Endothelial alterations during inhaled NO in lambs with pulmonary hypertension: implications for rebound hypertension

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Ross, Gregory A., Peter Oishi, Anthony Azakie, Sohrab Fratz, Robert K. Fitzgerald, Michael J. Johengen, Cynthia Harmon, Karen Hendricks-Munoz, Jie Xu, Stephen M. Black, and Jeffrey R. Fineman. Endothelial alterations during inhaled NO in lambs with pulmonary hypertension: implications for rebound hypertension. Am J Physiol Lung Cell Mol Physiol 288: L27–L35, 2005. First published September 3, 2004; doi:10.1152/ajplung.00144.2004.—Clinically significant increases in pulmonary vascular resistance (PVR) have been noted upon acute withdrawal of inhaled nitric oxide (iNO). Previous studies in the normal pulmonary circulation demonstrate that iNO decreases endothelin-1 (ET-1) levels and decreases endogenous nitric oxide synthase (NOS) activity, implicating an endothelial etiology for the increase in resistance upon iNO withdrawal. However, the effect of iNO on endogenous endothelial function in the clinically relevant pulmonary hypertension is unknown. The objective of this study was to determine the effects of iNO on endogenous NO-cGMP and ET-1 signaling in lambs with preexisting pulmonary hypertension secondary to increased pulmonary blood flow. Eight fetal lambs underwent in utero placement of an aortopulmonary vascular graft (shunt lambs). After delivery (4 wk), the shunt lambs were mechanically ventilated with iNO (40 ppm) for 24 h. After 24 h of inhaled NO, plasma ET-1 levels increased by 34.8% independently of changes in protein levels (P < 0.05). Contrary to findings in normal lambs, total NOS activity did not decrease during iNO. In fact, Western blot analysis demonstrated that tissue endothelial NOS protein levels decreased by 43% such that NOS activity relative to protein levels actually increased during iNO (P < 0.05). In addition, the β-subunit of soluble guanylate cyclase decreased by 70%, whereas phosphodiesterase 5 levels were unchanged (P < 0.05). Withdrawal of iNO was associated with an acute increase in PVR, which exceeded baseline PVR by 45%, and a decrease in cGMP concentrations to levels that were below baseline. These data suggest that the endothelial response to iNO and the potential mechanisms of rebound pulmonary hypertension are dependent upon the underlying pulmonary vasculature.

endothelium-derived factors; pulmonary heart disease; nitric oxide; endothelin-1

EXOGENOUSLY ADMINISTERED INHALED NO is currently utilized as an adjuvant therapy for a number of pulmonary hypertensive disorders, including persistent pulmonary hypertension of the newborn (PPHN) and perioperative pulmonary hypertension after repair of congenital heart disease (4, 12, 14). One of the more important issues regarding inhaled NO therapy is the safety of acute withdrawal. Several studies have noted a potentially life-threatening increase in pulmonary vascular resistance upon acute withdrawal of inhaled NO (1, 15). This “rebound pulmonary hypertension” is manifested by an increase in pulmonary vascular resistance, compromised cardiac output, and/or severe hypoxemia (1, 15). Recent data in normal animals demonstrate that exogenous NO exposure inhibits endogenous endothelial nitric oxide synthase (eNOS) activity and increases plasma endothelin (ET)-1 levels (8, 24). In addition, in vitro data suggest that the decrease in NOS activity during inhaled NO is mediated by an ET-1-induced increase in superoxide production, which results in peroxynitrite formation and the subsequent nitration and inactivation of eNOS (34, 42).

These studies in the normal pulmonary vasculature suggest that alterations in endogenous endothelial function during inhaled NO exposure mediate the rebound pulmonary hypertension associated with its acute withdrawal. However, clinically, inhaled NO is most often administered to patients with pulmonary vascular disorders that have associated preexisting alterations in endogenous pulmonary vascular endothelial function. For example, alterations in both endogenous NO-cGMP and ET-1 signaling have been demonstrated in newborns with persistent pulmonary hypertension and infants and children with increased pulmonary blood flow secondary to congenital heart disease (13, 22, 45). The potential effect of inhaled NO on endogenous endothelial function in the clinically relevant altered pulmonary vasculature has not been studied.

