Inhibition of neuronal nitric oxide synthase by 7-nitroindazole attenuates acute lung injury in an ovine model


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Nitric oxide (NO) has been shown to play a major role in acute lung injury (ALI) after smoke inhalation. In the present study, we developed an ovine sepsis model, created by exposing sheep to smoke inhalation followed by instillation of bacteria into the airway, that mimics human sepsis and pneumonia. We hypothesized that the inhibition of neuronal NO synthase (nNOS) might be beneficial in treating ALI associated with this model. Female sheep (n = 26) were surgically prepared for the study and given a tracheostomy. This was followed by insufflation of 48 breaths of cotton smoke (40°C) into the airway of each animal and subsequent instillation of live Pseudomonas aeruginosa [5 × 10^11 colony forming units (CFU)] into each sheep's lung. All sheep were mechanically ventilated using 100% O_2. Continuous infusion of 7-nitroindazole (7-NI), an nNOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), a nonspecific NOS inhibitor, or aminoguanidine (AG), an inducible NOS inhibitor, was started 1 h after insult. The administration of 7-NI improved pulmonary gas exchange (PaO_2/FIO_2; where PaO_2 is arterial PO_2 and FIO_2 is fractional inspired oxygen concentration) and pulmonary shunt fraction and attenuated the increase in lung wet-to-dry weight ratio seen in the nontreated sheep. Histologically, 7-NI prevented airway obstruction, which, along with pulmonary edema, is accompanied by impairment of respiratory gas exchange and bacterial colonization of the lungs, leading to the development of tracheobronchitis, atelectasis, and pneumonia.

Nitric oxide (NO) is formed from arginine by NO synthase (NOS). There are three isoforms of NOS. One isoform, induced by cytokines and bacterial products (inducible NOS, iNOS), is reported to be harmful in sepsis (22, 35). Other isoforms are endothelial NOS (eNOS) and neuronal NOS (nNOS). NO derived from eNOS regulates vascular tone (5). Despite a large number of reports, the role of NO in the pathogenesis of sepsis, particularly in sepsis-related acute lung injury (ALI), is controversial. Some investigators theorize that NO is protective while others believe it to be harmful. Although the three NOS isoforms are expressed by different cell types, all three are expressed in the lung (10). While numerous studies exist showing the involvement of iNOS in ALI, there are few reports demonstrating the participation of eNOS or nNOS in the pathogenesis of ALI. nNOS is expressed in both central and peripheral neurons (5, 32). However, it is also present in airway epithelium, airway smooth muscle, submucosal glands, blood vessels, and in the airway intrinsic parasympathetic plexus (12). Feletou et al. (11) described the presence of mRNA for nNOS in the trachea. De Sanctis et al. (7) found that nNOS contributes 40% of the NO measured in mixed expired air in mice. These studies suggest the possible participation of nNOS in the pulmonary vascular response to pathological insults. Thus, in the present study, we aimed to clarify the possible role of nNOS in ALI in sheep that received smoke inhalation injury followed by airway bacterial instillation.

SMOKE INHALATION is a major determinant in the mortality of fire victims. By themselves, inhalation injury and pneumonia increase mortality by a maximum of 20 and 40%, respectively; when both are present, mortality is increased by a maximum of −60% (33). Inflammatory exudates and aggregates of mucus, sloughed epithelial mucosa, and cellular debris, together with impaired mucociliary clearance, lead to airway obstruction, which, along with pulmonary edema, is accompanied by impairment of respiratory gas exchange and bacterial colonization of the lungs, leading to the development of tracheobronchitis, atelectasis, and pneumonia.
MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee of the University of Texas Medical Branch and conducted in compliance with the guidelines of the National Institutes of Health and the American Physiological Society for the care and use of laboratory animals.

