Inhaled nitric oxide inhibits NOS activity in lambs: potential mechanism for rebound pulmonary hypertension

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Black, Stephen M., R. Scott Heidersbach, D. Michael McMullan, Janine M. Bekker, Michael J. Johengen, and Jeffrey R. Fineman. Inhaled nitric oxide inhibits NOS activity in lambs: potential mechanism for rebound pulmonary hypertension. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1849–H1856, 1999.—Life-threatening increases in pulmonary vascular resistance have been noted on acute withdrawal of inhaled nitric oxide (NO), although the mechanisms remain unknown. In vitro data suggest that exogenous NO exposure inhibits endothelial NO synthase (NOS) activity. Thus the objectives of this study were to determine the effects of inhaled NO therapy and its acute withdrawal on endogenous NOS activity and gene expression in vivo in the intact lamb. Six 1-mo-old lambs were mechanically ventilated and instrumented to measure vascular pressures and left pulmonary blood flow. Inhaled NO (40 ppm) acutely decreased left pulmonary vascular resistance by 27.5 ± 4.7% (P < 0.05). This was associated with a 207% increase in plasma cGMP concentrations (P < 0.05). After 6 h of inhaled NO, NOS activity was reduced to 44.3 ± 5.9% of pre-NO values (P < 0.05). After acute withdrawal of NO, pulmonary vascular resistance increased by 52.1 ± 11.6% (P < 0.05) and cGMP concentrations decreased. Both returned to pre-NO values within 60 min. One hour after NO withdrawal, NOS activity increased by 19.1% to 70% of pre-NO values (P < 0.05). Western blot analysis revealed that endothelial NOS protein levels remained unchanged throughout the study period. These data suggest a role for decreased endogenous NOS activity in the rebound pulmonary hypertension noted after acute withdrawal of inhaled NO.

NITRIC OXIDE (NO) is a labile humoral factor synthesized from the oxidation of the guanidino nitrogen moiety of L-arginine after the activation of NO synthase (NOS) (27). Three isoforms of NOS are known. Constitutive forms are present in endothelial cells (endothelial NOS) and neurons (neuronal NOS), and a third, inducible isoform is present in macrophages (inducible NOS) (5, 24, 34). Pulmonary vascular endothelial cells synthesize NO after certain stimuli, such as shear stress and the receptor binding of specific vasodilators, activate endothelial NOS (19, 32). Once released from endothelial cells, NO diffuses into the adjacent vascular smooth muscle cells, where it activates soluble guanylate cyclase. The resulting increase in intracellular cGMP initiates a cascade leading to smooth muscle relaxation (20). Increasing data suggest that endogenously produced endothelium-derived NO is an important mediator of normal pulmonary vascular tone and vascular reactivity. For example, administration of NOS inhibitors produces significant increases in resting pulmonary vascular resistance and augments the response to pulmonary vasoconstricting stimuli (11, 12). In addition, recent data suggest that decreased endogenous NO production participates in the development of pulmonary hypertensive disorders (9, 14).

Exogenously administered inhaled NO is currently utilized under experimental protocol as an adjuvant therapy for a number of pulmonary hypertensive disorders. In both animal and human studies, inhaled NO [5–80 parts per million (ppm)] induces rapid and selective pulmonary vasodilation (3, 26a, 29, 31). When administered into the airways in its gaseous form, NO diffuses into pulmonary vascular smooth muscle cells, where it increases cGMP concentrations, causing selective pulmonary vasodilatation. No systemic vasodilatation occurs because NO is rapidly inactivated by binding with hemoglobin when it reaches the intravascular space (22). Two multi-centered, randomized trials have recently demonstrated that inhaled NO improves oxygenation and decreases the need for extracorporeal life support in newborns with persistent pulmonary hypertension (26a, 29). In addition, nonrandomized studies demonstrate that inhaled NO selectively decreases pulmonary arterial pressure and pulmonary vascular resistance in patients with congenital heart disease and decreases pulmonary vascular resistance and improves oxygenation in patients with acute lung injury (3, 31). Although these preliminary data are encouraging, several concerns regarding the safety of inhaled NO therapy remain.

