
<td>1.4 (0.6–1.8)</td>
<td>0.9 (0.7–1.6)</td>
<td>0.5 (0.2–1.2)</td>
<td>2.5 (2.4–2.7)</td>
</tr>
<tr>
<td>BAL fluid</td>
<td>2.3 (1.8–3.2)</td>
<td>0.8 (0.4–1.2)</td>
<td>8.0 (5.1–11.1)*</td>
<td>ND</td>
<td>1.7 (1.2–2.5)</td>
<td>0.6 (0.5–0.7)</td>
<td>3.5 (3.0–4.9)</td>
<td>6.2 (3.2–11.9)*</td>
</tr>
<tr>
<td>Alveolar unstimulated</td>
<td>4.0 (3.5–7.1)</td>
<td>0.8 (0.7–1.0)</td>
<td>7.6 (5.2–10.1)</td>
<td>ND</td>
<td>1.3 (1.1–2.1)</td>
<td>1.0 (0.7–1.5)*</td>
<td>4.9 (3.4–7.0)</td>
<td>5.4 (3.6–6.7)</td>
</tr>
<tr>
<td>Macrophage LPS</td>
<td>0.9 (0.4–2.5)</td>
<td>4.4 (2.5–4.9)</td>
<td>6.4 (4.8–7.5)†</td>
<td>1.9 (0.6–3.2)</td>
<td>0.4 (0.3–2.4)</td>
<td>0.6 (0.4–0.7)</td>
<td>0.6 (0.5–1.1)</td>
<td>0.7 (0.5–6.0)</td>
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</table>

Values are medians (25th–75th percentiles) for each group. NO, nitric oxide; NO, NO end products; H4–H72, 4–72 h time points after cecal ligation and puncture (CLP); BAL, bronchoalveolar lavage; ppb, parts per billion; LPS, lipopolysaccharide. *P < 0.05 compared with healthy group; †P < 0.01 compared with healthy group; ‡P < 0.001 compared with healthy group.

Exhaled NO After Peritonitis

Rats undergoing peritonitis were treated with either L-arginine (control condition) or L-arginine (both supplied by Sigma, Lille, France) at the end of surgical procedure for 23 h according to the experimental design that has been previously used by Horton and colleagues (17a), namely 300 mg/kg L- or D-arginine (intraperitoneal administration) at 30 min, 6 h, and 23 h after CLP. In a first set of experiment (L-arginine, n = 3; L-arginine, n = 5), rats were killed at the 24th hour after CLP to measure exhaled NO. In a second set of experiments (D-arginine, n = 3; L-arginine, n = 5), rats underwent endotoxin instillation at the 24th hour after CLP (as described above) and were killed 4 h later to assess neutrophil influx into BAL fluid (as described above). This additional experiment was conducted to evaluate whether L-arginine administration was able to restore NO synthesis (on the basis of exhaled NO measurement) and correct the inability of neutrophils to migrate into alveolar space in response to endotoxin instillation.

Statistical Analysis

All data are expressed as medians (25th–75th percentiles). Data were analyzed with Statview 5.0 (SAS Institute, Berkeley, NC). For between-group comparisons of continuous variables, we used the Mann-Whitney U-test or the Kruskal-Wallis test, as appropriate. Statistical significance was defined as P < 0.05.

RESULTS

Decreased Exhaled NO After Peritonitis

Experiments were designed to describe the time course of NO exhalation after mild peritonitis or a sham operation. The results are summarized in Table 1. An early decrease in exhaled NO was found after mild peritonitis that seemed related to a decrease in NO production, because NO-derived end products were not increased in BAL fluid or plasma. Furthermore, in addition to this decrease in NO production by the lungs, we found a decrease in NO production by isolated alveolar macrophages, which paralleled the decrease in exhaled NO (Table 1 and Fig. 1). By contrast, sham-operated animals exhibited a trend toward an early decrease in exhaled NO (4th hour), which was subsequently followed by a rapid increase in NO synthesis (24th hour).
Mechanisms of Decreased Lung NO Synthesis

NOS assays on lung homogenates. The decrease in NO output was not related to downregulation of type 2 or 3 NOS proteins, as shown by the Western blot assays on lung homogenates. Expression of NOS-3 was not significantly increased after CLP, whereas expression of NOS-2 was significantly increased (Fig. 2). We then assessed NOS activities and found a decrease in NOS-2 activity in lung homogenates of CLP rats compared with healthy rats (Fig. 3).