Materials. The selective nNOS inhibitor 7-nitroindazolazolo (7-NI) and aminoquinidine (AG) were purchased from Sigma-Aldrich (St. Louis, MO). N\textsuperscript{G}-monomethyl-L-arginine (l-NMMA) was obtained from Apex Biosciences (Durham, NC). The continuous infusion of 7-NI (1 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}) was begun 1 h after injury via the internal jugular vein and continued for 24 h. All other reagents used were analytical grade or better. *Pseudomonas aeruginosa* [5 × 10\textsuperscript{12} colony forming units (CFU)] was isolated and cultured from a male burn patient at Brook Army Medical Center (San Antonio, TX).

Experimental protocol. Our model of septic pneumonia has been described in detail previously (25). Briefly, 26 female sheep were prepared surgically for chronic study under halothane anesthesia. The right femoral artery was cannulated (Intracath, 16GA, 24IN, Becton Dickinson Vascular Access, Sandy, UT). A thermodilution catheter (Swan-Ganz, model 131F7, Baxter, Edwards Critical-Care Division, Irvine, CA) was introduced through the right common jugular vein into the pulmonary artery. A catheter (Duralastic Silicone Tubing DT08, 0.062 in inner diameter, 0.125 in outer diameter; Allied Biomedical, Paso Robles, CA) was also positioned in the left atrium through the fifth intercostal space. After 5 days of recovery, each animal received a tracheostomy, performed under ketamine-halothane anesthesia, and a smoke inhalation injury, produced by inhalation of cotton smoke (48 breaths, 40°C). Briefly, a bee smoker was filled with 40 g of burning cotton tallowing and then attached to the tracheostomy tube via a modified endotracheal tube containing an indwelling thermistor from Swan-Ganz catheter to monitor the temperature of the smoke. Four sets of 12 breaths of smoke were delivered, and the carboxyhemoglobin level was determined immediately after each set. After inhalation injury *Pseudomonas aeruginosa* was instilled into the airway through a bronchoscope. *Pseudomonas aeruginosa* was cultured overnight and centrifuged at 2,000 rpm for 15 min, after which the bacteria was mixed with saline and the number was adjusted to 1.5 × 10\textsuperscript{10} CFU/ml. From this solution, 10 ml was placed in the right middle part of lung, 10 ml was instilled in the right lower lobe, and 10 ml was placed in the left lower lung (25). After the injury, animals were maintained on mechanical ventilation (Servo Ventilator 900C, Siemens-Elema, Sweden) throughout the 24-h experimental period. Ventilation was performed with a positive end-expiratory pressure of 5 cmH\textsubscript{2}O and a tidal volume of 15 ml/kg. The inspiratory concentration of O\textsubscript{2} was maintained at 100%. The respiratory rate was maintained at 30 breaths/min, initially, and was adjusted further according to blood gas analysis. All animals received maintenance fluid resuscitation during the study period. Initially, animals were given fluid at 10 ml·kg\textsuperscript{-1}·h\textsuperscript{-1}; the rate was subsequently adjusted to maintain left atrial pressure above baseline and hematocrit at baseline levels. The animals were divided randomly into three groups: noninjured sham operated (n = 6), noninjured but not treated (n = 6), and treated (injured, but treated with 7-NI; n = 6). l-NMMA, a nonspecific NOS inhibitor (n = 3), was infused continuously (7 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}). AG, a specific inhibitor of iNOS, was infused with dose of 10 mg·kg\textsuperscript{-1}·h\textsuperscript{-1} (n = 5).

Measurements. The catheters inserted into the jugular vein, femoral artery, and left atrium were connected to the monitoring device (model 78304A, Hewlett Packard, Santa Clara, CA) by pressure transducers (model PX-1800, Baxter, Edwards Critical-Care Division, Irvine, CA) and measured the vascular pressures: pulmonary arterial pressure, central venous pressure, mean arterial pressure, and left atrial pressure. Cardiac output was measured by the thermodilution technique using a cardiac output computer (COM-1, Baxter, Edwards Critical-Care Division). A 5% dextrose solution (5°C) was used as the indicator. For the measurement of the blood gases, the blood was withdrawn through the catheter at different time points and analyzed using a blood gas analyzer (model IL 1600, Instrumental Laboratory, Lexington, MA). The blood gas results were corrected for the body temperature of the sheep. Hematocrit was measured in heparinized microhematocrit capillary tubes (Fisher Brand, Pittsburgh, PA).