One of the most important issues regarding inhaled NO therapy is the safety of acute withdrawal. Several studies have noted a serious increase in pulmonary vascular resistance on acute withdrawal of inhaled NO (2, 10, 25, 26). This “rebound pulmonary hypertension” is manifested by an increase in pulmonary vascular resistance, which may compromise cardiac output and/or result in severe hypoxemia in patients with underlying pulmonary vascular disease (2, 10, 25, 26). In vitro, exogenous NO exposure appears to inhibit endothelial NOS activity (1, 6, 16, 17, 28, 30, 33). These data suggest that transient decreases in endogenous NOS activity during inhaled NO therapy may be a potential mechanism for rebound pulmonary hyperten-
tion. However, the effects of inhaled NO on NOS activity in vivo have not been studied.

The purposes of this study were 1) to characterize the physiological effects of inhaled NO and its acute withdrawal after a 24-h treatment period and 2) to investigate the effects of inhaled NO and its acute withdrawal on lung NOS activity and gene expression. To determine the physiological effects of inhaled NO, the hemodynamic effects of inhaled NO therapy (40 ppm) and its acute withdrawal were determined in six 1-mo-old lambs. In addition, sequential determinations of plasma cGMP (the second messenger to NO-mediated vasodilation) and plasma nitrate (an indirect determinant of total body NO production) were made. To determine the effects of inhaled NO on NOS activity and gene expression, the conversion of [3H]arginine to [3H]citrulline (an indicator of NOS activity) and Western blot analysis were performed in sequential peripheral lung biopsies taken from lambs treated with inhaled NO.

METHODS

Surgical preparation. Six lambs (35.0 ± 8.5 days old) were fasted for 24 h with free access to water. The lambs were then anesthetized with ketamine hydrochloride (0.3 mg·kg⁻¹·min⁻¹) and diazepam (0.002 mg·kg⁻¹·min⁻¹), intubated with a 7.0-mm-OD cuffed endotracheal tube, and mechanically ventilated with a HealthyCare pediatric time-cycled, pressure-limited ventilator. Succinylcholine chloride (2 mg·kg⁻¹·dose⁻¹) was given intermittently for muscle relaxation. Under additional local anesthesia with 1% lidocaine hydrochloride, polyurethane catheters were placed in an artery and vein of a hind leg. These catheters were advanced to the descending aorta and the inferior vena cava, respectively. With the use of a strict aseptic technique, a midsternotomy incision was then performed and the pericardium was incised. With a purse-string suture technique, polyurethane catheters were placed directly into the right and left atrium and the main pulmonary artery. An ultrasonic flow probe (Transonic 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 gentamicin (3 mg/kg iv) were administered before the first surgical incision and every 8 h thereafter. The lambs were kept normothermic (39°C) with a heating blanket. To ensure that the lambs were adequately anesthetized throughout the study period, heart rate and systemic arterial blood pressure were continuously monitored. Increases in both heart rate and systemic arterial pressure were treated with the supplemental administration of ketamine and diazepam.

Experimental protocol. After a 30-min recovery, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, left pulmonary blood flow, and left and right atrial pressures) and systemic arterial blood gases and pH were measured (pre-NO). The hemodynamic variables were monitored continuously. Systemic arterial blood gases were determined intermittently, and ventilation was adjusted to achieve a PaO₂ between 35 and 45 Torr and a PaCO₂ > 50 Torr. Sodium bicarbonate was administered intermittently to maintain a pH > 7.30. Normal saline was administered to maintain stable atrial pressures and hemoglobin concentrations throughout the study period. A peripheral lung wedge biopsy was obtained for endothelial NOS activity and protein. 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 for each biopsy. Blood was collected from the femoral artery for plasma nitrate and cGMP determinations. All blood losses were replaced with maternal blood.

