Inhaled nitric oxide increases surfactant protein gene expression in the intact lamb

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Stuart, Regan B., Boaz Ovadia, Vincent V. Suzara, Patrick A. Ross, Stephan Thelitz, Jeffrey R. Fineman, and Jorge A. Gutierrez. Inhaled nitric oxide increases surfactant protein gene expression in the intact lamb. Am J Physiol Lung Cell Mol Physiol 285: L628–L633, 2003.—Inhaled nitric oxide (iNO) is used to treat a number of disease processes. Although in vitro data suggest that nitric oxide (NO) alters surfactant protein gene expression, the effects in vivo have not been studied. The objective of this study was to evaluate the effects of iNO on surfactant protein (SP)-A, -B, and -C gene expression in the intact lamb. Thirteen 4-wk-old lambs were mechanically ventilated with 21% oxygen and received iNO at 40 ppm (n = 7) or vehicle gas (n = 6) for 24 h. Peripheral lung biopsies were obtained at 0, 12, and 24 h and analyzed for surfactant mRNA, protein, and total DNA content. Inhaled NO increased SP-A and SP-B mRNA content by 80% from 0 to 12 h and by 78 and 71%, respectively, from 0 to 24 h. There was an increase in SP-A and SP-B protein content by 45% from 0 to 12 h, and a decrease by 70 and 65%, respectively, from 0 to 24 h. DNA content was unchanged. The mechanisms and physiological effects of these findings warrant further investigation.

Inhaled nitric oxide (iNO) is a free radical that serves as an important cellular messenger affecting vascular tone (14), platelet adhesion (17), immunological responses (8), and neurotransmission (27). Endogenous NO is synthesized in endothelial cells from arginine and activates soluble guanylyl cyclase, which catalyzes the formation of cGMP, relaxes smooth muscle cells, and activates protein kinases (14). Exogenously administered inhaled NO (iNO) has been shown clinically to be a potent, selective pulmonary vasodilator (11, 28). It is proven effective in reducing pulmonary vascular resistance in newborns with persistent pulmonary hypertension (5, 6, 10, 22) and in children with various congenital heart diseases who suffer from postoperative pulmonary hypertension (2, 19). Recently, iNO has also been used to improve oxygenation in acute lung injury by increasing ventilation and perfusion matching, thereby decreasing the shunt fraction (20, 23).

However, NO has been shown to damage surfactant proteins and alter their function (13). A recent in vitro study by Ayad and Wong (4) demonstrated that NO negatively affected pulmonary cell viability, surfactant protein expression, and modulated surfactant protein (SP)-A gene expression in a human lung tumor cell line (H441) known to have similar cellular properties as distal lung epithelium. Matalon et al. (18) ventilated newborn lambs with multiple concentrations of NO through tracheostomy tubes in an open ventilation circuit. Bronchoalveolar lavage (BAL) fluid from the lambs exposed to high doses of iNO (80–200 ppm) for 6 h showed damage to SP-A apoproteins and a decreased ability to aggregate lipids in vitro. Evaluation of BAL fluids from both rats and piglets after prolonged NO exposure (24–48 h) at 100 ppm demonstrated increased pulmonary inflammation, damage to epithelial fluid lining, and alteration in the surface active properties of surfactant (15, 21).

On the other hand, several studies have shown NO to have beneficial effects on surfactant function as well. In a study evaluating BAL fluid from rabbits treated with low-dose iNO (20 ppm), Sison et al. (25) showed that NO exposure improved alveolar surface activity, increased large surfactant aggregates, and was beneficial to gas exchange. NO at higher doses (80 ppm) has been shown to enhance the surface activity of surfactant (16).

The aim of our study was to determine the effect of iNO at a moderate dose (40 ppm) on SP-A, -B, and -C gene expression in the intact lamb. To this end, we ventilated 4-wk lambs with 21% oxygen and 40 ppm of iNO for 24 h. Intermittent lung biopsies were sampled for the determination of SP-A, -B, and -C mRNA content and SP-A and -B protein content.