Assays on isolated alveolar macrophages. To further explore the mechanism of decreased NO production by isolated alveolar macrophages, experiments using a specific arginase inhibitor (NOHA) were conducted (Table 2). Arginase inhibition caused a dose-dependent increase in NOx levels in alveolar macrophage supernatants of both healthy and CLP rats. Interestingly, arginase inhibition led to significantly greater enhancement of NOx production by macrophages from CLP rats than from healthy rats, suggesting enhanced NOS activity in the CLP rats.

Effects of Decreased Lung NO Production on Immune Function Regulation

In these experiments, we evaluated whether the decrease in NO production associated with mild CLP was involved in the inhibition of alveolar neutrophil migration that occurs after injury. After LPS instillation 24 or 72 h after CLP, the neutrophil influx into the alveolar spaces was less marked than in healthy rats. In vivo inhibition of NO synthesis by treatment of healthy rats with aminoguanidine led to a significant decrease in both exhaled NO [1.7 ppb (1.2–2.4), n = 3] and NOx in supernatants of isolated alveolar macrophages [unstimulated condition, 0.4 μM (0.2–0.7)] compared with healthy rats. Furthermore, aminoguanidine administration was associated with inhibition of neutrophil migration toward the alveolar spaces after LPS administration (Fig. 4).

To further explain the role of NO in macrophage functions, we assessed MIP-2 synthesis by isolated alveolar macrophages. MIP-2 is a chemokine involved in recruiting neutrophils to the alveolar spaces (Fig. 5). An increased MIP-2 secretion by isolated alveolar macrophages was evidenced in both the sham-operated and the CLP rats, despite the absence of alveolar neutrophil influx (data not shown). In vivo (aminoguanidine) inhibition of NO synthesis in healthy rats was followed by increased MIP-2 production into supernatants of isolated alveolar macrophages.

Effect of L-Arginine Supplementation

Administration of L-arginine was associated with an increase in exhaled NO compared with D-arginine administration [4.8

Fig. 1. Time course of exhaled nitric oxide (NO) and nitrites or nitrates in supernatants of unstimulated alveolar macrophages. This figure illustrates the parallel course of exhaled NO [parts per billion (ppb)] and ex vivo production of nitrites and nitrates by isolated alveolar macrophages (μM) in healthy animals (horizontal dashed lines), sham-operated animals, and rats with peritonitis via cecal ligation and puncture (CLP). For statistical comparison and percentile ranges, see Table 1.