Measurement of plasma nitrate/nitrite. The plasma NO levels were evaluated by measuring the intermediate and end products, nitrate/nitrite (NOx). For conversion of NOx to NO, the plasma samples were mixed with vanadium (III) and hydrochloric acid at 90°C in the NOx reduction assembly (ANTEK model 745, Antek Instruments, Houston, TX). Thereafter, the NO reacted with ozone in the reaction chamber of the chemiluminescent NO detector (ANTEK model 7020, Antek Instruments), and the emitted light signal was recorded by dedicated software as the NO content (μmol/l).

Measurement of tracheal blood flow. To determine the tracheal blood flow, colored (12 × 10\textsuperscript{6} fluorescent) microspheres (Interactive Medical Technologies, West Los Angeles, CA) were injected into the left atrium at different time points. Immediately after injection of microspheres, blood was withdrawn (Harvard Apparatus model 55–1143, South Natick, MA) from the femoral artery at the rate of 10 ml/min for 2 min. Tissue samples of trachea were obtained postmortem and used to quantify the tracheal blood flow (30).

Lung histology. After the animals were killed under anesthesia (24 h after insult), the lower lobe of the right lung of each animal was excised and inflated with 10% formalin. Fixed samples were embedded in paraffin, sectioned into 6-μm pieces, and stained with hematoxylin-eosin. A pathologist who was unaware of the group assignment analyzed the samples. The levels of airway obstruction were obtained with a standardized protocol. Fifteen bronchi and 50 bronchioles and respiratory bronchioles were investigated, and the percentage of total airway lumen obstructed by casts was estimated (0 to 100%) as previously described (25).

Measurement of lung wet-to-dry weight ratio. Animals were killed 24 h after insult, and lung tissues and blood were taken for measurement of lung wet-to-dry weight ratio (25). The lung tissues were weighed and then dried to a constant weight in an oven at 50°C. The wet-to-dry weight ratio was obtained by dividing the wet weight by the final weight of the dried lungs. In addition, we determined the blood wet-to-dry ratio to calculate the bloodless lung wet-to-dry ratio to exclude contribution of blood.

Statistical analysis. Data are presented as means ± SE. Results were compared through ANOVA and Scheffé’s post hoc test or the unpaired t-test. A value of P < 0.05 was accepted as statistically significant.

RESULTS

During the 24-h experimental period, all animals survived smoke inhalation injury followed by airway instillation of bacteria. The arterial carboxyhemoglobin levels immediately after smoke exposure were 71.0% in the nontreated group and 69.7% in the 7-NI-
treated group. There was no statistical difference between these values, indicating that both control and treated animals received similar injuries. The sham-operated group received 48 breaths of sham smoke (air from empty bee smoker), and the arterial carboxyhemoglobin level after the sham smoke was 4.7%.

**Effect of 7-NI on plasma NOx.** The plasma NOx levels began to increase 3 h after insult in the nontreated group and remained elevated throughout the experimental period (Fig. 1). Treatment with 7-NI resulted in marked inhibition of plasma NOx, especially the first 12 h. At this time period plasma NOx was reduced almost to the normal range.

**Effect of 7-NI on pulmonary gas exchange and pulmonary shunt.** Pulmonary gas exchange was stable in sham-operated animals (Fig. 2), but PaO/FIO2 (where PaO2 is arterial Po2 and FIO2 is fractional inspired concentration) began to decrease significantly in nontreated animals beginning 3 h after insult, reaching its lowest point at 12 h after injury. Treatment with 7-NI resulted in significant attenuation of this decrease in PaO/FIO2 ratio. In nontreated animals, pulmonary shunt fraction increased beginning 3 h after injury and peaked at 12 h. This increase was significantly attenuated by continuous infusion of 7-NI (Fig. 3).

