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Inhaled nitric oxide-induced rebound pulmonary hypertension: role for endothelin-1

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McMullan, D. Michael, Janine M. Bekker, Michael J. Johengen, Karen Hendricks-Munoz, Rene Gerrets, Stephen M. Black, and Jeffrey R. Fineman. Inhaled nitric oxide-induced rebound pulmonary hypertension: role for endothelin-1. *Am J Physiol Heart Circ Physiol* 280: H777–H785, 2001.—Clinically significant increases in pulmonary vascular resistance have been noted on acute withdrawal of inhaled nitric oxide (NO). Endothelin (ET)-1 is a vasoactive peptide produced by the vascular endothelium that may participate in the pathophysiology of pulmonary hypertension. The objectives of this study were to determine the effects of inhaled NO on endogenous ET-1 production in vivo in the intact lamb and to determine the potential role of ET-1 in the rebound pulmonary hypertension associated with the withdrawal of inhaled NO. Seven 1-mo-old vehicle-treated control lambs and six PD-156707 (an ET_A receptor antagonist)-treated lambs were mechanically ventilated. Inhaled NO (40 parts per million) was administered for 24 h and then acutely withdrawn. After 24 h of inhaled NO, plasma ET-1 levels increased by 119.5 ± 42.2% ($P < 0.05$). Western blot analysis revealed that protein levels of preproET-1, endothelin-converting enzyme-1α, and ET_A and ET_B receptors were unchanged. On acute withdrawal of NO, pulmonary vascular resistance (PVR) increased by 77.8% ($P < 0.05$) in control lambs but was unchanged (−5.5%) in PD-156707-treated lambs. Inhaled NO increased plasma ET-1 concentrations but not gene expression in the intact lamb, and ET_A receptor blockade prevented the increase in PVR after NO withdrawal. These data suggest a role for ET-1 in the rebound pulmonary hypertension noted on acute withdrawal of inhaled NO.

endothelium-derived factors; pulmonary heart disease; endothelin receptor; pulmonary hypertension of the newborn

EXOGENOUSLY ADMINISTERED inhaled nitric oxide (NO) is currently utilized as an adjuvant therapy for a number of pulmonary hypertensive disorders. In both animal and human studies (3, 9, 11, 30, 31), inhaled NO [5–80 parts per million (ppm)] induces rapid and selective pulmonary vasodilation. 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 vasodilation. No systemic vasodilation occurs because NO is rapidly inactivated by binding with hemoglobin when it reaches the intravascular space (19). Recent multicentered randomized trials (9, 11, 30) have demonstrated that inhaled NO improves oxygenation and decreases the need for extracorporeal life support in newborns with persistent pulmonary hypertension. In addition, nonrandomized studies (3, 31) 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. 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 (2, 12, 21, 24) have noted a potentially life-threatening increase in pulmonary vascular resistance on acute withdrawal of inhaled NO. This "rebound pulmonary hypertension" is manifested by an increase in pulmonary vascular resistance, compromised cardiac output, and/or severe hypoxemia (2, 12, 21, 24). Recent data demonstrate that exogenous NO exposure inhibits endogenous endothelial NO synthase (NOS) activity, suggesting that transient decreases in endogenous NOS activity during inhaled NO therapy may be a potential mechanism for rebound pulmonary hypertension (6, 8, 34).

Endothelin (ET)-1 is a 21-amino acid polypeptide produced by vascular endothelial cells whose potent vasoactive properties have been implicated in the pathophysiology of pulmonary hypertensive disorders

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(40). The gene for human ET-1 is located on chromosome 6 and is translated to a 203-amino acid peptide precursor (preproET-1), which is then cleaved to form proendothelin-1. Proendothelin (Big ET-1) is then cleaved by the metalloprotease endothelin converting enzyme-1 (ECE-1) into its functional form (37). The complex pulmonary vasoactive effects of ET-1, which may include either pulmonary vasoconstriction and/or pulmonary vasodilation, are mediated by at least two different receptors: ET_A and ET_B. ET_A receptors and a subpopulation of ET_B receptors mediate vasoconstriction and are located on vascular smooth muscle cells. A second subpopulation of ET_B receptors mediate vasodilation and are located on vascular endothelial cells (1, 33, 35). Increasing data suggest that endogenous ET-1 and NO participate in the regulation of each other through an autocrine feedback loop (22). For example, ET-1 stimulates endothelial NOS activity via ET_B receptor activation, whereas NO-cGMP production increases ET_A receptors in vascular smooth muscle cells and inhibits ET-1 secretion and gene expression in vascular endothelial cells (7, 28). However, the potential effects of inhaled NO on endogenous ET-1 production have not been studied in vivo.