Fig. 2. Time course of NO synthase (NOS)-2 and NOS-3 protein expression in the lungs, as assessed by immunoblotting. A, top: inducible NOS-2 expression (arrow shows the 130-kDa NOS-2 protein band). Shown is a representative (of 5 experiments) Western blotting analysis of lung homogenates obtained from rats at different time points after CLP to induce peritonitis, compared with healthy rats (lane LPS→) and a positive control [lane LPS+: healthy rat instilled intratracheally with lipopolysaccharide (LPS) and killed 4 h later]. In healthy rats, NOS-2 protein is almost undetectable and peritonitis induces NOS-2 protein, which is clearly evident from the 48th hour, as demonstrated elsewhere (27). A, bottom: endothelial NOS expression (arrow shows the 140-kDa NOS-3 protein band). Shown is a representative Western blotting analysis of lung homogenates obtained from rats at different time points after CLP to induce peritonitis, after aminoguanidine administration to a healthy rat (lane amino, see MATERIALS AND METHODS), after LPS instillation in a healthy rat (lane LPS+; healthy rat instilled intratracheally with LPS and killed 4 h later), and in a noninstilled healthy rat (lane LPS→). No significant modifications in endothelial NOS protein expression were evidenced except in the healthy rat given the NOS-2 inhibitor (aminoguanidine), as previously shown (26). B: densitometry analysis and statistical comparisons. Results (arbitrary units) are expressed as percentage of control condition (healthy rats = 100%). *P < 0.05 compared with control condition (dashed line).
isolated alveolar macrophages from healthy rats; † fi ed by L-arginine administration. A sim-recruitment was modi-stillation to assess whether the kinetic of alveolar neutrophil 1,089 (947 65) 590 (523 364) 630 (597 364) Healthy, % 100 330 (296 25 1.7) ppb, respectively, P < 0.05; however, despite increased NO production, alveolar neutrophil recruitment 4 h after endotoxin instillation was unmodified (l-arginine: 86 (69–122), 10^4 cells/ml vs. d-arginine: 75 (64–110), 10^4 cells/ml BAL fluid; P = NS). In an additional experiment, rats supplemented with either l-arginine or d-arginine (n = 4 per group) were killed 24 h after endotoxin instillation to assess whether the kinetic of alveolar neutrophil recruitment was modified by l-arginine administration. A similar number of alveolar neutrophil was demonstrated whatever the treatment (l-arginine: 426 (369–493), 10^4 cells/ml vs. d-arginine: 387 (276–456), 10^4 cells/ml BAL fluid; P = NS).

**DISCUSSION**

Increased levels of exhaled NO have been found during lung infections, as well as during intravenous endotoxin administration (1, 14). The contribution of alveolar macrophages to pulmonary NO production after endotoxin injection has been demonstrated by Fujii and colleagues (14). Unexpectedly, in our model of mild peritonitis, we found no increase in exhaled NO, but on the contrary, exhaled NO concentration was decreased after peritonitis. In keeping with our finding, a significant decrease in exhaled NO has been found in patients after cardiopulmonary bypass (18). Interestingly, L-arginine administration in this latter setting has been found to decrease the occurrence of infectious complications (16). The beneficial effects of immunonutrition using formulas with high content of L-arginine may be attributed to decreased blood concentration of L-arginine, which is a semissential amino acid (3). We found no increase in NO end products in plasma or BAL fluid, although NOS-2 protein was upregulated and NOS-3 protein unchanged. The decrease in lung NOS-2 activity and exhaled

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Basal</th>
<th>10 μM NOHA</th>
<th>100 μM NOHA</th>
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<tbody>
<tr>
<td>Healthy, %</td>
<td>100</td>
<td>330 (296–364)†</td>
<td>630 (597–671)‡</td>
</tr>
<tr>
<td>Peritonitis, %</td>
<td>37 (24–65)</td>
<td>590 (523–661)‡</td>
<td>1,089 (947–1,115)‡</td>
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</table>

Values are median (25th–75th percentiles) for each condition. NOHA, N-hydroxy-nor-l-arginine, diacetate salt, an arginase inhibitor. Results for all conditions are expressed as the percentage of basal (without NOHA) NO end product production by alveolar macrophages from healthy rats, n = 3 experiments per condition. *P < 0.05 compared with NO end-product production by isolated alveolar macrophages from healthy rats; †P < 0.05 compared with the basal condition.

Fig. 3. NOS activity measured in lung homogenates at predefined time points (1, 4, 24, 48, and 72 h) after CLP to induce peritonitis, compared with healthy rats (control, time 0) and sham-operated rats (studied at the 24th hour). NOS activities, in supernatant fraction (cytosol, mainly NOS-2) (A) and membrane fraction (mainly NOS-3) (B), were measured as the enzymatic conversion of L-[H^3]arginine to L-[H^3]citrulline as described in MATERIALS AND METHODS. The results are expressed in picomoles per milligram per protein per 45-min incubation; n = 4 rats in each group. Results are expressed as median (25th–75th percentiles). *P < 0.05 compared with control condition.