Previously, we developed a model of congenital heart disease with increased pulmonary blood flow in the lamb utilizing in utero placement of an aortopulmonary vascular graft (28). At 4 wk of age, these lambs have a mean pulmonary arterial pressure that is 35–75% of systemic values, a pulmonary-to-systemic blood flow ratio of ~2.5:1, and morphometric abnormalities of the pulmonary vasculature that include medial hypertrophy, abnormal extension of muscle to the periphery, and increased vessel density. In addition, these lambs have altered endothelial function, which include impaired endothelium-dependent pulmonary vasodilation, increased NOS activity and eNOS gene expression, increased ET-1 levels, decreased ETB receptor-mediated pulmonary vasodilation and protein levels, and increased ETA receptor-mediated vasoconstriction and protein levels (6, 7, 29, 43).

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We hypothesized that, compared with the normal vasculature, the endothelial response to inhaled NO exposure would be altered in the pulmonary hypertensive vasculature because of preexisting endothelial alterations. Therefore, the purpose of this study was to investigate the effects of inhaled NO on endogenous NO-cGMP and ET-1 signaling in the pulmonary hypertensive vasculature. To this end, inhaled NO (40 ppm) was administered to eight 4-wk-old shunt lambs for 24 h. To determine the effects of inhaled NO on endogenous NO-cGMP signaling, sequential plasma samples were taken for cGMP concentrations. In addition, sequential peripheral lung biopsies were taken for NOS activity determinations and protein determinations of eNOS, inducible NOS (iNOS), neuronal NOS (nNOS), soluble guanylate cyclase (sGC), and phosphodiesterase (PDE) 5 by Western blot analysis. To determine the effects of inhaled NO on endogenous ET-1 signaling, sequential plasma samples were taken for ET-1 concentrations, and sequential peripheral lung biopsies were taken for protein determinations of prepro-ET-1, endothelin converting enzyme (ECE)-1α, ETₐ receptors, and ETₐ receptors.

METHODS

Surgical preparation. Twelve mixed-breed Western pregnant ewes (137–141 days gestation, term = 145 days) were operated on under sterile conditions with the use of local anesthesia (2% lidocaine hydrochloride) and inhalational anesthesia (1–3% isoflurane). A midline incision was made in the ventral abdomen, and the pregnant horn of the uterus was exposed. Through a small uterine incision, the left fetal forelimb and chest were exposed, and a left lateral thoracotomy was performed in the third intercostal space. Additional fetal anesthesia consisted of local anesthesia with 1% lidocaine hydrochloride and ketamine hydrochloride (20 mg im). With the use of side-biting vascular clamps, an 8.0-mm Gore-tex vascular graft (~2 mm length; Gore, Milpitas, CA) was anastomosed between the ascending aorta and main pulmonary artery with 7.0 prolene (Ethicon, Somerville, NJ), using a continuous suture technique. The thoracotomy incision was then closed in layers. This procedure is previously described in detail (28). After surgery, antibiotics (1 million units penicillin G potassium and 100 mg gentamycin sulfate im) were administered to the ewe for 3 days. After spontaneous delivery, the lambs were weighed daily, and the respiratory rate and heart rate were obtained. Furosemide (1 mg/kg im) was administered daily. Elemental iron (50 mg im) was given weekly.

At 4 wk of age, the lambs were fasted for 24 h, with free access to water. The lambs were then anesthetized with ketamine hydrochloride (15 mg/kg im). Under additional local anesthesia with 1% lidocaine hydrochloride, polyurethane catheters were placed in an artery and vein of a hindleg. These catheters were advanced to the descending aorta and the inferior vena cava, respectively. The lambs were then anesthetized with ketamine hydrochloride (~0.3 mg·kg⁻¹·min⁻¹), diazepam (0.002 mg·kg⁻¹·min⁻¹), and fentanyl citrate (1.0 μg·kg⁻¹·h⁻¹), intubated with a 7.0-mm outer-diameter cuffed endotracheal tube, and mechanically ventilated with a Healthdyne pediatric time-cycled, pressure-limited ventilator. Pancuronium bromide (0.1 mg·kg⁻¹·dose⁻¹) was given intermittently for muscle relaxation. With the use of a strict aseptic technique, a midsternotomy incision was then performed, and the pericardium was incised. With the purse-string suture technique, polyurethane catheters were placed directly in the right and left atrium and main pulmonary artery. An ultrasonic flow probe (Transonics Systems, Ithaca, NY) was placed around the left pulmonary artery to measure pulmonary blood flow. The midsternotomy incision was then temporarily closed with towel clamps. An intravenous infusion of Lactated Ringer and 5% dextrose (75 ml/h) was begun and continued throughout the study period.

Cefazolin (500 mg iv) and gentamycin (3 mg/kg iv) were administered before the first surgical incision, and every 8 h thereafter. The lambs were maintained normothermic (39°C) with a heating blanket.

