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

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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) as a neurotransmitter (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 bacterial instillation in the airway.

acute respiratory distress syndrome; smoke inhalation; pneumonia

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.


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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-nitroindazole (7-NI) and aminoguanidine (AG) were purchased from Sigma-Aldrich (St. Louis, MO). NωNω-monomethyl-L-arginine (L-NMMA) was obtained from Apex Biosciences (Durham, NC). The continuous infusion of 7-NI (1 mg·kg⁻¹·h⁻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 × 10⁶ 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 toweling 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 × 10¹⁰ 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 cmH₂O and a tidal volume of 15 ml/kg. The inspiratory concentration of O₂ was maintained at 100%.

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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 × 10⁶ 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-

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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 non-treated 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 PaO2/FI02 (where PaO2 is arterial Po2 and FI02 is fractional inspired concentration) began to decrease significantly in non-treated 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 PaO2/FI02 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 non-treated 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 non-treated 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 blood flow. The changes in airway blood flow are shown in Fig. 5. In the non-treated 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 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

**Fig. 1.** Effect of 7-nitroindazole (7-NI) on plasma levels of nitrate/nitrite (NOx). The plasma nitric oxide levels were evaluated by measuring its intermediate and end products, nitrate/nitrite. Data are expressed as means ± SE. †P < 0.05 vs. control.

**Fig. 2.** Effect of 7-NI on PaO2/FI02 ratio, where PaO2 is arterial Po2 and FI02 is fractional inspired oxygen concentration. Data are expressed as means ± SE. *P < 0.05 vs. sham; †P < 0.05 vs. control.

**Fig. 3.** 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.
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

**Fig. 4.** Effect of 7-NI on airway obstruction. Lung histology was evaluated by a pathologist to determine the percent airway obstruction. Data are shown as percentages and are expressed as means ± SE. *P < 0.05 vs. sham (bronchi); †P < 0.05 vs. control (bronchi); ‡P < 0.05 vs. sham (bronchiole); §P < 0.05 vs. control (bronchiole).

**Fig. 5.** Effect of 7-NI on tracheal blood flow. Tracheal blood flow was measured by using colored microspheres. Data are expressed as means ± SE. †P < 0.05 vs. control.

**Fig. 6.** Effect of 7-NI on peak (A) and pause (B) airway pressures. Data are expressed as means ± SE. *P < 0.05 vs. control.

**Fig. 7.** Effect of 7-NI on changes in lung wet-to-dry weight ratio. The bloodless lung wet-to-dry weight ratio was obtained by measuring the blood wet-to-dry weight ratio. Data are expressed as means ± SE. *P < 0.05 vs. sham; †P < 0.05 vs. control.
Table 1. Effect of l-NMMA and AG on pulmonary derangements induced by smoke inhalation and airway bacterial instillation

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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 aminoguanidine, a specific inducible NOS (iNOS) inhibitor (n = 5). *P < 0.05 vs. control.

Characteristics of hyperdynamic sepsis (25). These animals also displayed marked increases in plasma NOx levels, suggesting the possible involvement of NO in the pathogenesis of lung injury in this model. The increase in plasma NOx was significantly inhibited by 7-NI, especially the first 12 h. This result suggests that the increase in plasma NOx is mainly due to nNOS. However, the plasma NOx was not completely inhibited by 7-NI over the second 12 h, suggesting a possible upregulation of other isoforms of NOS, particularly iNOS, which has been shown previously to participate in the pathogenesis of sepsis-related ALI (21). However, the fact that 7-NI inhibits the elevation of NO significantly may suggest that there is an increased activity of the nNOS isoform. nNOS has been shown to be expressed in the airway epithelium, especially in lower bronchi (7, 36), and pulmonary vascular endothelium. It has also reported that ~40% of exhaled NO is derived from nNOS in mice (7). Inhibition of nNOS resulted in significant attenuation of acute inflammatory response and brain injury during bacterial meningitis (27). De Sanctis et al. (6) demonstrated an important role of nNOS in airway hyperresponsiveness in an allergic asthma model using nNOS knockout mice. These observations suggest that nNOS may participate in the pathogenesis of ALI.

There is debate as to whether 7-NI is a specific nNOS inhibitor. 7-NI inhibits both nNOS and eNOS in vitro, but it does not influence the endothelium-dependent relaxation of blood vessels in vivo (3, 24). Thus it is considered that 7-NI does not affect eNOS in vivo. Based on this finding, many investigators use 7-NI as an nNOS inhibitor.

In the present study, the inhibition of nNOS by 7-NI resulted in improved pulmonary gas exchange that was markedly depressed in animals with smoke inhalation injury followed by airway instillation of Pseudomonas aeruginosa. The decrease in Pao2/Fio2 ratio was associated with a sevenfold increase in pulmonary shunt fraction. It is known that an intrapulmonary shunting is the predominant gas exchange abnormality in acute respiratory distress syndrome (29). Excessive production of NO causes a loss of hypoxic vasoconstriction and vasodilatation in the low or nonventilated areas of the lung, thus leading to poor oxygenation. Hopkins et al. (16) reported that inhaled NO increased blood flow to areas of low ventilation/perfusion ratio and thereby worsened gas exchange. 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 ~40% of bronchi and 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). Ischiropoulos 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


