Inhaled nitric oxide induced NOS inhibition and rebound pulmonary hypertension: a role for superoxide and peroxynitrite in the intact lamb

Peter Oishi, Albert Grobe, Eileen Benavidez, Boaz Ovadia, Cynthia Harmon, Gregory A. Ross, Karen Hendricks-Munoz, Jie Xu, Stephen M. Black, and Jeffrey R. Fineman. Inhaled nitric oxide induced NOS inhibition and rebound pulmonary hypertension: a role for superoxide and peroxynitrite in the intact lamb. *Am J Physiol Lung Cell Mol Physiol* 290: L359–L366, 2006. Previous in vivo studies indicate that inhaled nitric oxide (NO) decreases nitric oxide synthase (NOS) activity and that this decrease is associated with significant increases in pulmonary vascular resistance (PVR) upon the acute withdrawal of inhaled NO (rebound pulmonary hypertension). In vitro studies suggest that superoxide and peroxynitrite production during inhaled NO therapy may mediate these effects, but in vivo data are lacking. The objective of this study was to determine the role of superoxide in the decrease in NOS activity and rebound pulmonary hypertension associated with inhaled NO therapy in vivo. In control lambs, 24 h of inhaled NO (40 ppm) decreased NOS activity by 40% (P < 0.05) and increased endothelin-1 levels by 64% (P < 0.05). Withdrawal of NO resulted in an acute increase in PVR (60.7%, P < 0.05). Associated with these changes, superoxide and peroxynitrite levels increased more than twofold (P < 0.05) following 24 h of inhaled NO therapy. However, in lambs treated with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) during inhaled NO therapy, there was no change in NOS activity, no increase in superoxide or peroxynitrite levels, and no increase in PVR upon the withdrawal of inhaled NO. In addition, endothelial NO nitration was 18-fold higher (P < 0.05) in control lambs than in PEG-SOD-treated lambs following 24 h of inhaled NO. These data suggest that superoxide and peroxynitrite participate in the decrease in NOS activity and rebound pulmonary hypertension associated with inhaled NO therapy. Reactive oxygen species scavenging may be a useful therapeutic strategy to ameliorate alterations in endogenous NO signaling during inhaled NO therapy.

THE USE OF EXOGENOUSLY ADMINISTERED inhaled nitric oxide (NO) as adjuvant therapy for a number of pulmonary hypertensive disorders is increasing. By virtue of its selective pulmonary vasodilatation, inhaled NO has been utilized successfully in the treatment of neonates with persistent pulmonary hypertension, patients with congenital heart disease, and patients with acute lung injury (2, 20, 26). Although many patients benefit from inhaled NO, several problems associated with its use have emerged, including unpredictable or nonsustained responses to therapy, and rapid, even life-threatening, increases in pulmonary vascular resistance (PVR) following its acute withdrawal (1, 9). This “rebound pulmonary hypertension” manifests variably between patient populations but often results in a clinically significant decrease in systemic arterial oxygen saturation and/or cardiac output.

These clinical observations and recent laboratory data suggest that exogenously administered inhaled NO may alter endogenous pulmonary endothelial function (4, 21, 29, 35). For example, both in vitro and in vivo data demonstrate that exogenous NO exposure alters the endogenous NO-cGMP and endothelin (ET)-1 cascades (4, 35). Endogenously produced NO is integral to normal endothelial function and vascular tone, and alterations in its production have been implicated in the pathophysiology of pulmonary hypertensive disorders (10, 12). When exposed to specific stimuli, such as mechanical shear stress or the binding of specific vasodilators, endothelial nitric oxide synthase (eNOS) is activated within endothelial cells, resulting in the synthesis and release of NO from the precursor l-arginine (22, 27). NO then diffuses into adjacent smooth muscle cells, where it activates the enzyme soluble guanylate cyclase (sGC), resulting in cGMP production and, ultimately, vasodilation (16). Both in vitro and in vivo studies demonstrate that exogenous NO decreases endogenous NOS activity, independently of changes in gene expression (4, 21, 35).