Inhaled NO (40 ppm) was then delivered in nitrogen into 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). Peripheral lung wedge biopsies were performed, and blood was obtained for plasma nitrate and cGMP determinations after 2, 6, 12, and 24 h of therapy. The inhaled NO was then stopped, and the hemodynamic variables were monitored for 2 h. Blood was obtained for plasma nitrate and cGMP determinations 10, 30, 60, and 120 min after discontinuation of inhaled NO; peripheral lung biopsies were obtained 60 and 120 min after discontinuation of inhaled NO.

In preliminary studies, lambs were mechanically ventilated without inhaled NO therapy over an 8 (n = 2)- and 24 (n = 1)-h study period. These lambs were surgically prepared and treated in the same manner as those receiving inhaled NO treatment. The hemodynamic variables were monitored, sequential lung biopsies were obtained, and blood was collected for plasma cGMP and nitrate concentrations as previously described.

At the end of the protocol, all lambs were killed with a lethal injection of pentobarbital sodium followed by bilateral thoracotomy as described in Guidelines for the Care and Use of Laboratory Animals [National Institutes of Health (NIH)]. 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 Statham P23 Db pressure transducers (Statham Instruments, Hato Rey, PR). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiotachometer triggered from the phasic systemic arterial pressure pulse wave. Left pulmonary blood flow was measured on an ultrasonic flowmeter (Transonic 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 Corning 158 pH-blood gas analyzer (Corning Medical and Scientific, Medfield, MA). Hemoglobin concentration and oxygen saturation were measured by a hemoximeter (model OSM 2, Radiometer, Copenhagen, Denmark). Pulmonary vascular resistance was calculated using standard formulas. Body temperature was monitored continuously with a rectal temperature probe.

Plasma cGMP determinations. Blood samples (1–2 ml) were collected in iced vacutainer tubes containing EDTA (7.5 mM). The samples were centrifuged (4,000 g for 15 min), and the resulting plasma was stored in polypropylene storage tubes at -70°C. At the time of the assay, the plasma (200 µl) was diluted 1:1 with 0.2 M HCl, vortexed, and the acidified plasma was passed through a 0.2-µm CentrFlex filter (Schleicher and Schuell, Keene, NH) by centrifugation (4,000 g for 15 min). The samples were assayed with a [25S]-cGMP assay kit (Amersham International, Amersham, UK) according to manufacturer’s instructions. Briefly, the extracted plasma (50 µl) was diluted with 450 µl of assay buffer. Samples (500 µl) along with standards (2–128 fm) were acetylated with 25 µl of acetylation reagent. Aliquots (100 µl) of samples and standards were pipetted into assay tubes, 100 µl of antiserum
were added, and the tubes were incubated for 1 h at room temperature. After incubation, 100 µl of 125I-cGMP were pipetted to all tubes, covered with Parafilm, and incubated for 12–18 h at 2–8°C. Amerlex-M 2nd antibody reagent (500 µl) was added to each tube, vortexed, and incubated at room temperature for 10 min. The antibody bound fraction was separated by placing the tubes in a magnetic rack for 15 min. The supernatant was poured off, and the tubes were counted for 1 min in a gamma scintillation counter (Beckman Gamma 5500, Beckman Instruments, Palo Alto, CA). Cross-reactivity for other nucleotides is <0.001%.

Plasma nitrate determinations. Blood samples (1–2 ml) were collected in iced vacutainer tubes. The samples were centrifuged (4,000 g for 15 min), and the resulting plasma was stored in polypropylene storage tubes at −70°C. In solution, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. The nitrite and nitrate were reduced using vanadium(III) and HCl at 90°C. NO was then purged from solution, resulting in a peak of NO. Therefore, this value represents total NO, nitrite, and nitrate (NOx). This peak was then detected by chemiluminescence (NOA 280, Sievers Instruments, Boulder CO). The detection limit is 1 nM/ml of nitrate.