Fig. 4. Alveolar cell recruitment after endotoxin instillation in rats with peritonitis induced by CLP, compared with healthy rats treated by aminoguanidine (amino) or with healthy rats. Alveolar cell recruitment was assessed 4 h after endotoxin (LPS) instillation by bronchoalveolar lavage (BAL). Differential cell counts demonstrated that almost all recruited cells were neutrophils (>95% of total cells in LPS-instilled conditions). In healthy rats without LPS instillation, alveolar macrophages were the predominant cell type (>97%). Results are expressed as median (25th–75th percentiles). *P < 0.05 compared with healthy plus LPS condition. Median (25th–75th percentiles) exhaled NO concentration in each group: healthy plus LPS, 23.9 ppb (15.2–32.1); CLP-24 h plus LPS, 13.5 ppb (7.2–16.5); CLP-2 h plus LPS, 13.3 ppb (6.2–15.9); healthy plus LPS and aminoguanidine, 35.1 ppb (19.2–43.4).

Fig. 5. Macrophage inflammatory protein-2 (MIP-2) concentration in isolated alveolar macrophage supernatants. MIP-2 concentrations were measured in supernatants of isolated alveolar macrophages (4-h incubation) by ELISA (see MATERIALS AND METHODS). MIP-2 concentrations increased after both surgeries, i.e., sham surgery or CLP, and after treatment of healthy rats with an inducible NOS-2 inhibitor [aminoguanidine (amino)]. Results are expressed as median (25th–75th percentiles). *P < 0.05 compared with healthy condition.
NO further suggests that NO production was decreased in our model.

There is controversy in the literature over whether NO released during the inflammatory process favors or inhibits neutrophil migration. However, exposure of neutrophils to exogenous NO induces a rapid and persistent morphological hyperpolarization followed by migrational arrest due to reduction of the F-actin content (22). Moreover, NO supplied exogenously into reactions containing activated endothelium downregulates proinflammatory activity, such as the secretion of chemokines, and functional activity, such as transendothelial migration of neutrophils (13). These in vitro experiments may explain that the inhibition of neutrophil migration by NO is due to inhibition of a neutrophil-endothelium adhesion mechanism. In vivo experiments further support the role of NO in the inhibition of neutrophil migration at the time of severe sepsis (4) or subsequent to sepsis (for instance after intravenous endotoxin injection; Ref. 32). These effects of NO have previously been demonstrated to be mediated via inducible NOS-2 activity (29). Eventually, inhaled NO has also been demonstrated to reduce neutrophil emigration in some studies (15) (however, we found an opposite response in a bacterial pneumonia model; Ref. 19). Consequently, we were surprised to find an inhibition of neutrophil migration toward alveolar spaces after a mild peritonitis that was not associated with increased NO synthesis (at least in the pulmonary compartment from the first hour of injury). Furthermore, we have previously shown using the same animal model an increase in blood proinflammatory cytokines (2), which have been shown to inhibit subsequent neutrophil emigration via NO synthesis (31). On the other hand, in a model of trinitrobenzene sulfonic acid-induced colitis in rats, nitro-L-arginine methyl ester reduced neutrophil influx (17). Therefore, reduced NO synthesis has also been associated with inability for neutrophil to migrate.