ET-1 is a 21-amino acid polypeptide produced by vascular endothelial cells (39). Its vasoreactive properties are complex, but the most striking is its intense vasoconstrictive response mediated by the G protein-coupled ETA receptor located on vascular smooth muscle cells (19). Upregulation of the ET-1 cascade has also been implicated in the pathophysiology of pulmonary hypertensive disorders (13). Recent studies demonstrate increases in plasma ET-1 levels during inhaled NO therapy and suggest a role for ET-1 in the pulmonary vasoconstriction associated with the withdrawal of NO therapy. Moreover, these studies suggest a link between ETA-receptor activation and decreased NO activity, as ETA-receptor antagonism was shown to block the decrease in NOS activity observed during inhaled NO exposure (21).

More recently, in vitro studies demonstrate a role for superoxide anion in the link between increases in ET-1 and decreases in NOS activity during NO exposure (35). Reactive oxygen species (ROS) appear to participate in the regulation of vascular tone under normal conditions. However, mounting evidence also implicates oxidant stress in the pathophysiology...
of a wide array of cardiovascular disorders (7). Superoxide is a relatively weak oxidant but can react rapidly with NO to produce peroxynitrite, a strong oxidizing agent. To summarize, in vitro data indicate that exogenous inhaled NO results in ET<sub>A</sub> receptor-mediated increases in superoxide production, resulting in the formation of peroxynitrite and subsequent nitration and inactivation of eNOS (35). However, the role that ROS play in the development of these NO/ET-1 interactions during inhaled NO therapy in vivo has not yet been evaluated.

Therefore, the purposes of this study were 1) to determine potential changes in superoxide production during inhaled NO exposure in the intact lamb and 2) to examine the role of superoxide in the physiological alterations and NO/ET-1 interactions induced by exogenous inhaled NO. To determine potential changes in superoxide production, sequential peripheral lung biopsies were taken for quantification of ROS by ROS-sensitive dyes and fluorescence microscopy in 13 1-mo-old lambs during 24 h of inhaled NO (40 ppm) therapy. These lambs were treated with either polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), the enzyme responsible for the in vivo dismutation of superoxide to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), or its vehicle, PEG. To examine the role of superoxide in the physiological alterations and NO/ET-1 interactions induced by exogenous inhaled NO, the hemodynamic effects of inhaled NO and its acute withdrawal were determined in lambs treated with and without PEG-SOD. In addition, lung tissue NOS activity, eNOS protein, eNOS nitration, and plasma ET-1 levels were determined and compared in lambs treated with and without PEG-SOD.

METHODS

Surgical preparation and experimental protocol. Thirteen 1-mo-old lambs were instrumented to measure vascular pressures and pulmonary blood flow. The surgical preparation used was as previously described (4, 21). After a 30-min recovery, either PEG diluted in 5 ml of normal saline (n = 6, vehicle control) or PEG-SOD (n = 7) was delivered through the left pulmonary artery catheter. The dose of PEG-SOD (1,000–2000 U/kg every 6 h) was based upon previous studies that demonstrate a sustained significant increase in plasma SOD activity (8, 32). Either PEG or PEG-SOD was given every 6 h to complete a total of four doses. Thirty minutes after the first dose, 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. Systemic arterial blood was drawn for ET-1 determinations. A peripheral lung wedge biopsy was obtained as previously described (4, 21), to determine tissue NOS activity and eNOS protein levels, and for ROS quantification.

Inhaled NO (40 ppm) was then delivered in nitrogen into the inspiratory limb of the ventilator (Inovent; Ohmeda, Liberty, NJ) and cryosectioned at 20 μm. Sections were collected onto Superfrost Plus slides and a pH >7.30. Normal saline was administered intermittently to maintain stable atrial pressures throughout the study period. Peripheral lung wedge biopsies were performed after 24 h of therapy and systemic arterial blood samples were obtained.

After 24 h of therapy, inhaled NO was acutely withdrawn, and the hemodynamic variables were monitored for two additional hours.