Assay for NOS activity. This assay was performed using the conversion of [153H]arginine to [153H]citrulline as a measure of NOS activity essentially as described by Bush et al. (7). Briefly, lung tissues 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 flavin adenine dinucleotide, 1 mM MgCl2, 5 µM unlabeled L-arginine, 15 nM [153H]arginine, calmodulin (25 units), 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 N'-nitro-L-arginine methyl ester. Assays were incubated for 60 min so that no more than 20% of the [153H]arginine was metabolized to ensure that the substrate was not limiting. Activities were found to be proportional to the quantity of protein used in the assay. The reactions were stopped by the addition of iced stop buffer (20 mM sodium acetate, pH 5.1, 1 mM citrulline, 2 mM EDTA, and 0.2 mM EGTA) and then applied to columns containing 1 ml of Dowex AG50W-X8 resin, Na+ form, preequilibrated with 1 N NaOH. [153H]citrulline was then quantitated by scintillation counting.

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, 20% glycerol) containing a protease inhibitor cocktail. Extracts were then clarified by centrifugation (15,000 g for 10 min 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 (4). Briefly, lung protein extracts (100 µg) were separated on 7.5% SDS-polyacrylamide gels, and either electrophoretically transferred to Hybond-polyvinylidene difluoride membranes (Amersham, Arlington Heights, IL) or stained with Coomassie brilliant blue to normalize for any differences in protein loading. The membranes were blocked with 5% nonfat dry milk in Tris-base buffered saline (TBS) containing 0.1% Tween. After blocking, the membranes were incubated at room temperature with an endothelial NOS specific monclonal antibody (1:2,500 dilution; Transduction Laboratories, Lexington, KY) and washed with TBS containing 0.1% Tween. The membranes were then incubated with an anti-mouse IgG-horseradish peroxidase conjugate (1:1,000 dilution). After washing, chemiluminescence (Pierce Laboratories) was used to detect the protein bands of interest. Statistical analysis. Means ± SD were calculated for the baseline hemodynamic variables, systemic arterial blood gases and pH, plasma nitrate and cGMP concentrations, and NOS activity. The general hemodynamic variables, systemic arterial blood gases and pH, NOS activity, and nitrate and cGMP concentrations were determined over time by ANOVA for repeated measures. To determine differences between times, Student-Newman-Kuels post hoc testing was performed. Quantitation of autoradiographic results were performed by scanning (Hewlett Packard SCA Jet IIIX; Hewlett-Packard, Palo Alto, CA) the bands of interest into an image-editing software program (Adobe Photoshop; Adobe Systems, Mt. View, CA). For Western blot analysis, to ensure that band intensities were measured within the dynamic range of the autoradiographic film, different concentrations of lung protein extract were analyzed. Densitometric analysis was carried out, results were plotted graphically, and the linear range was determined. When the inhaled NO extracts were analyzed, different exposure times were obtained and only those within the linear range were used to analyze endothelial NOS protein expression.

All band intensities from Western blot analysis were analyzed densitometrically on a Macintosh computer (model 9500; Apple Computer, Cupertino, CA) using the public domain NIH Image program (developed at NIH and available on the Internet at http://rsb.info.nih.gov/nih-image). For Western blot analysis, to ensure equal protein loading, duplicate polyacrylamide gels were run. One was stained with Coomassie blue. Results from pre-NO lungs were assigned the value of 1 (relative endothelial NOS protein). Means ± SE were calculated for the relative protein at each time point after the start of inhaled NO therapy. Comparisons over time were made by ANOVA for repeated measures. A P < 0.05 was considered statistically significant.

RESULTS

The experimental protocol without inhaled NO did not change the hemodynamic variables, NOS activity, or plasma cGMP and nitrate concentrations (data not shown).

Inhaled NO (40 ppm) rapidly decreased mean pulmonary arterial pressure and left pulmonary vascular resistance (P < 0.05; Fig. 1). Left pulmonary blood flow, mean systemic arterial pressure, heart rate, right and left atrial pressures, and systemic arterial blood gases and pH were all unchanged. Throughout the 24-h treatment course, these hemodynamic variables did not change further (Table 1).

When inhaled NO was discontinued, there was a rapid increase in both mean pulmonary arterial pressure and left pulmonary vascular resistance (P < 0.05; Fig. 1). These variables returned to pre-NO values within 60 min of discontinuation of NO. Left pulmonary blood flow, mean systemic arterial pressure, heart rate, right and left atrial pressures, and systemic arterial blood gases and pH remained unchanged (Table 1).