The purposes of this study were *1*) to investigate the effects of inhaled NO on ET-1 production and gene expression and *2*) to investigate the role of ET-1 in the rebound pulmonary hypertension associated with NO withdrawal. To determine the effects of inhaled NO on endogenous ET-1, sequential plasma samples were taken for ET-1 concentrations in seven 1-mo-old lambs during 24 h of inhaled NO (40 ppm) therapy. In addition, sequential peripheral lung biopsies were taken for protein determinations of preproET-1, ECE-1α, and ET_A and ET_B receptors by Western blot analysis. To determine the role of ET-1 in rebound pulmonary hypertension, the hemodynamic effects of inhaled NO and its acute withdrawal were determined and compared with an additional six lambs pretreated with an infusion of PD-156707 (1.0 $mg \cdot kg^{-1} \cdot h^{-1}$), a selective ET_A receptor antagonist.

METHODS

Surgical preparation. Thirteen lambs (30.1 ± 4.3 days old) 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 hind leg. These catheters were advanced to the descending aorta and inferior vena cava, respectively. The lambs were then anesthetized with ketamine hydrochloride (~0.3 $mg \cdot kg^{-1} \cdot min^{-1}$), diazepam (0.002 $mg \cdot kg^{-1} \cdot h^{-1}$), and fentanyl citrate (1.0 $\mu g \cdot kg^{-1} \cdot h^{-1}$), 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 per dose) was given intermittently for muscle relaxation. With the use of strict aseptic technique, a midsternotomy incision was then performed, and the pericardium was incised. With the use of a purse-string suture technique, polyurethane catheters were placed directly into 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 gentamicin (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 30-min recovery period, an intravenous infusion of normal saline ($n = 7$, vehicle control) or PD-156707 (a selective ET_A receptor antagonist; 1.0 $mg \cdot kg^{-1} \cdot h^{-1}$, $n = 6$) was begun and continued throughout the study period. The dose of PD-156707 was chosen after several previous studies (18, 27, 29, 32) showed that a 30-min infusion completely blocked the vasoconstricting effects of exogenous ET-1 and resulted in steady-state plasma concentrations that blocked ET_A receptors in vivo. Thirty minutes after initiation of the infusion, 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). Blood was collected from the femoral artery for plasma ET-1 determinations, and a peripheral lung wedge biopsy was obtained for preproET-1, ECE-1, and ET_A and ET_B receptor protein determinations. 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.

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 NO_2 were continuously quantified by electrochemical methodology (Inovent). The hemodynamic variables were monitored continuously. Systemic arterial blood gases were determined intermittently, and ventilation was adjusted to achieve a Pa_{CO_2} between 35 and 45 Torr and a $Pa_{O_2} > 50$ Torr. Sodium bicarbonate was administered intermittently to maintain a pH > 7.30. Normal saline was administered intermittently to maintain stable atrial pressures throughout the study period. Peripheral lung wedge biopsies were performed, and blood was obtained for plasma ET-1 determinations after 2, 6, and 24 h of therapy. The inhaled NO was then stopped, and the hemodynamic variables were monitored for an additional 2 h. Blood was obtained 60 and 120 min after discontinuation of inhaled NO. All blood losses were replaced with maternal blood.

To ensure that potential changes demonstrated resulted from inhaled NO and not from mechanical ventilation alone, two additional lambs were intubated, sedated, and mechanically ventilated for 24 h as described above without inhaled NO therapy.

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 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 pressures and right and left atrial pressures were measured using Sorenson neonatal transducers (Abbott 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. Left pulmonary blood flow was measured on an

ultrasonic flow meter (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 Radiometer ABL5 pH/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin 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 ET-1 determinations. Systemic arterial blood (4 ml) was collected and placed in iced polypropylene tubes containing 330 μl aprotinin 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 C18 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% acetronitrile. The eluant was dried in a Savant speed vac and stored at −70°C or assayed immediately for immunoreactive endothelin (ET-1). The ET-1 standard, ^{125}I-labeled ET-1, anti-ET antibody, and secondary antibody were purchased from Peninsula Laboratories. Cross-reactivity for measured human and bovine ET-1 antiserum is 100% for human ET-1, 7% for human ET-2 and ET-3, and 0% for bovine ET-2 and ET-3. Inter- and intra-assay variabilities were 10 and 4% respectively. Each sample was assayed in duplicate. This assay was modified from a previously published method (39).

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. 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 (6). Briefly, protein extracts (25 μg) were separated on 7.5% denaturing polyacrylamide gels for ECE-1α, 10% denaturing polyacrylamide gels for ET_A and ET_B receptors, or 15% denaturing polyacrylamide gradient gels for preproET-1. Positive controls were also included for the ECE-1α and ET_A Western blots. These consisted of protein extracts (10 μg) prepared from COS-7 cells transiently transfected with a mammalian expression vector containing the full-length bovine ECE-1α cDNA (a generous gift from Dr. M. Yanagisawa, Howard Hughes Medical Institute, UT and Southwestern Medical Center, Dallas, TX) or a full-length rat ET_A receptor (a generous gift from Dr. C. Miyamoto, Department of Molecular Genetics, Nippon Roche research Center, Kamakura, Japan). All gels were electrophoretically transferred to Hybond-polyvinylidene fluoride membranes (Amersham). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween. After blocking, the membranes were incubated at room temperature with the appropriate dilution of the antiserum of interest (1:1,000 for ECE-1α, 1:1,000 for ET_A and ET_B, or 1:500 for preproET-1), washed with Tris-buffered saline containing 0.1% Tween, and then incubated with either goat anti-rabbit IgG-horseradish peroxidase conjugate (for ECE-1α and ET_A and ET_B receptors) or goat anti-sheep IgG-horseradish peroxidase conjugate (for preproET-1). After washing, chemiluminescence was used to detect the protein bands.