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 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 cardiotachometer triggered from the phasic systemic arterial pressure pulse wave. Left pulmonary blood flow was measured on an ultrasonic flow meter (Transonic Systems, Ithaca, NY). All hemodynamic variables were recorded continuously on a Gould multichannel electronic 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). PVR was calculated using standard formulas. Body temperature was monitored continuously with a rectal temperature probe.

Preparation of protein extracts and Western blot analysis. Lung protein extracts were prepared by homogenizing peripheral lung tissues in Triton lysis buffer and used for Western blot analysis of eNOS as previously described (4, 21). 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). To normalize for protein loading in the Western blot analyses, blots were reprobed with the housekeeping protein, β-actin. Relative eNOS expression was then determined as a ratio of the eNOS:β-actin signals.

Assay for NOS activity. The formation of [3H]-citrulline from [3H]-arginine was determined in lung tissue by methods described by Bush et al. and modified as previously described (3–5). In brief, 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 mM [3H]-l-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 [3H]-l-arginine was metabolized, to ensure that substrate was not limiting. The reactions were stopped by the addition of iced stop buffer (20 mM sodium acetate, pH 5, 1 mM l-citrulline, 2 mM EDTA, and 0.2 mM EGTA) and then applied to columns containing 1 ml of Dowex AG50W-X8 resin, Na⁺ form, pre-equilibrated with 1 N NaOH. [3H]Citrulline was then quantified by scintillation counting. All activities were normalized to the amount of protein in each lysate.

Measurement of ET-1. Plasma ET-1 levels were determined using an I<sub>125</sub> radioimmunoassay as we have previously described (36).

ROS quantification. To quantify lung tissue superoxide levels, dihydroethidium (DHE) staining and fluorescence microscopy were performed on lung tissue biopsies. To quantify lung tissue peroxynitrite levels, 3-nitrotyrosine (3-NT) levels were determined utilizing immunohistologic staining and fluorescence microscopy.

Snap-frozen lung tissue samples stored at −80°C were embedded in Tissue-Tek OCT Compound (Sakura Finetek USA, Torrance, CA) and cryosectioned at 20 μm. Sections were collected onto Superfrost Plus slides (VWR Scientific, West Chester, PA), allowed to air-dry at room temperature, and stored at −80°C until needed. For staining, slides were blocked in PBS-T for 30 min at room temperature, antibody to 3-NT (2 μg/ml; EMD/Cabiochem, San Diego, CA) in PBS-Tween was added to each slide and incubated for 30 min at room temperature. Slides were rinsed with PBS-T and incubated with goat antibody to 3-NT (1:100 dilution; EMD/Cabiochem). Slides were then incubated with a 1:200 dilution of biotinylated sheep anti-mouse secondary antibody (Vector Laboratories) for 30 min at room temperature. Slides were rinsed with PBS-T and incubated with a 1:200 dilution of biotinylated horse anti-sheep antibody (Vector Laboratories) for 30 min at room temperature. Slides were then rinsed and incubated with a 1:500 dilution of Streptavidin-Cy3 (Jackson ImmunoResearch) for 30 min at room temperature. Slides were rinsed and counterstained with 10 μg/ml DAPI (Molecular Probes) for 10 min at room temperature. Coverslips were mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen).
anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) in PBS-T for 30 min at room temperature in the dark. The slides were washed with PBS and counterstained with DHE (10 μM) in PBS for 30 min in a moist chamber in the dark. The sections were rinsed extensively with PBS and coverslipped, and multiple random fields were photographed with an Olympus IX51 inverted microscope in both red (DHE) and green (3-NT) fluorescence channels. To focus the quantification of ROS on the vasculature within tissue samples, for each image, we defined the area of the blood vessel(s) using the AOI tool (area of interest), and the mean IOD fluorescence for DHE and 3-NT of each vessel was quantified with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Pre- and postinhalation NO samples from each individual animal (on separate slides) were stained and imaged together to minimize potential differences due to time or staining intensity. IOD values generated from multiple fields were adjusted to means ± 2 SD to remove outliers.