Inhaled NO induced a rapid increase in plasma cGMP concentrations (the secondary messenger of NO-mediated vasodilation; P < 0.05; Fig. 2). The increase in plasma cGMP was sustained during the 24-h treat-
ment course. On discontinuation of inhaled NO, there was a rapid decrease in plasma cGMP concentrations (P < 0.05). Within 60 min of discontinuing NO, plasma cGMP concentrations had decreased to values that were not significantly different from pre-NO values (Fig. 2).

Inhaled NO induced a gradual increase in plasma NOx concentrations (an indirect determinant of total body NO production) over the 24-h treatment course (P < 0.05; Fig. 3). When inhaled NO was discontinued, plasma NOx concentrations began to decrease. Over the study period, NOx concentrations did not return to pre-NO values, correlating with its biological half-life of 3.8 h (35) (Fig. 3).

Over the initial 6 h of inhaled NO therapy, NOS activity, as determined using the conversion of [3H]arginine to [3H]citrulline, decreased from 0.467 ± 0.19 to 0.194 ± 0.07 pmol·min⁻¹·mg lung protein⁻¹ (P < 0.05; Fig. 4). Between 6 and 24 h of inhaled NO therapy, NOS activity was reduced to ~45% of pre-NO values. When inhaled NO was discontinued, NOS activity increased to ~70% of pre-NO values at 1 and 2 h (P < 0.05; Fig. 4).

In the experimental protocol without inhaled NO, there was no change in the hemodynamic variables (data not shown) or NOS activity (0.234 pmol·min⁻¹·mg

**Table 1. Hemodynamic changes during and after 24 hours of inhaled NO**

<table>
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<tr>
<th>Pulmonary arterial pressure, mmHg</th>
<th>Left pulmonary vascular resistance, mmHg·m⁻¹·min⁻¹·kg⁻¹</th>
<th>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</th>
<th>Systemic arterial pressure, mmHg</th>
<th>Heart rate, beats/min</th>
<th>Left atrial pressure, mmHg</th>
<th>Right atrial pressure, mmHg</th>
<th>pH, units</th>
<th>PaO₂, Torr</th>
<th>PaCO₂, Torr</th>
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<tr>
<td>Pre-NO</td>
<td>15 min</td>
<td>6 h</td>
<td>12 h</td>
<td>24 h</td>
<td>10 min off</td>
<td>30 min off</td>
<td>60 min off</td>
<td>120 min off</td>
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<tr>
<td>18.3 ± 1.6</td>
<td>148 ± 3.2†</td>
<td>16.4 ± 2.3</td>
<td>16.0 ± 2.3</td>
<td>16.5 ± 1.5</td>
<td>23.4 ± 6.5†</td>
<td>19.3 ± 3.2</td>
<td>20.6 ± 2.8</td>
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<td>0.318 ± 0.07</td>
<td>0.226 ± 0.05‡</td>
<td>0.241 ± 0.09</td>
<td>0.250 ± 0.06</td>
<td>0.254 ± 0.10</td>
<td>0.418 ± 0.04†</td>
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<td>70.8 ± 20.0</td>
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<td>53.6 ± 13.2*</td>
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Values are means ± SD; n = 6 lambs. NO, nitric oxide; ppm, parts per million; PaCO₂ and PaO₂, arterial PCO₂ and PO₂, respectively. *P < 0.05 vs. pre-NO. †P < 0.05 vs. previous column (ANOVA).
lung protein$^{-1}$ pre-NO vs. 0.223 pmol·min$^{-1}$·mg lung protein$^{-1}$ 24 h after NO). In addition, nitrate concentrations (41.4 µM before, 37.2 µM at 12 h, and 35.5 µM at 24 h after NO) did not increase.

Western blot analysis demonstrated no change in endothelial NOS levels throughout the 26-h study period (Fig. 5). Additional Western blot analysis determined that neither the neuronal nor the inducible NOS isoform was expressed in these lung homogenates (data not shown).