METHODS

Experimental protocol. Thirteen 4-wk-old lambs were anesthetized with intravenous infusions of ketamine hydrochloride (1 mg·kg⁻¹·min⁻¹) and diazepam (0.002 mg·kg⁻¹·h⁻¹), intubated with a 6.0–7.0-mm outer diameter endotracheal tube, and mechanically ventilated with a Healthdyne pediatric time-cycled, pressure-limited ventilator (Health-
The lambs were ventilated with 21% oxygen, and the rate were monitored continuously to ensure adequate anesthesia. The lambs were ventilated with an ultrasonic and RA, respectively) and main pulmonary artery (PA). An ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the left PA to measure left pulmonary blood flow (Q). After a 60-min recovery, baseline hemodynamic variables (LA, RA, PA, heart rate, systemic arterial pressure, and oxygen saturations) were continuously monitored. A side-biting vascular clamp was utilized to isolate peripheral lung tissue from a randomly selected lobe, and the incision was cauterized. Approximately 300 mg of peripheral lung were obtained. iNO (40 ppm) was then delivered in nitrogen into the inspiratory limb of the ventilator (INOvent; Datex-Ohmeda, Andover, MA) in seven of the lambs and was continued for 24 h. The other six lambs were ventilated, without iNO, for 24 h. Peripheral lung biopsies were repeated at 12 and 24 h. Body temperatures were maintained between 38 and 24°C and maintained between 38° and 24°C with a warming blanket. The midline incisions were approximated and closure maintained with Kelly clamps.

At the end of the study period, the lambs were euthanized by an intravenous injection of pentobarbital sodium (Euthasol CII; Central City Medical, Union City, CA). The committee on Animal Research of the University of California, San Francisco (UCSF), approved all procedures and protocols.

Lung tissue preparation. Lung tissue biopsies were weighed for wet weight values and snap frozen in liquid nitrogen. Tissue samples were stored at −80°C until use.

Hemodynamic measurements. PA, LA, RA, and systemic pressures were measured by Sorenson Neonatal Transducers (Abbot Critical Care Systems, Chicago, IL). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiotachometer triggered from the phasic systemic arterial pressure pulse wave. Q was measured on an ultrasonic flow meter (Transonic). All hemodynamic variables were recorded continuously on a Gould multichannel electrostatic recorder (Gould, Cleveland, OH). Systemic arterial blood gases and pH were measured on a Radiometer ABL5 pH and blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxygen saturation were measured by a hemoximeter (model 270, Ciba-Corning). The pulmonary-to-systemic blood flow ratio was calculated using the Fick principle.

Preparation of RNA, Northern blotting, and hybridization. Lung tissue was pulverized and then briefly homogenized in RNA-STAT (Tel-Test, Friendswood, TX). Total cellular RNA was extracted with phenol-chloroform, precipitated with isopropanol, and quantitated spectrophotometrically. RNA integrity was assessed by electrophoresis. Total RNA (10 μg/sample) was separated electrophoretically on 1% agarose gels. Total RNA was transferred to positively charged nylon membranes (BrightStar-Plus; Ambion, Austin, TX) and cross-linked with ultraviolet light (UV Stratalinker 2400; Stratagene, La Jolla, CA). Filters were probed with cDNAs for ovine SP-A, SP-B, and SP-C (a kind gift of Dr. Phillip Ballard) and 18s rRNA, labeled with [α-32P]dCTP (NEN Research Products, Boston, MA) by random-primer second strand synthesis (Random Primer Labeling Kit; Gibco-BRL, Gaithersburg, MD). Filters were prehybridized for 10 min in QuikHyb hybridization solution (Stratagene) at 68°C and then hybridized in 10 ml of QuikHyb solution containing 1.25 × 10⁶ dpm/ml for 18 h. Hybridized filters were washed under high stringency conditions and subjected to autoradiography (Hyperfilm; Amersham CEA, Uppsala, Sweden). Radioabeled bands were quantified by volume integration of pixels measured by phosphorimage analysis (Imagequant software; Molecular Dynamics, Sunnyvale, CA). Filters were also probed for 18s rRNA as a control measure to ensure equal loading of samples.

*Significant increase in mRNA content between NO-treated samples; ‡Significant increase in mRNA content between NO-treated lambs and the time-matched controls; P < 0.05.
Protein measurement. Protein content was measured by the bicinchoninic acid method (Pierce, Rockford, IL) (26).

Lung tissue dry weight measurement. To estimate dry weight, we dried a sample of peripheral lung tissue for 72 h in a vacuum oven at 86°C and weighed it. Dry weight measurements were used in determining surfactant protein and DNA content.