Experimental protocol. After a 60-min recovery, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, left pulmonary blood flow, left and right atrial pressures) and systemic arterial blood gases and pH were measured (pre-NO). Blood was collected from the femoral artery for plasma ET-1 and cGMP determinations, and a peripheral lung wedge biopsy was obtained for NOS activity and eNOS, iNOS, nNOS, sGC, PDE-5, prepro-ET-1, ECE-1, and ETα and ETβ receptor protein determinations. A side-biting vascular clamp was used to isolate peripheral lung tissue from a randomly selected lobe, and the incision was cauterized. Approximately 300 mg peripheral lung were obtained for each biopsy.

In eight of the lambs, inhaled NO (40 ppm) was then delivered in nitrogen in the inspiratory limb of the ventilator (Inovent; Ohmeda, Liberty, NJ) and continued for 24 h. The inspired concentrations of NO and nitrogen dioxide were continuously quantified by electrochemical methodology (Inovent; Ohmeda). The hemodynamic variables were monitored continuously. Systemic arterial blood gases were determined intermittently, and ventilation was adjusted to achieve a PaCO₂ between 35 and 45 Torr and a PaO₂ > 50 Torr. Sodium bicarbonate was administered intermittently to maintain a pH > 7.30. Normal saline was administered intermittently to maintain stable arterial pressures throughout the study period. Peripheral lung wedge biopsies were performed, and blood was obtained after 24 h of therapy. The inhaled NO was then stopped, and the hemodynamic variables were monitored for two additional hours. Blood and lung tissue was obtained 120 min after discontinuation of inhaled NO. All blood losses were replaced with maternal blood (~15 ml/kg over the study period). Four additional shunt lambs underwent the identical protocol without inhaled NO administration.

At the end of the protocol, all lambs were killed with a lethal injection of pentobarbital sodium followed by bilateral thoracotomy, as described in the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco.

Measurements. Pulmonary and systemic arterial and right and left atrial pressures were measured using Sorenson Neonatal Transducers (Abbott Critical Care Systems, N. Chicago, IL). Mean pressures were obtained by electrical integration. Heart rate was measured by a cathodochamber triggered from the phasic systemic arterial pressure pulse wave. Left pulmonary blood flow was measured on an ultrasonic flowmeter (Transonics Systems). 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/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hb concentration and oxygen saturation were measured by a hemoximeter (model 270; Ciba-Corning). Pulmonary vascular resistance was calculated using standard formulas. Body temperature was monitored continuously with a rectal temperature probe.

Plasma cGMP determinations. Plasma samples were assayed with a cGMP 125I RIA kit (Amersham International) according to the manufacturer’s instructions. Cross-reactivity for other nucleotides is <0.001.

Assay for NOS activity. This was performed using the conversion of [1⁴C]arginine to [1⁴C]citrulline as a measure of NOS activity, essentially as described by Bush et al. (11). Briefly, peripheral lung tissues and isolated fifth-generation pulmonary arteries were homogenized in NOS assay buffer (50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 0.1 mM EGTA) with a protease inhibitor cocktail. Enzyme reactions were carried out at 37°C in the presence of total lung protein extracts (500 μg), 1 mM NADPH, 14 μM tetrahydrobiopterin, 100 μM FAD, 1 mM MgCl₂, 5 μM unlabeled l-arginine, 15 mM [1⁴C]arginine, 25 units calmodulin, and 5 mM calcium to produce conditions
that drive the reaction at maximal velocity. Duplicate assays were run in the presence of the NOS inhibitor Nω-nitro-l-arginine methyl ester. Assays were incubated for 60 min at 37°C such that no more than 20% of the [14C]arginine was metabolized, to ensure that the substrate was not limiting. The reactions were stopped by the addition of ice cold stop buffer (20 mM sodium acetate, pH 5.1, 1 mM l-citrulline, 2 mM EDTA, and 0.2 mM EGTA) and then applied to columns containing 1 ml Dowex AG50W-X8 resin, Na+ form, preequilibrated with 1 N NaOH. [14C]citrulline was then quantitated by scintillation counting.