Immunoprecipitation-Western blot analysis for eNOS nitration. To confirm that changes in peroxynitrite generation were associated with eNOS nitration, we determined the level of eNOS protein nitration utilizing an immunoprecipitation-Western blot technique as we have previously described (35). Frozen lung tissue from PEG (n = 3) and PEG-SOD (n = 3)-treated animals was homogenized in 3× volume per tissue weight of immunoprecipitation (IP) buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM MgCl2, 1 mM EDTA, 2% glycerol supplemented with protease inhibitors), centrifuged at 14,000 rpm at 4°C for 10 min, the supernatant collected and the protein concentration quantified by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). To 1,000 μg of total protein, 1 μg of anti-eNOS antibody was added, the volume was brought to 1 ml with immunoprecipitation buffer, and the mixture was nuted at 4°C overnight. To precipitate the bound eNOS, 10 μl of protein G-Agarose (EMD/Calbiochem) was added, and the samples incubated at 4°C for 1 h at 4°C. To collect the bead-bound antibody, the samples were centrifuged at 14,000 rpm for 5 s, the supernatant was removed, and the beads were washed with 500 μl of IP buffer. The wash step was repeated two additional times and 20 μl of 2× Laemmli sample buffer was added to the samples and boiled for 5 min. The samples were then divided equally and loaded onto duplicate Life 4–20% Tris-SDS-HEPES gels (Gradipore, Frenchs Forest, Australia) and run to completion according to the manufacturer’s instructions. The proteins were transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories), and the membrane was blocked with 5% skim milk in TBS-T for 1 h to overnight. The membranes were probed with antibodies to either eNOS (to normalize for the immunoprecipitation efficiency) or 3-NT (EMD/Calbiochem), and reactive bands were visualized with the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce, Rockford, IL) and Kodak 440CF image station (Kodak, New Haven, CT). The image was optimized with the public domain program NIH Image, and band intensity was quantified. Relative nitrated eNOS was then determined as a ratio of the 3-NT-eNOS/total eNOS signals.

Statistical analysis. The means ± SD were calculated for the baseline hemodynamic variables, systemic arterial blood gases and pH, and NOS activities. The general hemodynamic variables, systemic arterial blood gases, and pH were compared over time within each group by ANOVA for repeated measures. Comparisons of NOS activity and plasma ET-1 levels, before and after inhaled NO, were made by paired t-test.

Band intensities from Western blot analysis were analyzed densitometrically on a Macintosh computer (model G4, Apple Computer) using the public domain NIH Image program (developed at the National Institutes of Health 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. Comparisons were made by paired t-test. For nitrated eNOS, comparisons between treatment groups were made by the unpaired t-test.

The relative fluorescent intensity was calculated for both DHE and 3-NT and expressed as mean ± SD. Comparisons before and after inhaled NO were made by the paired t-test. A P < 0.05 was considered statistically significant.

RESULTS

Six control lambs (PEG-alone) and seven PEG-SOD-treated lambs were exposed to inhaled NO (40 ppm) for 24 h. There were no differences in age, weight, sex distribution, or baseline hemodynamic variables between control and PEG-SOD-treated lambs (data not shown).

To evaluate the effects of inhaled NO on endogenous NO production, NOS activity and eNOS protein levels were determined from sequential peripheral lung biopsies. Inhaled NO therapy decreased NOS activity by 47 ± 15% (P < 0.05) in control lambs (Fig. 1). These changes were independent of changes in eNOS protein levels (Fig. 1). ET-1 levels increased from 13.8 ± 3.1 to 22.7 ± 8.7 pg/ml (n = 4, P < 0.05), following 24 h of inhaled NO.