Quantification of SP-A and SP-B by dot-blot analysis. Lung tissue was thawed at room temperature (RT) and homogenized to powder consistency and dounced in 1 ml 50 mM NaHCO₃ (pH 9). The samples were sonicated on ice for 30 s each and centrifuged at 14,000 revolutions/min for 1 min. The supernatant was removed, and protein content was measured as above. SP-A and SP-B were assayed by quantitative dot blotting. Appropriate amounts of supernatant were diluted 1:10 with 50 mM NaHCO₃, pH 9. Duplicate dots of serial dilutions were assayed on the same piece of nitrocellulose (Bio-Dot Slot Format; Bio-Rad Laboratories, Richmond, CA). A gentle vacuum was applied to the dot-blot apparatus until all the solution was pulled through. The nitrocellulose was removed from the apparatus, and endogenous peroxidase activity was quenched with 15% H₂O₂ for 5 min. Nonspecific binding was blocked with a solution of 5% nonfat dry milk, 0.3% bovine serum albumin, 0.4% gelatin, and 20 mM Tris-buffered saline (TBS), pH 7.4, at RT for 60 min. The blots were washed in Tris-buffered saline with 0.5% Tween 20 (TBS-T), five washes for 5 min each, and probed separately for SP-A and SP-B proteins. The blots were incubated in primary rabbit antibody (1:3,000 dilution in TBS-T) against ovine sheep SP-A and SP-B (a kind gift of Dr. Sam Hawgood, UCSF Medical Center, San Francisco, CA) for 30 min. The blots were incubated in peroxidase-labeled, secondary donkey antibody (1:2,000 dilution in TBS-T) against rabbit Ig (Amersham CEA) for 20 min. Specificity and sensitivity of these...
Table 1. Hemodynamic data

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>NO Treated</th>
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<tr>
<td></td>
<td>Time 0</td>
<td>12 h</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>17 ± 4</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>72 ± 11</td>
<td>79 ± 13</td>
</tr>
<tr>
<td>LAP, mmHg</td>
<td>7 ± 4</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>6.5 ± 4</td>
<td>6 ± 1</td>
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<tr>
<td>PVR, mmHg/min</td>
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<td>0.48 ± 0.4</td>
</tr>
<tr>
<td>PBF, ml·kg⁻¹·min⁻¹</td>
<td>38.3 ± 8</td>
<td>36.1 ± 13</td>
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<tr>
<td>HR, beats/min</td>
<td>160 ± 20</td>
<td>164 ± 13</td>
</tr>
<tr>
<td>pH</td>
<td>7.38 ± 0.04</td>
<td>7.41 ± 0.04</td>
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<tr>
<td>Pao2, Torr</td>
<td>39.5 ± 10</td>
<td>38 ± 5</td>
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Values are means ± SE. Shown is a summary of the measured and calculated hemodynamic data for nitric oxide (NO)-treated and ventilated-only lambs. PAP, pulmonary arterial pressure; SAP, systemic arterial pressure; LAP, left atrial pressure; RAP, right atrial pressure; PVR, pulmonary vascular resistance; PBF, pulmonary blood flow; HR, heart rate. *P < 0.05.

RESULTS

Effects of iNO on mRNA content of SP-A, SP-B, and SP-C. Figure 1 demonstrates the effects of iNO on surfactant protein mRNA content at 12 and 24 h. SP-A mRNA content increased between 50 and 85% (P < 0.05) in the NO-treated lambs (n = 3) at 12 h and between 60 and 77% (P < 0.05) at 24 h, compared with pre-NO treatment (time 0) and with the time-matched, ventilated-only lambs (n = 6). SP-B mRNA content increased between 60 and 80% (P < 0.05) in the NO-treated lambs at 12 h and between 30 and 71% (P < 0.05) at 24 h, compared with time 0 and with the time-matched, ventilated-only lambs. Although there was an increase in SP-C mRNA content at both 12 and 24 h, these differences were significant only at 12 h, increasing between 30 and 40% (P < 0.05), compared with time 0 and with the time-matched, ventilated-only lambs. There was no change in the SP-A, SP-B, or SP-C mRNA content of the ventilated-only lambs at 12 and 24 h compared with time 0 values (Fig. 2).

Effects of iNO on protein levels of SP-A and SP-B. Figure 3 demonstrates the effects of iNO on surfactant protein content at 12 and 24 h. SP-A protein content increased between 30 and 45% (P < 0.05) at 12 h and decreased between 40 and 70% (P < 0.05) at 24 h, compared with time 0 and with the time-matched, ventilated-only lambs. SP-B protein content increased between 44 and 50% (P < 0.05) at 12 h and decreased between 30 and 64% (P < 0.05) at 24 h, compared with time 0 and with the time-matched ventilated-only lambs. There was no change in SP-A or SP-B protein content of the ventilated-only sheep at 12 and 24 h compared with time 0 values (Fig. 4).