**Effect of 7-NI on airway obstruction.** Both bronchi and bronchioles were markedly obstructed in nontreated animals, but inhibition of nNOS resulted in significant reduction of the airway obstruction, especially in bronchioles. The airway obstruction scores were ~40% of both bronchi and bronchioles in the nontreated group and 25 and 10% of bronchi and bronchioles, respectively, in animals treated with 7-NI (Fig. 4).

**Effect of 7-NI on tracheal blood flow.** The changes in airway blood flow are shown in Fig. 5. In the nontreated group, the tracheal blood flow was dramatically increased after smoke inhalation and bronchial instillation of bacteria. However, this increase was significantly attenuated by 7-NI treatment.

**Effect of 7-NI on pulmonary shunt fraction (Qs/Qt).** Data are expressed as means ± SE. Qs, shunt; Qt, total blood flow. *P < 0.05 vs. sham; †P < 0.05 vs. control.

**Effect of 7-NI on airway pressures.** The airway pressures (peak and pause airway pressure) were stable in sham-operated animals but were markedly increased in control animals beginning from 6 h after insult. Treatment with 7-NI significantly reduced this increase in both peak (Fig. 6A) and pause pressures (Fig. 6B).

**Effect of 7-NI on lung wet-to-dry ratio.** An estimate of the degree of pulmonary edema was provided by the lung wet-to-dry ratio of the right lung (Fig. 7). The ratio was significantly increased in the nontreated group compared with noninjured sham animals. In the treated group, however, treatment with 7-NI significantly inhibited this increase.

**Effect of L-NMMA and AG on pulmonary functions.** In Table 1, we showed the effects of two different NOS inhibitors on derangements in pulmonary gas exchange and oxygen saturation of arterial blood seen in control animals. Treatment with a nonspecific NOS
inhibitor (l-NMMA) reversed these changes induced by smoke inhalation and airway instillation of bacteria while a specific inhibitor of iNOS (AG) did not.

DISCUSSION

In this study we showed the beneficial effect of 7-NI, a selective inhibitor of nNOS, in sheep receiving smoke inhalation injury followed by bacterial instillation into the airway. The advantages of this sepsis model over other animal models include the ability to create a response to smoke inhalation injury associated with pneumonia that is quite similar to that noted in humans and the capacity to monitor cardiopulmonary changes over a long time period (≥24 h). During the experimental time, sheep were placed on a ventilator with 100% oxygen supply to 1) allow for a rapid disappearance of carboxyhemoglobin after smoke inhalation, and 2) maintain the arterial oxygen saturation above 90% throughout the experimental period (which was necessary). Mechanical ventilation with 100% oxygen for 24 h did not alter cardiopulmonary variables in sham-operated animals, and the instillation of 20 and 10 ml normal saline in the right and left lung, respectively, did not result in noticeable changes in this group, either.

Control animals showed an increase in cardiac index, heart rate, and body temperature, all of which are
Table 1. Effect of L-NMMA and AG on pulmonary derangements induced by smoke inhalation and airway bacterial instillation

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Control</th>
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<th>AG</th>
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<tbody>
<tr>
<td>0</td>
<td>503 ± 26</td>
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<td>359 ± 19*</td>
<td>147 ± 25</td>
</tr>
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<td>65 ± 14</td>
<td>281 ± 51*</td>
<td>120 ± 23</td>
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<td>192 ± 41*</td>
<td>119 ± 30</td>
</tr>
<tr>
<td>24</td>
<td>100 ± 19</td>
<td>265 ± 31*</td>
<td>113 ± 22</td>
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</tbody>
</table>

Values are means ± SE. PaO2/FiO2, pulmonary gas exchange, where PaO2 is arterial Po2 and FiO2 is fractional inspired oxygen concentration; SaO2, arterial oxygen saturation; shunt, pulmonary shunt fraction. Control: injured, nontreated (n = 6); L-NMMA: injured, but treated with Nω-monomethyl-L-arginine, a nonspecific nitric oxide synthase (NOS) inhibitor (n = 3); AG: injured, but treated with aminoxyguanidine, a specific inducible NOS (iNOS) inhibitor (n = 5). *P < 0.05 vs. control.