The ET_A receptor antiserum was generated as previously described (4). The ET_B receptor antiserum was obtained from Maine Biotechnology Services (Portland, ME). The preproET-1 antibody was obtained from Affinity Bioreagents (Golden, CO). The specificity of the preproET-1 antibody was verified with a preincubation step with purified ET-1 (50 ng ET-1 per 15 μl of antiserum) protein. The purified ET-1 was purchased from Sigma (St. Louis, MO).

Positive controls were run to demonstrate antibody specificity. The methodology and exposure times used were those that we have previously demonstrated to be within the linear range of the autoradiographic film and able to detect changes in lung protein expression (4).

Generation of ECE-1α antisera. This was undertaken commercially (Biosynthesis, Lewisville, TX). A peptide was designed that was specific for the ECE-1α protein [the predominant isoform of ECE-1 in the lung (32)]. This peptide (SYKRATLDEEDL) corresponded to amino acids 4–15 of the rat ECE-1α protein and was synthesized at >90% purity. The peptide was then conjugated, via the addition of a COOH-terminal cysteine, to KLH. Two female New Zealand White rabbits (12 wk of age and 2 kg in weight) were then injected with 200 μg of conjugated peptide and 200 μg of Fruend's complete adjuvant. This injection was repeated after 14, 28, 42, and 56 days with the exception that Fruend's incomplete adjuvant was used. Bleeds (15 ml) were taken at 42, 56, and 70 days, and IgG purification and ELISA analysis were then carried out. Aliquots of antisera were then stored at −20°C until used.

Statistical analysis. The mean ± SD was calculated for the baseline hemodynamic variables, systemic arterial blood gases and pH, and plasma ET-1 concentrations. The general hemodynamic variables, systemic arterial blood gases and pH, and ET-1 concentrations were compared over time within each group by ANOVA for repeated measures. Comparisons between treatment groups (PD-156707 vs. control) were made by unpaired *t*-test.

Quantitation of autoradiographic results was performed by scanning (Hewlett-Packard SCA Jet IICX, Hewlett-Packard, Palo Alto, CA) the bands of interest into an image editing software program (Adobe Photoshop, Adobe Systems, Mt. View, CA). 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. The mean ± SD was calculated for the relative protein at each time point after the start of inhaled NO therapy. Comparisons over time were made by paired *t*-test. A $P < 0.05$ was considered statistically significant.

RESULTS

There were no differences in age, weight, sex distribution, or baseline hemodynamic variables between control and PD-156707-treated lambs (data not shown).

In control lambs, inhaled NO (40 ppm) rapidly decreased mean pulmonary arterial pressure and left pulmonary vascular resistance (from 0.242 ± 0.04 to 0.179 ± 0.02 mmHg/ml per min/kg) ($P < 0.05$). 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. During the 24-h treatment course, pulmonary arterial pressure returned to the pre-NO value. Systemic arterial pressure slightly decreased and systemic Pa_{CO_2} increased compared with pre-NO values (Table 1). On 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). Right atrial pressure increased, and systemic Pa_{O_2} decreased ($P < 0.05$). Left pulmonary blood flow, mean systemic arterial pressure, heart rate, left atrial pressures, and systemic Pa_{CO_2} and pH remained unchanged from 24 h NO values (Table 1).

To begin 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 and remained elevated 60 min after discontinuation ($P < 0.05$) (Fig. 2). In addition, Western blot analysis demonstrated no change in preproET-1, ECE-1α, or ET_A or ET_B receptor protein levels throughout the study period (Figs. 3–6).

The infusion of PD-156707 decreased mean pulmonary arterial pressure (from 14.0 ± 3.3 to 12.0 ± 2.1 mmHg, $P < 0.05$) and mean systemic arterial pressure (from 64.0 ± 9.9 to 50.7 ± 4.7 mmHg, $P < 0.05$). Left pulmonary vascular resistance, left pulmonary blood flow, heart rate, and right and left atrial pressures were unchanged.

In PD-156707-treated lambs, the initiation of inhaled NO did not change the hemodynamic variables. Systemic Pa_{O_2} increased ($P < 0.05$) (Table 2). During the 24-h treatment course, systemic arterial pressure decreased (Table 2). On discontinuation of inhaled NO, mean pulmonary arterial pressure and left pulmonary vascular resistance remained unchanged (Table 2 and Fig. 1). During the 2-h study period after the discontinuation of inhaled NO, left pulmonary vascular resistance was greater in control lambs than PD-156707-treated lambs ($P < 0.05$) (Fig. 1).

In PD-156707-treated lambs, plasma ET-1 concentrations increased during inhaled NO therapy (from 6.5 ± 1.5 to 25.2 ± 19.1 pg/ml, $P < 0.05$) to values that were similar to control lambs.