Plasma ET-1 determinations. Systemic arterial blood (4 ml) was collected and placed in iced polypropylene tubes containing 330 μl propranolol and 100 μl EDTA. The tubes were immediately centrifuged at 4,000 g for 20 min. Collected plasma was treated with equal volumes of 0.1% trifluoroacetic acid and stored at −70°C. The acidified supernatant was centrifuged at 1,000 g for 20 min and loaded on a 3 × 18 Ci Sep-Pak column (Peninsula Laboratories, Belmont, CA) equilibrated with 0.1% trifluoroacetic acid. The adsorbed material was eluted with 3 ml of 0.1% trifluoroacetic acid/60% acetonitrile. The eluant was dried in a Savant Speed Vac, and on ice at 4°C. Supernatant fractions were then assayed for protein concentration using the Bradford reagent (Bio-Rad, Richmond, CA) and used for Western blot analysis. Western blot analysis was performed as previously described (6, 7, 9, 24, 34, 42). Briefly, protein extracts (25 μg) were separated on 4–20% polyacrylamide gradient gels for ETA and ETB receptors, sGC, and PDE5, and Western blot analysis was performed as previously published method (44).

Preparation of protein extracts and Western blot analysis. Lung protein extracts were prepared by homogenizing peripheral lung tissues in Triton lysis buffer (50 mM Tris HCl, pH 7.6, 0.5% Triton X-100, and 20% glycerol) containing a protease inhibitor cocktail. Protein extracts were prepared by homogenizing peripheral lung tissues in Triton lysis buffer (50 mM Tris HCl, pH 7.6, 0.5% Triton X-100, and 20% glycerol) containing a protease inhibitor cocktail. Tissue homogenates were centrifuged at 10,000 × g for 30 min at 4°C. Supernatant fractions were then applied to columns containing 1 ml Dowex AG50W-X8 resin, Na+ form, preequilibrated with 1 N NaOH. [14C]citrulline was then quantitated by scintillation counting.

RESULTS

In shunt lambs, inhaled NO (40 ppm) rapidly decreased mean pulmonary arterial pressure (P < 0.05). Left pulmonary blood flow, left pulmonary vascular resistance, mean systemic arterial pressure, heart rate, right and left atrial pressures, and systemic arterial blood gases and pH were all unchanged. During the 24-h treatment course, pulmonary arterial pressure remained below pre-NO values. Systemic arterial pressure, pulmonary blood flow, heart rate, and systemic arterial Po2 all decreased slightly, whereas left and right atrial pressures increased (Table 1). Upon discontinuation of inhaled NO, there was a rapid increase in both mean pulmonary arterial pressure and left pulmonary vascular resistance (P < 0.05; Table 1 and Fig. 1). Left pulmonary blood flow, mean systemic arterial pressure, heart rate, left and right atrial pressures, and systemic arterial Po2, PCO2, and pH remained unchanged from 24-h NO values (Table 1). Pulmonary vascular resistance remained above pre-NO values for the remaining 2-h study period (Fig. 1).

To determine the effects of inhaled NO on endogenous NO-cGMP signaling, we determined plasma cGMP concentrations, total NOS activity, and eNOS, iNOS, nNOS, sGC, and PDE5 protein levels. We found that plasma cGMP levels increased during inhaled NO, but rapidly decreased after NO withdrawal and remained below pre-NO values for the remaining 2-h study period (Fig. 2). As opposed to our findings in normal lambs, total NOS activity did not decrease in shunt lambs during NO exposure. In fact, eNOS protein levels decreased by 43% such that NOS activity relative to protein levels actually increased during inhaled NO by 227% (P < 0.05). These changes returned to pre-NO values 2 h after NO withdrawal (Fig. 3). iNOS and nNOS protein levels were not
detectable during the study period. The α-sGC subunit and PDE5 protein levels were unchanged during inhaled NO, but the β-sGC subunit decreased by 70% (P < 0.05; Fig. 4).

To determine the effects of inhaled NO on endogenous ET-1 production, we determined plasma ET-1 concentrations and lung protein levels. We found that plasma ET-1 concentrations were increased after 24 h of inhaled NO but returned to pre-NO values 2 h after discontinuation (P < 0.05; Fig. 5). In addition, Western blot analysis demonstrated no change in prepro-ET-1, ECE-1α, ET_{A} receptor, or ET_{B} receptor protein levels throughout the study period (Fig. 6).

In four additional shunt lambs, 24 h of mechanical ventilation alone, without inhaled NO therapy, did not change mean pulmonary arterial pressure. Similar to NO-treated lambs, mean systemic arterial pressure, left pulmonary blood flow, and heart rate decreased (Table 2; P < 0.05). Left and right atrial pressures and systemic arterial blood gases and pH were not changed (Table 2). Plasma cGMP levels, tissue NOS activity, and eNOS, iNOS, and nNOS protein levels were unchanged. In addition, plasma ET-1 levels were unchanged after 24 h of ventilation without inhaled NO. This was associated with no changes in prepro-ET-1, ECE-1α, ET_{A} receptor, or ET_{B} receptor protein levels (data not shown).