In the present study, the inhibition of nNOS by 7-NI results in inhibition of airway cast formation by 10.2 ± 0.3 mm on July 9, 2017. Furthermore, the histological analysis of lung tissue showed that the decrease in PaO2/FiO2 ratio was significantly inhibited by 10.2 ± 0.3 mm on July 9, 2017. Additionally, the inhibition of NO has been reported to reduce sepsis-induced lung injury and decrease pulmonary shunt fraction (13, 26). In the present study, treatment with 7-NI after smoke inhalation injury and instillation of bacteria resulted in significant inhibition of the increased intrapulmonary shunt fraction. These observations suggest that 7-NI may improve the pulmonary gas exchange through inhibiting the shunt fraction.

The histological analysis of lung tissue showed that 38% of bronchioles were obstructed in nontreated animals. Tracheobronchial casts are composed of a thick, tenacious coagulum consisting of sloughed tracheobronchial epithelium, leukocytes, and ultrafiltrate that leaks across the damaged microvascular barrier as a result of smoke inhalation injury (15, 4). Airway occlusion results in edema, congestion, atelectasis, and pneumonia (17). In addition, airway obstruction leads to higher airway resistance and, hence, greater pressure requirements that cause barotrauma of nonobstructed portions of the lung (20, 8, 19). Sheep treated with 7-NI showed a significantly lower airway obstruction score, especially in small airways, compared with the nontreated group. In addition, treatment with 7-NI significantly reduced the increase in both peak and pause airway pressures seen in nontreated animals. Thus inhibition of nNOS by 7-NI results in inhibition of airway cast formation and improvement in pulmonary gas exchange. A reduction in tracheal blood flow produced by 7-NI might be the mechanism of reduction of cast formation, since this circulation is the main source of airway exudate.
Consistent with these beneficial effects of 7-NI, lung edema formation, as measured by the lung wet-to-dry weight ratio, was decreased in treated animals compared with controls. Although increased lung water content seen in control animals may have been caused by direct bacterial effects and smoke insufflation, it is likely that the inhibition of airway blood flow by 7-NI resulted in less edema formation in the lung interstitial space. Bronchial blood flow has been shown to increase immediately after smoke inhalation injury (2, 34). Because bronchial venous drainage enters the pulmonary vasculature through various bronchopulmonary anastomoses (31), it has been suggested that the bronchial circulation plays a significant role in the spread of injury from the airway of the lung to the parenchyma (1, 14). We have previously shown that the bronchial circulation plays a significant role in lung edema formation after smoke inhalation (30). Efimova et al. (9) showed that ligation of the bronchial artery, but not the pulmonary artery, reduced lung edema formation after smoke inhalation. Thus treatment with 7-NI may attenuate the increase in lung wet-to-dry ratio by preventing airway hyperemia through the inhibition of NO production.

Finally, in the present study, we showed that a nonspecific NOS inhibitor, L-NMMA, reduced pulmonary derangements in sheep that received smoke inhalation and bacterial instillation in airway while AG, a specific inhibitor of iNOS, did not (Table 1). Ischipouroulos et al. (18) demonstrated a beneficial effect of L-NAME in ALI induced by smoke inhalation. Park et al. (28) reported that 7-NI, but not AG, attenuated the inflammatory responses and brain injury during Escherichia coli meningitis in piglets. The results of our present study suggest that 7-NI, a selective nNOS inhibitor, may attenuate ALI induced by smoke inhalation followed by bacterial instillation in sheep.

REFERENCES