Mechanical ventilation and sequential lung biopsy sampling without inhaled NO therapy did not alter pulmonary arterial pressure or pulmonary blood flow. Mean systemic arterial pressure decreased from 60 to 51 mmHg after 24 h of mechanical ventilation. In addition, plasma ET-1 concentrations were unchanged (data not shown).

Table 1. *Hemodynamic changes during and after 24 h of inhaled NO in control lambs*

	Inhaled NO (40 ppm)					Off NO			
	Pre-NO	15 min	2 h	6 h	24 h	10 min off	30 min off	60 min off	120 min off
Pulmonary arterial pressure, mmHg	13.2 ± 1.3	11.6 ± 1.8*†	12.9 ± 2.7	12.0 ± 2.0	12.0 ± 1.2	16.8 ± 0.6*†	17.2 ± 1.6*	17.1 ± 1.6*	16.4 ± 2.1*
Left pulmonary blood flow, ml·kg^{-1}·min^{-1}	33.3 ± 7.3	33.8 ± 6.9	33.7 ± 6.5	33.2 ± 9.8	29.1 ± 6.1	28.1 ± 5.4*	31.8 ± 4.3	30.5 ± 3.3	31.1 ± 4.4
Systemic arterial pressure, mmHg	63.7 ± 6.9	61.7 ± 9.5	62.9 ± 10.4	60.2 ± 10.7	45.7 ± 6.9*†	48.9 ± 15.3*	49.1 ± 10.7*	48.3 ± 9.2*	48.2 ± 13.8*
Heart rate, beats/min	152.0 ± 17.7	142.7 ± 14.1	146.4 ± 16.4	144.0 ± 16.5	137.9 ± 17.5	135.9 ± 12.6*	144.8 ± 21.2	143.8 ± 21.5	143.9 ± 18.6
Left atrial pressure, mmHg	5.1 ± 1.9	5.6 ± 2.0	5.1 ± 2.3	4.1 ± 1.1	5.7 ± 0.5	6.0 ± 1.7	5.5 ± 1.5	5.3 ± 1.9	4.7 ± 1.4
Right atrial pressure, mmHg	4.2 ± 1.7	4.0 ± 1.5	4.0 ± 1.4	3.0 ± 1.2	4.0 ± 1.9	5.7 ± 2.1*†	5.1 ± 1.6	5.0 ± 1.5	4.7 ± 1.3
pH, units	7.41 ± 0.03	7.42 ± 0.03	7.44 ± 0.02*	7.41 ± 0.02	7.39 ± 0.05	7.38 ± 0.03	7.36 ± 0.03*	7.38 ± 0.04	7.37 ± 0.03
Pa_{CO_2}, Torr	33.1 ± 3.2	32.7 ± 2.1	33.1 ± 1.3	38.4 ± 2.4*†	36.7 ± 2.0*	37.7 ± 3.7*	39.0 ± 5.6*	37.7 ± 4.7*	37.9 ± 2.5*
Pa_{O_2}, Torr	77.4 ± 6.1	86.4 ± 6.4	84.7 ± 7.6	73.7 ± 5.7†	83.6 ± 17.9†	61.6 ± 7.0*†	60.7 ± 4.8	59.6 ± 4.7	57.6 ± 6.4*

Values are means ± SD; $n = 7$. NO, nitric oxide; ppm, parts per million; Pa_{CO_2} and Pa_{O_2}, systemic arterial P_{O_2} and P_{CO_2}. *$P < 0.05$ vs. pre-NO; †$P < 0.05$ vs. previous column (by ANOVA).

DISCUSSION

Increasing evidence suggests that vascular endothelial function is a vital mediator of pulmonary vascular tone and growth. Both NO and ET-1 are potent vasoactive factors produced by the vascular endothelium and are important mediators of the fetal, transitional, and postnatal pulmonary circulations (13). In addition, aberrations in endothelial function have been impli-

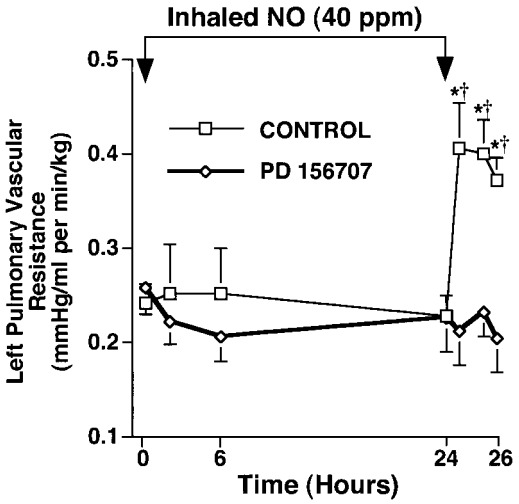

Fig. 1. Changes in left pulmonary vascular resistance before, during, and after 24 h of inhaled nitric oxide [NO; 40 parts per million (ppm)] therapy. $n = 7$ control lambs and 6 PD-156707-treated lambs. PD-156707 blocks the increase in left pulmonary vascular resistance after acute withdrawal of inhaled NO. Values are means ± SE. *$P <$ 0.05 vs. 0 h; †$P < 0.05$ vs. previous data point (by ANOVA).