The lambs required 1–3 meq/kg bicarbonate during the study period to maintain normal acid-base status and 30–50 ml/kg volume to maintain atrial pressures. There were no differences between NO-treated lambs and vehicle-treated lambs in the amount of volume, buffer, or ventilatory support required.

**DISCUSSION**

Exogenous inhaled NO is increasingly utilized as an adjunct therapy for pediatric pulmonary hypertension disorders, including infants and children with congenital heart disease (4). It produces potent, selective pulmonary vasodilation that is independent of endothelial cell function. Therefore, it has been employed in the preoperative assessment of pulmonary hypertension, the perioperative management of pulmonary hypertension, and the postoperative assessment of pulmonary hypertension (2, 4, 5). Although many studies demonstrate a clear benefit in patient outcome with inhaled NO use, several safety concerns remain, including unpredictable and nonsustained responses to inhaled NO and a clinically significant rapid increase in pulmonary vascular resistance upon its acute withdrawal (1, 15–17). Recent data suggest that alterations in endogenous endothelial function mediate this rebound pulmo-

**Table 1. Hemodynamic changes during and after 24 h of increased pulmonary blood flow**

<table>
<thead>
<tr>
<th>Pulmonary arterial pressure, mmHg</th>
<th>Pre-NO</th>
<th>2</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120</th>
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<tr>
<td>Left pulmonary blood flow, ml/kg·min⁻¹</td>
<td>123.0±63.8</td>
<td>117.3±64.4</td>
<td>114.7±58.1</td>
<td>108.2±60.9</td>
<td>93.3±49.3</td>
<td>87.5±46.0</td>
<td>91.1±49.9</td>
<td>91.0±49.1</td>
<td>92.2±56.3</td>
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<td>Systemic arterial pressure, mmHg</td>
<td>60.2±11.8</td>
<td>55.3±13.2</td>
<td>54.8±10.5</td>
<td>47.9±9.9</td>
<td>40.5±7.2</td>
<td>39.4±9.2</td>
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<td>40.9±11.7</td>
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<td>Heart rate, beats/min</td>
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<td>152.9±24.1</td>
<td>154.1±21.5</td>
<td>146.5±28.8</td>
<td>124.1±14.9</td>
<td>117.1±14.3</td>
<td>120.3±13.8</td>
<td>124.8±14.9</td>
<td>121.9±10.3</td>
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<tr>
<td>Left atrial pressure, mmHg</td>
<td>6.0±0.9</td>
<td>6.2±1.1</td>
<td>5.8±1.0</td>
<td>6.0±1.3</td>
<td>7.8±2.1</td>
<td>7.2±0.9</td>
<td>7.2±1.6</td>
<td>7.2±1.4</td>
<td>8.0±2.7</td>
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<tr>
<td>Right atrial pressure, mmHg</td>
<td>4.6±1.5</td>
<td>5.0±1.2</td>
<td>4.7±1.3</td>
<td>5.2±1.5</td>
<td>6.7±2.3</td>
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<td>7.2±2.4</td>
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<td>7.3±2.9</td>
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<td>pH, units</td>
<td>7.40±0.05</td>
<td>7.44±0.05</td>
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<td>PACO₂, Torr</td>
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<td>35.8±3.9</td>
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<td>PAO₂, Torr</td>
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Values are means ± SD; n = 8 lambs. *P < 0.05 vs. pre-NO; †P < 0.05 vs. previous column (ANOVA).
nary hypertension. Indeed, recent in vitro and in vivo animal data in the normal pulmonary circulation suggest that exogenous NO decreases endogenous NOS activity via an ET-1-mediated increase in superoxide production. Superoxide then binds to available NO, resulting in peroxynitrite production, which phosphorylates and inactivates NOS (8, 24, 34, 42). The net result of these changes, increased ET-1 activity and decreased NOS activity, may mediate the clinically significant increases in pulmonary vascular resistance noted upon inhaled NO withdrawal. However, the potential effects of inhaled NO on endogenous NO-cGMP and ET-1 signaling have not been studied previously in the pulmonary hypertensive circulation.