A limitation of our study is the fact that sham-operated rats were not studied at all time points. These experiments showed a transient decrease in exhaled NO (4th hour) after sham surgery followed by an unexpected rebound of NO synthesis (24th hour). We did not subsequently performed experiments in sham rats inasmuch as statistical calculations demonstrated that we were unable to demonstrate significant differences between both surgeries using small number of animals (<30 per group). Consequently, from this study we cannot infer whether our results are related to surgical injury and/or septic injury. However, we previously showed that both surgeries were characterized by an increase in plasma IL-10 (2), an anti-inflammatory cytokine involved in immune paralysis (25). To further explore the decrease in exhaled NO in our rats with mild peritonitis, we conducted experiments on isolated alveolar macrophages, because these cells also showed a decrease in NO synthesis, suggesting a link between cellular and organ impairment of NO production. Several lines of evidence led us to hypothesize that stress, via increased production of catecholamines and their cellular effects mediated by cAMP, leads to arginine upregulation and decreased arginine availability for NOS, the end result being impaired NO production (5, 8, 28). We chose to explore this hypothesis in isolated alveolar macrophages because arginase regulation in these cells has been extensively described (7, 24, 33). In macrophages, l-arginine can be used by NOSs and arginases to form NO and urea, respectively. A similar balance between arginases and NOSs exists in endothelial cells (21, 38). Thus activation of arginases may be an effective mechanism for regulating NO production through substrate competition. Support for this possibility was provided by our experiments showing an increase in NOX production in the presence of a specific arginase inhibitor. The results of these ex vivo experiments are in agreement with in vitro evidence that there is impairment in NO production by macrophages or endothelial cells when both NOS-2 and arginase are induced by endotoxin (7, 21).

Although the reason for the decrease in lung NO production remains speculative, besides a decrease in substrate availability either relative (competition) or absolute (deficiency), several proteins have been shown to block the dimerization and activity of NOS isoforms (6). Furthermore, NOS-2 enzyme activity has been shown to be dependent on molecular oxygen levels (12); conceivably, a decrease in partial pressure of oxygen due to lung edema could also have reduced NO synthesis. The decrease in neutrophil emigration was not clearly related to defective substrate availability because L-arginine administration failed to improve neutrophil migration despite restoring exhaled NO concentration.

Inhibition of NOS-2 activity by treatment of healthy rats with aminoguanidine, which reduced both exhaled NO and NOX production by alveolar macrophages, reproduced the impairment of neutrophil migration to alveolar spaces. The impairment in neutrophil migration was not related to a decrease in MIP-2 secretion by alveolar macrophages, because MIP-2 secretion was stimulated after both CLP and aminoguanidine administration. This last finding may be ascribable to decreased NO production by alveolar macrophages because NO is known to inhibit nuclear factor-κB (via nitrosylation), a transcription factor responsible for proinflammatory cytokine upregulation (34). Given the lack of selectivity of the high dose of aminoguanidine administered, it is also conceivable that the inhibitor may have inhibited endothelial NOS-3 remote from the inflammatory locus, resulting in vasoconstriction, reduced blood flow delivery, and reduced neutrophil emigration toward alveolar spaces.

We previously showed that peritonitis was characterized by an increase in plasma IL-10 (2), a major anti-inflammatory cytokine involved in immune paralysis (25) and macrophage arginase upregulation (24). Interestingly, Tsuei and colleagues (35) have demonstrated an increased arginase expression in blood monocytes that may have contributed to postsurgical immune dysfunction, which may have been regulated via plasma IL-10 secretion. These results suggest that plasma IL-10 and exhaled NO may be markers for immune paralysis. NO exhalation measurement has the advantage of being noninvasive. Clinical studies are ongoing in our institution to determine whether the decrease in exhaled NO is related to the alveolar or to the bronchial compartment, both of which contribute to exhaled NO (10), and whether exhaled NO may be a prognosis factor in critically ill patients.

In summary, mild peritonitis (local infection) is associated with an early decrease in exhaled NO that is related to decreased lung production. This impaired NO production is associated with impaired neutrophil migration into the alveolar spaces that may contribute to immune paralysis in critically ill patients. Thus exhaled NO may represent a noninvasive marker for immune paralysis.
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