cated in the pathophysiology of many pulmonary hypertensive disorders. For example, decreased NO gene expression and increased ET-1 gene expression have been demonstrated in patients with advanced pulmonary vascular disease (15, 16). Recently, exogenous inhaled NO has been utilized as an adjunct therapy for pulmonary hypertension. It produces potent selective pulmonary vasodilation that is independent of endothelial cell function (3, 9, 11, 19, 30, 31). Although many studies (2,12, 21, 24) demonstrate a clear benefit in patient outcome with inhaled NO use, several safety concerns remain, including its potential acute and chronic adverse effects on endogenous endothelial function. For example, recent in vitro and in vivo data suggest that exogenous NO decreases endogenous NOS activity and that the resulting decrease in NO production may mediate the clinically significant increases in

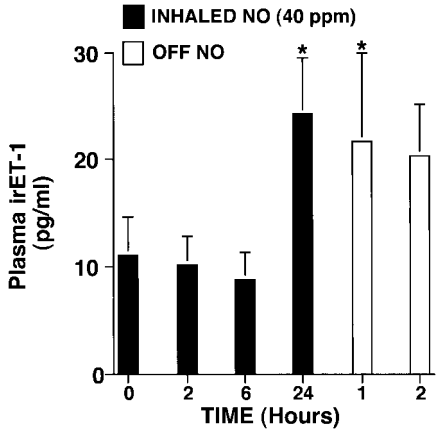

Fig. 2. Changes in plasma endothelin (ET)-1 concentrations before, during, and after 24 h of inhaled NO (40 ppm) therapy. $n = 7$ control lambs. Values are means ± SE. * $P < 0.05$ vs. 0 h. irET-1, immunoreactive ET-1.

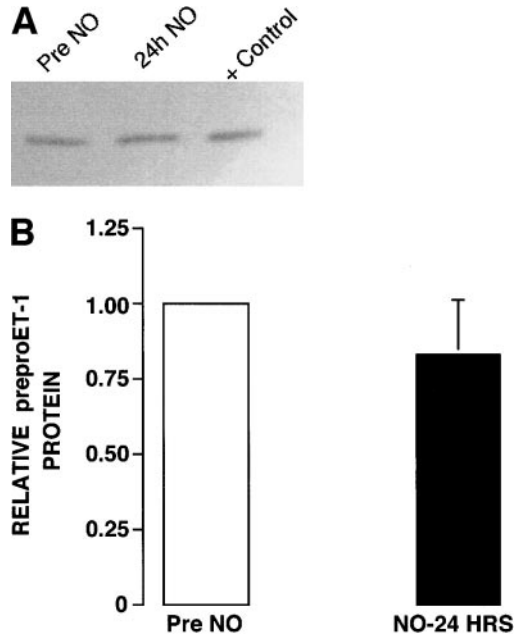

Fig. 3. Western blot analysis for preproET-1 protein in lung tissue before and after 24 h of inhaled NO (40 ppm) therapy. *A*: representative Western blot is shown from protein extracts (25 μg) prepared from lung tissue from a 1-mo-old lamb, separated on a 15% denaturing polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against preproET-1. *B*: densitometric values for relative preproET-1 protein (normalized to *time 0*) from 5 control lambs. Values are means ± SE. PreproET-1 protein expression was unchanged during inhaled NO therapy.

pulmonary vascular resistance noted on inhaled NO withdrawal (6, 34). Despite increasing evidence that NO and ET-1 coregulate each other within the pulmonary circulation, the potential effects of inhaled NO on endogenous ET-1 have not been previously investigated (7, 22, 28). To our knowledge, the present study is the first in vivo investigation of the effects of exogenous inhaled NO therapy on endogenous ET-1 and gene expression. In the intact 1-mo-old lamb, we found that inhaled NO increases plasma ET-1 concentrations independent of changes in lung protein expression and that pretreatment with a selective ET_A receptor antagonist completely blocks the acute increase in pulmonary vascular resistance associated with inhaled NO withdrawal.

Two previous investigations (10, 26) have measured plasma ET-1 concentrations during inhaled NO administration. In newborns with persistent pulmonary hypertension, plasma ET-1 concentrations decreased in all neonates, but NO-treated neonates displayed a greater decrease in ET-1 than conventionally treated neonates (10). Conversely, a preliminary investigation in children with pulmonary hypertension after cardiac surgery demonstrates an increase in plasma ET-1 concentrations in inhaled NO-treated patients (26). These conflicting results are difficult to interpret given the dynamic changes in these patients and their potential differences in endogenous endothelial dysfunction. In the present study, we demonstrate a clear increase in