**DISCUSSION**

The present study is the first in vivo investigation of the effects of exogenous inhaled NO therapy and its acute withdrawal on endogenous NOS activity and gene expression. In the intact 1-mo-old lamb, we found that inhaled NO therapy decreased pulmonary vascular resistance over a 24-h treatment period. This was associated with significant increases in plasma cGMP (the second messenger of NO-mediated vasodilation) and NOx (an indirect determinant of total body NO) concentrations (20, 35). Abrupt discontinuation of therapy resulted in a significant increase in pulmonary vascular resistance that exceeded the pre-NO pulmonary vascular resistance. This rebound pulmonary hypertension was associated with decreases in both plasma cGMP and nitrate concentrations. With the use of sequential lung biopsies for the sequential determination NOS activity, we also demonstrated that inhaled NO significantly decreased endogenous NOS activity. 
Over the first 6 h of therapy, NOS activity decreased to ~45% of pre-NO values and remained decreased over the 24-h treatment period. One and two hours after discontinuation of inhaled NO, NOS activity increased to ~70% of pre-NO values. Endothelial NOS protein expression remained unchanged throughout the study period. These data demonstrate that inhaled NO reversibly inhibits endogenous NOS activity but not endothelial NOS protein levels in vivo and strongly suggest that decreased endogenous NOS activity mediates at least in part the recently described rebound pulmonary hypertension associated with termination of inhaled NO therapy.

In the current study we employed a novel study design, sequential lung biopsy sampling during a prolonged course of inhaled NO therapy. This enabled the sequential determination of NOS activity and gene expression during inhaled NO therapy and its withdrawal in the same animal. However, a few concerns regarding this study design are noteworthy. For example, frequent lung biopsy sampling could potentially alter the hemodynamic variables and/or NO production, independent of inhaled NO therapy. To minimize these potential problems, we minimized the amount of lung tissue obtained in each biopsy and found that pulmonary vascular resistance at the end of the study period was not significantly different from the baseline pulmonary vascular resistance. In addition, we performed preliminary studies that demonstrated that 8 h of mechanical ventilation and sequential lung biopsy sampling without inhaled NO therapy did not alter the hemodynamic variables, plasma cGMP or nitrate concentrations, or NOS activity. These data demonstrate that the alterations observed in the present study were secondary to inhaled NO therapy and independent of the unique study design.

Rebound pulmonary hypertension is one of the most significant safety issues regarding inhaled NO therapy. Sudden increases in pulmonary vascular resistance on acute withdrawal of therapy have been described in patients with a variety of pulmonary vascular disorders (2, 10, 25, 26). In newborns with persistent pulmonary hypertension, it is manifested as a sudden decrease in systemic oxygenation with or without hypotension; in children with congenital heart disease, it is manifested by an increase in pulmonary vascular resistance that may compromise cardiac output; and in patients with acute lung injury, it is manifested by a sudden decrease in systemic oxygenation and an increase in pulmonary vascular resistance, which may impair cardiac output. In general, these effects can occur after only hours of therapy, can last between 15 and 60 min, can be alleviated by reinstitution of NO therapy, and are independent of the initial response; patients with no initial pulmonary vasodilatory response can have clinically significant pulmonary vasoconstriction on withdrawal (2, 10, 25, 26). In addition to these cardiopulmonary events, rebound pulmonary hypertension may prolong the need for mechanical ventilation and impede the ability to transport patients (15). Therefore, a better understanding of its mechanism and the potential development of prevention strategies may decrease morbidity of patients treated with inhaled NO.