In the present study, we found that, similar to normal lambs, plasma levels of ET-1 increased in shunt lambs during inhaled NO independent of changes in protein levels (24). However, as opposed to normal lambs in which NOS activity decreased and eNOS protein levels were unchanged during NO exposure, NOS activity did not change in shunt lambs, and eNOS protein levels decreased, suggesting a posttranslational increase in NOS activity (8). These data suggest that the effects of inhaled NO on endogenous endothelial function, and the potential mechanisms of the rebound pulmonary hypertension associated...
with NO withdrawal, are dependent upon the preexisting status of the pulmonary circulation.

Although initially considered to be a constitutively expressed enzyme, an increasing body of literature demonstrates that eNOS is dynamically regulated at the transcriptional and posttranslational levels (20, 25). For example, laminar shear stress increases eNOS transcription, whereas factors such as intracellular location, protein-protein interactions (e.g., calmodulin, caveolin, and heat shock protein 90), phosphorylation, oxidant stress, and substrate and cofactor availability may all dynamically regulate eNOS activity (20, 25, 27, 33). The effect of exogenous NO exposure on endogenous NO gene expression has been previously studied in vitro and has yielded conflicting results. For example, in fetal intrapulmonary vascular endothelial cells, exposure to NO donor compounds increased eNOS protein and mRNA levels, whereas in pulmonary endothelial cells isolated from the fetal main pulmonary artery, exposure to NO donors did not change eNOS protein or mRNA (34, 46). In the normal 4-wk-old lamb, we have previously demonstrated that eNOS protein levels were unchanged during inhaled NO exposure (8). However, in the present study inhaled NO significantly decreases eNOS protein levels in 4-wk-old shunt lambs. The disparity in the regulation of eNOS protein levels with NO exposure may be explained, in part, by differences in basal eNOS protein and mRNA levels between normal and shunt lambs. For example, we have previously demonstrated that, under conditions of high pulmonary blood flow and pressure, shunt lambs have increased eNOS protein and mRNA levels compared with normal control lambs (7). We speculate that these conditions alter the regulatory response of eNOS to exogenous NO. However, these and potential other regulatory mechanisms will require further investigation.

Previously, we have also demonstrated a posttranslational decrease in NOS activity during NO exposure in the normal lamb secondary to ET-1-mediated superoxide production and subsequent peroxynitrite production (8). However, in the present study, total NOS activity was unchanged during inhaled NO. In fact, relative to the decrease in eNOS protein, NOS activity actually increased during NO exposure. Because total NOS activity may represent changes in eNOS, iNOS, and nNOS activity, we determined iNOS and nNOS protein levels and found no detectable changes in either during the treatment protocol, suggesting that the preservation of NOS activity represented a posttranslational increase in total NOS activity. We have previously demonstrated that baseline NOS activity is significantly increased in shunt lambs compared with controls (7). In addition, we have previously demonstrated that several known regulators of NOS activity, such as oxidant stress and cofactor availability, are altered in shunt lambs. For example, shunt lambs have decreased plasma levels of L-arginine (the substrate for NOS) and increased superoxide levels (28, 36). We speculate that these altered baseline conditions secondary to the chronic stimulus of increased flow and pressure have altered the posttranslational response of NOS to exogenous NO.

Fig. 5. Changes in plasma endothelin (ET)-1 concentrations before, during, and after 24 h of inhaled NO (40 ppm) therapy; n = 8 shunt lambs. Values are means ± SE. *P < 0.05 vs. pre-NO.

Fig. 6. A: Western blot analysis for prepro-ET-1 protein in lung tissue from shunt lambs before and after inhaled NO. Top: representative Western blot. Bottom: densitometric values for prepro-ET-1 normalized to pre-NO. Values are means ± SE; n = 7. Prepro-ET-1 protein expression is unchanged during inhaled NO therapy. B: Western blot analysis for ECE-1α protein in lung tissue from shunt lambs before and after inhaled NO. Top: representative Western blot. Bottom: densitometric values for endothelin converting enzyme (ECE)-1α normalized to pre-NO. Values are means ± SE; n = 7. *P < 0.05. ECE-1α protein expression is unchanged during inhaled NO therapy. C: Western blot analysis for ETα receptor protein in lung tissue from shunt lambs before and after inhaled NO. Top: representative Western blot. Bottom: densitometric values for ETα receptor normalized to pre-NO. Values are means ± SE; n = 7. ETα receptor protein expression is unchanged during inhaled NO therapy. D: Western blot analysis for ETβ receptor protein in lung tissue from shunt lambs before and after inhaled NO. Top: representative Western blot. Bottom: densitometric values for ETβ receptor normalized to pre-NO. Values are means ± SE; n = 7. ETβ receptor protein expression is unchanged during inhaled NO therapy.
inhaled NO therapy. During the 24-h study period, cGMP demonstrated that the expression of PDE5 is unchanged during alterations in animal models of pulmonary hypertension. As with sGC, little is known about the regulation of specific PDE, PDE5, is prevalent, especially early in development (32). As with sGC, little is known about the regulation of mammalian lung, there are a number of PDEs, but the cGMP-are the enzymes responsible for cGMP degradation (3). In the lambs may be secondary, in part, to these differences in basal differential response to inhaled NO between shunt and control age-matched controls (10). The potential mechanisms for the exact mechanisms for these differences are unclear and warrant further study.