To determine changes in ROS production during inhaled NO exposure, we used ROS-sensitive dyes and fluorescent microscopy on sequential lung biopsy samples. Control lambs displayed a twofold increase in fluorescence intensity in DHE-treated samples and greater than a twofold increase in fluorescence intensity in 3-NT-stained samples at 24 h following inhaled NO exposure compared with baseline, suggesting a

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**Fig. 1.** Lung tissue nitric oxide synthase (NOS) activity and eNOS protein levels following inhaled nitric oxide (NO) therapy in control lambs. **A:** relative NOS activity is decreased following 24 h of inhaled NO. **B:** lung tissue endothelial NOS (eNOS) protein levels, as determined by Western blot analysis, are not changed following 24 h of inhaled NO. **Top:** representative Western blots are shown for protein extracts prepared from lung tissue separated on a 7.5% SDS-polyacrylamide gel, electrochemically transferred to Hybond membranes, and analyzed using a specific antiserum raised against eNOS. Blots were then stripped and reprobed for β-actin as a loading control. **Bottom:** densitometric values for relative eNOS protein (normalized to β-actin) from control lambs. Values are means ± SD; n = 6. *P < 0.05 vs. pre-NO.
Fig. 2. In situ detection of superoxide and peroxynitrite in lung segments before and after inhaled NO in control lambs. A: representative fluorescent images of pulmonary vessels within lung segments treated with dihydroethidium (DHE, top) and 3-nitrotyrosine (3-NT, bottom); conversion of DHE by superoxide to ethidium results in red nuclear fluorescence, and 3-NT staining results in green fluorescence. 3-NT levels are an indirect determinant of peroxynitrite. B: fluorescent intensity was quantified using Image-Pro Plus software and normalized to pre-NO values. Superoxide and peroxynitrite levels are increased following 24 h of inhaled NO exposure in control lambs. Values are means ± SD; n = 6. *P < 0.05 vs. pre-NO.

significant increase in superoxide and peroxynitrite production respectively (P < 0.05, Fig. 2).

Control animals displayed a rapid decrease in pulmonary arterial pressure, from 17.7 ± 3.25 to 14.4 ± 3.11 mmHg (P < 0.05), and left PVR, from 0.38 ± 0.14 to 0.25 ± 0.10 mmHg·ml⁻¹·min⁻¹·kg⁻¹ (P < 0.05), upon the initiation of inhaled NO. Left pulmonary blood flow, mean systemic arterial pressure, heart rate, right and left atrial pressures, systemic arterial blood gases, and pH were all unchanged (Table 1). Upon the discontinuation of inhaled NO, pulmonary arterial pressure increased from 14.35 ± 2.17 to 17.7 ± 3.07 mmHg (n = 4, P < 0.05), and PVR increased from 0.28 ± 0.09 to 0.45 ± 0.18 mmHg·ml⁻¹·min⁻¹·kg⁻¹ (n = 4, P < 0.05, Table 1).

Unlike controls, PEG-SOD-treated lambs displayed no change in NOS activity (Fig. 3) following 24 h of inhaled NO therapy. Similar to control lambs, eNOS protein levels were