Previous in vitro data suggest that NO exposure inhibits NOS activity. For example, exogenously added NO or NO-donor compounds inhibit the activity of neuronal NOS in rat cerebellum, endothelial NOS in bovine aortic endothelial cells, and inducible NOS in activated rat alveolar macrophages (6, 17, 28, 30). Our laboratory has previously demonstrated that NO exposure inhibits NOS activity in cultured fetal pulmonary artery endothelial cells, without associated changes in endothelial NOS gene expression (33). The present in vivo study supports this previous in vitro data; inhaled NO decreased lung NOS activity to ~45% of pre-NO values independent of changes in gene expression. On discontinuation of inhaled NO, NOS activity was restored to 70% of pre-NO values after a 1- and 2-h recovery period. Although complete recovery of NOS activity following a longer recovery period cannot be ruled out, these data suggest both a reversible and an irreversible component of NOS inhibition. Similarly, in cultured endothelial cells, exogenous NO-induced NOS inhibition appears to have both a reversible and irreversible component. For example, removal of exogenous NO from the culture medium only partially restores endothelial NOS activity (28, 33). The exact mechanisms by which NO inhibits NOS activity remain unclear. However, preliminary in vitro data suggest that reversible NOS inhibition can occur by the binding of NO to the heme moiety of NOS, and an irreversible NOS inhibition can occur via peroxynitrite formation due to the reaction of NO with superoxide (18, 33). A previous in vivo study in rats has suggested that 1–3 wk of exposure to 20 ppm inhaled NO does not alter NOS activity or protein levels (13). It is unclear why the results of this study conflict with our own. However, it is possible that the presence of reducing agents in their assay restored the reversible NOS inactivation caused either by NO binding to the heme moiety or by increased levels of disulfides in the protein (13). In addition differences in species, duration, dose, and administration of inhaled NO also have to be taken into account. Further studies will be needed to investigate these differences and to identify the potential mechanisms of inhaled NO-induced NOS inactivation in vivo.

Two 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 to produce rebound pulmonary hypertension and their effect on endogenous NOS activity. In addition, these studies were performed in lambs with normal pulmonary circulations. Patients with pulmonary hypertension, who are currently treated with inhaled NO, often have preexisting aberrations in the NO-cGMP cascade (9, 14). Further studies are warranted to determine the effects of inhaled NO in the abnormal pulmonary circulation. Finally, because peripheral lung tissue was obtained for NOS activity determinations, changes in NOS activity cannot be isolated to a specific cell type. However,
we performed additional Western blot analysis on these lung samples that failed to reveal significant expression of either neuronal or inducible NOS, suggesting that the changes noted are reflective of changes in endothelial NOS activity.

Although inhaled NO therapy is currently limited to investigational use in the United States, its use in patients with pulmonary hypertensive disorders is increasing dramatically. Rebound pulmonary hypertension can result in significant increases in pulmonary vascular resistance and decreases in systemic oxygenation (2, 10, 25, 26). Current prevention strategies include slow weaning protocols for inhaled NO and the addition of other vasodilatory therapy at the time of NO discontinuation (21, 23). Although effective, these strategies may prolong the need for mechanical ventilation, impede the ability to urgently transport patients, and do not protect against inadvertent discontinuation of inhaled NO therapy (15). The present study is the first in vivo investigation of the effects of inhaled NO therapy on endogenous NOS activity. In support of previous in vitro investigations, we found that exogenous inhaled NO induces a significant inhibition of endogenous lung NOS activity in the intact lamb. Abrupt withdrawal of inhaled NO resulted in a dramatic increase in pulmonary vascular resistance, at a time when endogenous NOS activity was significantly decreased. Resolution of the pulmonary hypertension was associated with increases in endogenous NOS activity. These data suggest that decreased endogenous NOS activity mediates in part the recently described rebound pulmonary hypertension associated with withdrawal of inhaled NO therapy. A better understanding of the mechanism by which inhaled NO alters endogenous NO production could lead to more effective treatment and prevention strategies for rebound pulmonary hypertension and thereby decrease potential morbidity of patients treated with inhaled NO.

This research was supported by Grant 97–212 from the American Heart Association, Western States Affiliate (S. M. Black), HL-60190 from the National Heart, Lung, and Blood Institute (S. M. Black), 9640010N E1A from the American Heart Association (J. R. Fineman), and FY97–0175 from the March of Dimes (J. R. Fineman). Address for reprint requests and other correspondence: S. M. Black, Div. of Neonatology, Northwestern University Medical School, Ward 12–191 (MS W-140), 303 E. Chicago Ave., Chicago, IL 60611-3008 (E-mail: steveblack@nwu.edu).

Received 25 January 1999; accepted in final form 10 June 1999.

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