NO induces vasodilation by activating sGC. sGC is a heterodimeric enzyme comprising two subunits termed α and β that generate cGMP from GTP (21). Although much focus has been placed on potential alterations in NOS expression in pulmonary hypertensive disorders, data also suggest that changes in sGC expression and activity may be important in the pathophysiology of pulmonary hypertension. For example, decreased sGC protein levels and activity have been demonstrated in animal models of PPHN (37). In vitro, this is associated with impaired pulmonary vasodilation in response to NO donors and may play a role in the decreased responsiveness of some newborns with PPHN (40). Decreased sGC gene expression and activity have also been implicated in other vascular disorders, including essential hypertension (30). Although little data are available on the regulation of sGC, limited in vitro studies suggest that exogenous NO does alter sGC expression and activity. For example, in rat medullary interstitial cells, NO donor compounds decreased mRNA levels of both α1- and β1-sGC subunits (41). In addition, in rat pulmonary artery smooth muscle cells, exposure to NO donors induced a cGMP-dependent decrease in sGC mRNA and protein levels (18). Previously, we demonstrated that inhaled NO decreases pulmonary α1- and β1-sGC protein levels in normal lambs (38). In the current study, inhaled NO decreased the β1-subunit protein levels by 70%, whereas the α1-subunit protein levels were unchanged. At baseline, both the α1- and β1-sGC subunits are upregulated in shunt lambs compared with age-matched controls (10). The potential mechanisms for the differential response to inhaled NO between shunt and control lambs may be secondary, in part, to these differences in basal regulation, but require further investigation.

Intracellular cGMP concentrations are not simply determined by the accumulation of cGMP, but rather by a balance between synthesis and degradation. Cyclic nucleotide PDEs are the enzymes responsible for cGMP degradation (3). In the mammalian lung, there are a number of PDEs, but the cGMP-specific PDE, PDE5, is prevalent, especially early in development (32). As with sGC, little is known about the regulation of PDE5 gene expression. However, developmental regulation and alterations in animal models of pulmonary hypertension have been demonstrated (31, 32). In this study, we have demonstrated that the expression of PDE5 is unchanged during inhaled NO therapy. During the 24-h study period, cGMP levels were increased despite a decrease in β1-sGC protein levels. This is most likely secondary to the increase in enzyme substrate provided during inhaled NO treatment. However, the net effect of decreased sGC without a compensatory decrease in PDE5 will result in decreased cGMP levels after the withdrawal of exogenous NO. This decrease in cGMP levels below baseline values demonstrated in Fig. 2 may contribute to the physiological increase in pulmonary vascular resistance noted upon NO withdrawal.

Although the effects of NO exposure on ET-1 regulation in vitro have yielded conflicting results, previous reports in vivo have demonstrated that inhaled NO increases ET-1 levels. For example, in both children with pulmonary hypertension after cardiac surgery and normal 4-wk-old lambs, plasma ET-1 concentrations increased during inhaled NO exposure (24, 26). Similarly, in the current study, plasma ET-1 concentrations increased in lambs with preexisting increased pulmonary blood flow during inhaled NO exposure. Increases in plasma ET-1 levels may result from increases in ET-1 production, ET-1 release, and/or decreased ET-1 clearance. The production of ET-1 begins with the cleavage of the translational product prepro-ET-1 into a nonactive 38-amino-acid residue known as big ET-1. Big ET-1 is then cleaved into its functional form, ET-1, by the endopeptidase ECE-1 (39). ECE-1 exists in two isoforms, ECE-1α and ECE-1β, with ECE-1α considered to be the most biologically important (35). Because many studies suggest that ET-1 production is regulated at the transcriptional level of prepro-ET-1 and/or ECE-1, we performed sequential lung biopsies to determine potential changes in prepro-ET-1 and ECE-1α protein levels. We found that both prepro-ET-1 and ECE-1α protein levels were unchanged during inhaled NO therapy, suggesting that the increased plasma concentrations are independent of changes in gene expression. In addition, the ETα receptor has been implicated in the clearance of ET-1 from the circulation, but we found no changes in protein levels of the ETα receptor during inhaled NO (19). Rapid ET-1 release from intracellular secretory granules has been demonstrated after such stimuli as cytokines and stretch (23). Therefore, the increase in plasma ET-1 induced by inhaled NO may represent an increase in ET-1 release. However, potential changes in ECE-1 activity, NO-induced displacement of ET-1 from its receptors, and/or potential changes in ETα binding affinity represent additional potential mechanisms that were not studied but warrant investigation. These findings are similar to those seen in our previous study in the normal pulmonary vasculature (24). However, as opposed to normal lambs, exogenous ET-1 induces potent pulmonary vasoconstriction in shunt lambs secondary to increased ETα receptor expression and/or decreased ETβ receptor expression (6, 43). Therefore, a
similar increase in plasma ET-1 levels in shunt lambs may have greater physiological significance than control lambs and sug-
gests that ET-1-induced vasoconstriction may play a greater role in the increase in pulmonary vascular resistance after NO withdrawal in shunt lambs.