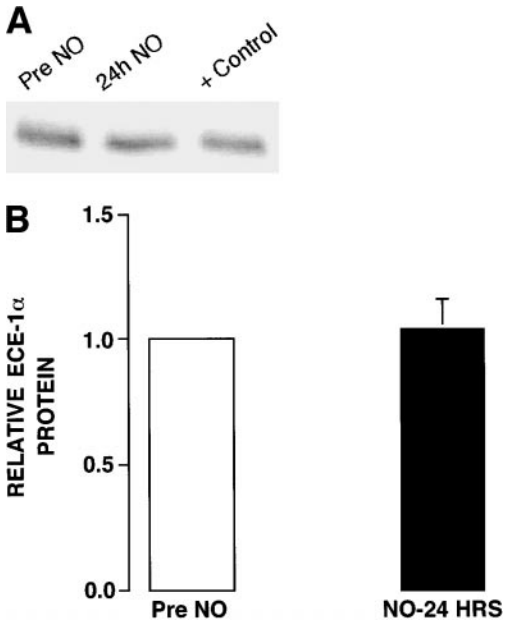

Fig. 4. Western blot analysis for endothelin-converting enzyme (ECE)-1α protein in lung tissue before and after 24 h of inhaled NO (40 ppm) therapy. *A*: representative Western blot is shown from protein extracts (25 μg) prepared from lung tissue from a 1-mo-old lamb, separated on a 7.5% SDS-polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against ECE-1α. Also included is a positive control consisting of a protein extract (10 μg) prepared from COS-7 cell transiently transfected with a mammalian expression vector containing the full-length bovine ECE-1α cDNA. *B*: densitometric values for relative ECE-1α protein (normalized to *time 0*) from 5 control lambs. Values are means ± SE. ECE-1α protein expression was unchanged during inhaled NO therapy.

plasma ET-1 concentrations in normal 1-mo-old lambs during inhaled NO administration. After 24 h of therapy, ET-1 concentrations more than doubled and began to decline after NO withdrawal.

Increases in plasma ET-1 concentrations 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 preproET-1 into a nonactive 38-amino acid residue known as Big ET-1. Big ET-1 in then cleaved into its functional form, ET-1, by the endopeptidase ECE-1 (37). ECE-1 exists in two isoforms, ECE-1α and ECE-1β, with ECE-1α considered to be the most biologically important (36). Because many studies suggest that ET-1 production is regulated at the transcriptional level of preproET-1 and/or ECE-1, we performed sequential lung biopsies to determine potential changes in preproET-1 and ECE-1α protein levels. We found that both preproET-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 ET_B receptor has been implicated in the clearance of ET-1 from the circulation, but we found no changes in protein levels of the ET_B receptor during inhaled NO (14). Rapid ET-1 release from intracellular secretory granules has been demonstrated after such stimuli as cytokines and stretch (23, 25). 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 ET_B binding affinity represent additional potential mechanisms that were not studied but warrant investigation.

Several previous in vitro studies (17, 20, 38) have investigated the effects of endogenous and exogenous NO-cGMP on ET-1 production. The majority of studies demonstrate that endogenous NO production downregulates ET-1 production. Although these data may appear to conflict with our present study, the effects of exogenous NO on ET-1 production in vitro is less clear. In fact, some in vitro investigations (17, 38) demonstrate a differential effect between endogenous and exogenous NO on ET-1 production, with no downregulation of ET-1 demonstrated on exposure to exogenous NO. In addition, to our knowledge, there are no in vitro investigations of exogenous NO on pulmonary vascular endothelial cells, which may behave quite differently than other derived cell lines. It is also interesting to note that we (5) have previously demonstrated that endogenous NOS activity is decreased in these lambs during inhaled NO therapy. Whether this resultant decrease in endogenous NO production participates in the increase in plasma ET-1 concentrations during NO is unclear and warrants further study.

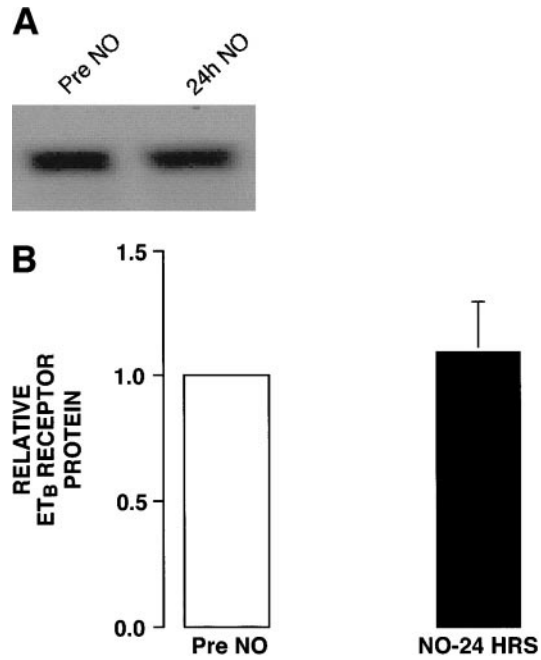

Fig. 5. Western blot analysis for ET_B receptor protein in lung tissue before and after 24 h of inhaled NO (40 ppm) therapy. *A*: representative Western blot is shown from protein extracts prepared from lung tissue from a 1-mo-old lamb, separated on a 10% SDS-polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against ET_B receptor. *B*: densitometric values for relative ET_B receptor protein (normalized to *time 0*) from 6 control lambs. Values are means ± SE. ET_B receptor protein expression was unchanged during inhaled NO therapy.