Effects of iNO on DNA content. To determine whether changes in surfactant protein mRNAs were due to changes in cell numbers, we measured DNA content at all time points. Figure 5 demonstrates that there was no significant change in DNA content in the NO-treated lambs at 12 and 24 h compared with time 0 values. There was no difference between the NO-treated lambs and their time-matched controls. There was no difference in DNA content in ventilated-only lambs at 12 and 24 h.

DISCUSSION

Surfactant is necessary in epithelial lung fluid to decrease surface tension at the alveolar air–liquid interface and prevent alveolar collapse at low lung volumes (3, 7, 30). There have been many recent in vitro and in vivo studies regarding NO and its effects on surfactant protein homeostasis in animals. The aim of
our study was to determine the effect of inhaled NO at a moderate dose (40 ppm) on SP-A, -B, and -C gene expression in the intact lamb. This study demonstrates that SP-A, SP-B, and SP-C mRNA content was significantly elevated in NO-treated lambs at 12 h and remained so for SP-A and SP-B at 24 h. Interestingly, these findings differ from the results of Ayad and Wong (4). In their study, the authors demonstrate that NO exposure negatively affects SP-A gene expression at the transcriptional level in distal respiratory epithelial cells in vitro. There are many potential reasons for these disparate findings. First, the model systems used in the two studies are markedly different. Ayad and Wong conducted in vitro studies utilizing a transformed cell line, whereas the current study was conducted in vivo utilizing a whole animal model. Another major difference is the delivery of NO. The cells were treated with the NO donor S-nitroso-N-acetyl penicillamine, whereas in the present study, lambs received 40 ppm of iNO. SP-A and SP-B protein content was increased at 12 h of iNO treatment. The increase in surfactant protein content at 12 h could be in response to the increase in mRNA expression at 12 h of NO treatment. Interestingly, we found that SP-A and SP-B proteins were decreased at 24 h compared with pretreatment levels. The decrease in surfactant proteins seen at 24 h does not appear to be due to cell loss, as our DNA values were not significantly different in the iNO-treated lambs at 12 and 24 h compared with time 0 values. Although we think that the cells most likely to be injured by iNO include type I and type II alveolar epithelial cells because they are exposed to the highest concentration of NO, the small increase in DNA, as well as the increase in surfactant protein mRNA at 24 h of treatment, would argue against type II cell injury. However, alterations in the epithelial cell surface can be subtle (9), and if our small increase in DNA represents an increase only in type II cells, which contribute ~16% of whole lung DNA pool (9), this might explain the increase in surfactant mRNA seen at 24 h of therapy but not the decrease in surfactant protein content.

It is possible that iNO resulted in oxidation or reduction of the proteins, which may have rendered them less detectable to our antibodies. NO may have damaged the surfactant proteins directly or by the formation of peroxynitrite (ONOO⁻) and lipid peroxidation. ONOO⁻ is an oxidant formed rapidly by the reaction of NO and O₂ in vivo. Several in vitro studies by Haddad et al. (13) have shown that ONOO⁻ inhibits pulmonary surfactant function and ability to aggregate lipids by lipid peroxidation and damage to surfactant proteins. In these studies, rat alveolar type II cells were exposed to an NO donor in the presence of superoxide anion. This resulted in a 60% decrease in the rate of surfactant synthesis, most likely due to the formation of ONOO⁻.

We studied NO effects on gene expression by obtaining peripheral lung biopsies from lambs ventilated with 21% oxygen. Oxygen use has been frequently studied in association with NO therapy as the two are commonly used together clinically. Hyperoxia is known to cause varying damage to surfactant proteins and alter their function (1, 21). We chose to ventilate the lambs with 21% oxygen to more accurately assess the sole effects of inhaled NO on distal respiratory epithelium. Ventilation alone has been shown to alter the pulmonary surfactant system (29). The 13 4-wk-old lambs were ventilated with similar support for the same length of time. There was no difference between groups in the tidal volume, rate, or mean airway pressure used. None of the lambs had known pulmonary abnormalities or illness. There were no statistically significant changes in mRNA, protein, or DNA content in the lung biopsies of the ventilated-only lambs.

In summary, iNO at 40 ppm resulted in an increase of SP-A, SP-B, and SP-C mRNA content and SP-A and SP-B protein content at 12 h of therapy. Interestingly, SP-A and SP-B proteins were decreased at 24 h of therapy compared with controls despite the persistent elevation of the mRNA. The mechanisms by which iNO affect surfactant protein gene expression remain to be elucidated. Further in vivo studies into the mechanisms of these alterations may lead to a better understanding of the effects of NO on alveolar epithelial function.

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DISCLOSURES

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