After the 24-h treatment period, mean systemic arterial pressure was decreased. The etiology of this hypotension is unclear. However, it was associated with a decrease in pulmonary blood flow and heart rate, suggesting a decrease in cardiac output secondary to long cumulative doses of anesthetics. Identical hemodynamic changes occurred in those lambs ventilated without inhaled NO, suggesting that these changes were independent of inhaled NO treatment. Furthermore, because nontreated lambs did not undergo the biochemical and protein changes noted in NO-treated lambs, despite having similar decreases in systemic arterial pressure, blood flow, and heart rate, the biochemical and protein changes were likely secondary to inhaled NO treatment and independent of these physiological changes.

Three limitations of the current study are noteworthy. Only one dose of inhaled NO (40 ppm) and one treatment duration (24 h) were studied. Further investigations are needed to determine the potential of different doses and treatment durations on endogenous ET-1. In addition, these studies were performed in 21% oxygen. Clinically, inhaled NO is most often administered with supplemental oxygen. We performed our studies in 21% oxygen to isolate the changes to NO, but further studies are warranted to determine the potential effect of supplemental oxygen. Last, these studies were performed in shunt lambs with the vascular graft left open. Although inhaled NO is used in the preoperative evaluation of pulmonary hypertension secondary to increased pulmonary blood flow, the majority of its use occurs in the postoperative period after pulmonary blood flow has been decreased by surgical repair (4). We did perform preliminary data that demonstrate a similar preservation of NOS activity when inhaled NO was initiated after graft closure (n = 2; data not shown). This suggests that the changes we observe are independent of graft patency and rather the chronic underlying endothelial function. However, further studies are warranted to better determine potential alterations in the endothelial response to inhaled NO when administered after normalization of pulmonary blood flow.

The present study is the first in vivo investigation of the effects of inhaled NO therapy on endogenous endothelial function in the pulmonary hypertensive circulation. We found that alterations in the ET-1 cascade induced by inhaled NO, increased plasma ET-1 levels that occurred independently of changes in protein levels, were similar to those found in the normal circulation. However, the response of endogenous eNOS to inhaled NO in the pulmonary hypertensive circulation differed from the response in the normal circulation. As opposed to a decrease in NOS activity without changes in protein levels, eNOS protein levels decreased during NO in shunt lambs, whereas activity increased from a presumed posttranslational modification. Similar to normal lambs, sGC decreased, PDE5 was unchanged during NO exposure, and cGMP concentrations decreased in shunt lambs after NO withdrawal. These data suggest that the decreases in cGMP levels are predominantly secondary to changes in sGC in shunt lambs as opposed to normal lambs where both decreases in NOS activity and sGC protein may contribute. In addition, the increases of ET-1 in shunt lambs likely have a greater primary role in inducing pulmonary vasoconstriction upon inhaled NO withdrawal in shunt lambs in which ETA receptor protein levels are increased, especially given the net increase in NOS activity in these lambs (6). We conclude that the endothelial response to inhaled NO therapy is dependent on the baseline endothelial function of the pulmonary vasculature. Therefore, the pathophysiolo-gy of rebound pulmonary hypertension may be different in the normal vs. the abnormal pulmonary circulation. A better understanding of these different mechanisms may lead to improved treatment strategies for rebound pulmonary hypertension and the preservation of endothelial function during chronic NO usage in pulmonary vascular disease states.

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