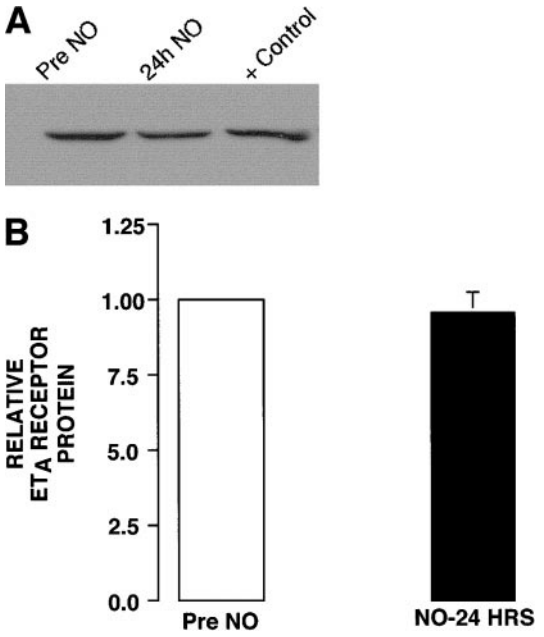

Fig. 6. Western blot analysis for ET_A receptor protein in lung tissue before and after 24 h of inhaled NO (40 ppm) therapy. *A*: representative Western blot is shown from protein extracts prepared from lung tissue from a 1-mo-old lamb, separated on a 10% SDS-polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against ET_A receptor. Also included is a positive control consisting of a protein extract prepared from COS-7 cell transiently transfected with a mammalian expression vector containing the full-length rat ET_A cDNA. *B*: densitometric values for relative ET_A receptor protein (normalized to *time 0*) from 6 control lambs. Values are means ± SE. ET_A receptor protein expression was unchanged during inhaled NO therapy.

Rebound pulmonary hypertension is one of the most significant safety issues regarding inhaled NO therapy. Clinically significant increases in pulmonary vascular resistance on acute withdrawal of therapy have been described in patients with a variety of pulmonary vascular disorders (2, 12, 21, 24). In general, these effects can occur after only hours of therapy and are independent of the initial response; patients with no initial pulmonary vasodilatory response can have life-threatening pulmonary vasoconstriction on withdrawal (2, 12, 21, 24). In addition to these life-threatening events, rebound pulmonary hypertension may prolong the need for mechanical ventilation and impede the ability to transport patients. Therefore, a better understanding of the mechanism and potential development of prevention strategies may decrease morbidity of patients treated with inhaled NO. Our laboratory (5) has previously demonstrated that inhaled NO decreases endogenous NOS activity, suggesting that decreased endogenous NOS activity mediates, at least in part, the rebound pulmonary hypertension associated with withdrawal of inhaled NO therapy. Because we initially found that plasma ET-1 concentrations were increased during NO therapy, we then pretreated six additional lambs with an ET_A receptor antagonist to determine the potential role of ET-1 during rebound pulmonary hypertension. To selec-

Table 2. *Hemodynamic changes during and after 24 h of inhaled NO in PD-156707-treated lambs*

	Inhaled NO (40 ppm)					Off NO			
	Pre-NO	15 min	2 h	6 h	24 h	10 min off	30 min off	60 min off	120 min off
Pulmonary arterial pressure, mmHg	12.0 ± 2.2	11.7 ± 1.5	12.0 ± 2.2	11.0 ± 1.1	11.5 ± 1.5	11.5 ± 1.4	12.3 ± 2.3	13.7 ± 3.4	12.3 ± 0.8
Left pulmonary blood flow, ml·kg^{-1}·min^{-1}	29.8 ± 8.1	28.3 ± 7.1	31.9 ± 7.2	32.4 ± 8.3	33.5 ± 8.0	33.5 ± 7.5	35.3 ± 9.6	35.9 ± 9.6	36.2 ± 8.0
Systemic arterial pressure, mmHg	50.7 ± 4.7	50.3 ± 8.7	62.7 ± 17.7*†	49.0 ± 8.6†	33.3 ± 3.9*†	30.7 ± 2.0*	30.3 ± 2.3*	32.3 ± 1.5*	32.7 ± 6.0*
Heart rate, beats/min	147.0 ± 16.7	155.8 ± 13.2	152.0 ± 12.0	154.5 ± 15.6	143.3 ± 14.7	138.2 ± 25.3	140.3 ± 36.3	142.5 ± 27.3	141.8 ± 24.1
Left atrial pressure, mmHg	4.7 ± 2.7	4.7 ± 2.9	5.0 ± 2.7	4.7 ± 1.4	4.5 ± 2.6	4.8 ± 2.5	4.7 ± 2.3	5.5 ± 2.6	5.3 ± 2.3
Right atrial pressure, mmHg	3.8 ± 2.2	4.3 ± 2.1	3.3 ± 1.5	3.5 ± 1.0	4.2 ± 1.8	4.0 ± 1.9	4.5 ± 1.9	4.8 ± 2.5	4.2 ± 1.7
pH, units	7.43 ± 0.02	7.44 ± 0.03	7.41 ± 0.01	7.42 ± 0.03	7.38 ± 0.05	7.36 ± 0.04*	7.36 ± 0.03*	7.34 ± 0.04*	7.39 ± 0.05†
Pa_{CO_2}, Torr	33.8 ± 1.2	34.0 ± 3.7	36.3 ± 1.9	34.5 ± 2.3	33.2 ± 5.5	36.0 ± 3.5	36.7 ± 4.8	39.0 ± 9.7*	33.0 ± 4.0†
Pa_{O_2}, Torr	67.0 ± 3.5	84.0 ± 31.3*	77.0 ± 17.1	67.8 ± 9.7	70.0 ± 9.7	71.8 ± 16.6	67.7 ± 18.5	60.5 ± 7.9	65.0 ± 16.3

Values are means ± SD; $n = 6$. *$P < 0.05$ vs. pre-NO; †$P < 0.05$ vs. previous column (by ANOVA).

tively block ET_A receptor activity during and after inhaled NO, we utilized PD-156707, a nonpeptide ET_A receptor antagonist. PD-156707 is highly selective for the ET_A receptor and inhibits the binding of ^{125}I-labeled ET-1 to cloned human ET_A and ET_B receptors with inhibitory constant values of 0.17 and 133.8 nM, respectively (29). In rabbits, PD-156707 infusion rates of 0.03 $mg \cdot kg^{-1} \cdot h^{-1}$ completely blocked the vasoconstricting effects of exogenous ET-1, with corresponding plasma concentrations that were <0.05 μg/ml (10^{-7} M) (18, 32). We have also performed several preliminary studies in lambs that demonstrate that PD-156707 infusion rates of 1.0 $mg \cdot kg^{-1} \cdot h^{-1}$ completely and selectively block the vasoconstricting effects of exogenous ET-1 (250 ng/kg) and produce stable plasma concentrations of >500 ng/ml within 30 min of initiating the infusion (27). Therefore, in the present study, we utilized an infusion rate of 1.0 $mg \cdot kg^{-1} \cdot h^{-1}$ that was initiated 30 min before the initiation of inhaled NO. Interestingly, we found that ET_A receptor blockade completely blocked the rebound pulmonary hypertension, suggesting an important role for ET-1-mediated vasoconstriction in inhaled NO-induced rebound pulmonary hypertension. A previous in vitro study (28) has demonstrated that exogenous NO upregulates the ET_A receptor in cultured vascular smooth muscle cells. Therefore, we determined the protein levels of the ET_A receptor in sequential lung biopsies and found no changes in protein levels during inhaled NO. These data suggest that increased ET-1-mediated pulmonary vasoconstriction results from the increase in plasma ET-1 levels without changes in gene expression of the ET_A receptor. However, changes in receptor binding affinity may participate and cannot be excluded.

It is interesting to note that, despite an increase in plasma ET-1 concentrations during the study period, systemic arterial pressure did not increase. In fact, after 24 h of NO, systemic arterial pressure was lower than pre-NO values. Because ET-1 is known to produce systemic as well as pulmonary vasoconstriction, these data were surprising, and the etiology remains unclear. However, possible explanations include changes in ET receptor gene expression and/or binding affinities in the systemic circulation during the study period and the possible accumulation of anesthesia effects. Systemic arterial pressure also decreased in our two lambs that were studied without inhaled NO, and a previous lamb investigation (12a), unrelated to inhaled NO, has demonstrated a decrease in systemic vascular resistance after prolonged study periods, suggesting that this systemic effect is unrelated to inhaled NO.

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 on endogenous ET-1. 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 and ET-1 cascades (15, 16). Further studies are warranted to determine the effects of inhaled NO in the abnormal pulmonary circulation.

Inhaled NO was recently approved by the Food and Drug Administration for use in neonates with hypoxemic respiratory failure and persistent pulmonary hypertension. Associated with this approval, we can expect an increase in not only the acute usage of inhaled NO for patients with pulmonary hypertension but potential chronic usage as well. The present study is the first in vivo investigation of the effects of inhaled NO therapy on endogenous ET-1 production. We found that exogenous inhaled NO induces a significant increase in plasma ET-1 concentrations in the intact lamb and that ET_A receptor blockade prevented the rebound pulmonary hypertension. These data suggest that increased ET-1-mediated pulmonary vasoconstriction mediates, at least in part, the recently described rebound pulmonary hypertension associated with withdrawal of inhaled NO therapy. Rebound pulmonary hypertension can result in life-threatening increases in pulmonary vascular resistance and decreases in systemic oxygenation (2, 12, 21, 24). A better understanding of the mechanism by which inhaled NO alters endogenous endothelial function is important in not only developing effective treatment and prevention strategies for rebound pulmonary hypertension but also for learning about the potential modulating effects of chronic NO usage on underlying pulmonary vascular disease states.

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