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

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<tr>
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<th>Inhaled NO, 40 ppm</th>
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<tr>
<td></td>
<td>pre-NO</td>
<td>15 min</td>
<td>6 h</td>
<td>12 h</td>
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<tr>
<td>Pulmonary arterial pressure, mmHg</td>
<td>17.70±3.25</td>
<td>14.44±3.11*</td>
<td>17.39±5.13</td>
<td>16.56±3.46</td>
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<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>35.51±9.20</td>
<td>37.57±11.22</td>
<td>46.15±17.28*</td>
<td>42.93±13.91*</td>
</tr>
<tr>
<td>Left pulmonary vascular resistance, mmHg</td>
<td>0.38±0.14</td>
<td>0.25±0.10*</td>
<td>0.29±0.11*</td>
<td>0.26±0.10*</td>
</tr>
<tr>
<td>Systemic arterial pressure, mmHg</td>
<td>77.32±14.82</td>
<td>77.95±10.78</td>
<td>73.78±11.96</td>
<td>72.57±10.81</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>151.50±18.51</td>
<td>150.50±12.40</td>
<td>146.83±21.3</td>
<td>143.67±20.22</td>
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<tr>
<td>Left atrial pressure, mmHg</td>
<td>4.95±2.54</td>
<td>5.68±2.00</td>
<td>5.26±3.42*</td>
<td>6.16±3.08</td>
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<tr>
<td>Right atrial pressure, mmHg</td>
<td>4.18±1.91</td>
<td>5.09±3.47</td>
<td>4.47±1.91</td>
<td>4.43±2.02</td>
</tr>
<tr>
<td>pH units</td>
<td>7.44±0.17</td>
<td>7.43±0.19</td>
<td>7.41±0.07</td>
<td>7.40±0.32</td>
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<tr>
<td>PaO₂ Torr</td>
<td>33.87±4.32</td>
<td>41.23±3.13</td>
<td>43.76±6.21</td>
<td>39.98±7.21</td>
</tr>
<tr>
<td>PaCO₂ Torr</td>
<td>82.45±13.23</td>
<td>73.45±12.56</td>
<td>71.76±18.07</td>
<td>73.37±18.31</td>
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|                          | 24 h inhaled NO |                          |                          |                          |
| Pulmonary arterial pressure, mmHg | 17.39±2.17       | 17.86±2.57†             | 18.18±3.79†              | 19.22±3.00†              |
| Left pulmonary blood flow, ml·kg⁻¹·min⁻¹ | 24.54±6.41†       | 35.66±8.14†             | 29.29±13.45              | 29.39±13.31†             |
| Left pulmonary vascular resistance, mmHg | 0.45±0.18         | 0.39±0.10*              | 0.40±0.14†               | 0.45±0.15†               |
| Systemic arterial pressure, mmHg | 49.35±3.94         | 49.75±5.68*             | 51.25±7.89*              | 49.0±2.16*               |
| Heart rate, beats/min | 102.75±5.19†       | 108.25±5.97*             | 101.75±10.21†            | 106.75±11.27†            |
| Left atrial pressure, mmHg | 7.44±3.63*         | 8.38±2.98*               | 7.77±3.28†               | 7.45±3.35*               |
| Right atrial pressure, mmHg | 6.09±2.06*         | 5.97±1.88*               | 5.80±2.05*               | 5.60±1.57*               |
| pH units                  | 7.42±0.09          | 7.44±0.03               | 7.36±0.11                | 7.39±0.08                |
| PaO₂ Torr                 | 36.78±9.43         | 43.54±6.44              | 35.33±5.55               | 39.54±3.34               |
| PaCO₂ Torr                | 71.23±15.87*       | 69.78±17.23*             | 68.73±14.33‡             | 71.43±13.22              |

Values are means ± SD. Inhaled nitric oxide (NO); n = 6; Off NO, n = 4. *P < 0.05 vs. pre-NO; †P < 0.05 vs. previous column; ‡P < 0.05 vs. 24 h (ANOVA).
unchanged (Fig. 3), and plasma ET-1 levels increased (from 14.5 \pm 2.9 to 16.8 \pm 3.3 pg/ml, n = 7, P < 0.05), following 24 h of inhaled NO therapy in PEG-SOD-treated lambs. Furthermore, DHE and 3-NT fluorescence was not changed following 24 h of inhaled NO exposure in PEG-SOD-treated lambs, suggesting that PEG-SOD treatment prevented the increase of both superoxide and peroxynitrite (Fig. 4).

In PEG-SOD-treated lambs, inhaled NO (40 ppm) rapidly decreased mean pulmonary arterial pressure (from 15.7 \pm 3.32 to 13.34 \pm 3.46 mmHg) and left PVR (from 0.40 \pm 0.15 to

Fig. 4. In situ detection of superoxide and peroxynitrite in lung segments before and after inhaled NO in PEG-SOD-treated lambs. A: representative fluorescent images of pulmonary vessels within lung segments treated with DHE (top) and 3-NT (bottom); conversion of DHE by superoxide to ethidium results in red nuclear fluorescence, and 3-NT staining results in green fluorescence. 3-NT levels are an indirect determinant of peroxynitrite. B: fluorescent intensity was quantified using Image-Pro Plus software and normalized to pre-NO values. PEG-SOD treatment prevents increases in superoxide and peroxynitrite levels during inhaled NO. Values are means \pm SD; n = 7.
ET-1 levels and decreased NOS activity that is mediated by vivo, that prolonged NO exposure is associated with increased findings (4, 21, 29, 35). More specifically, we have shown, in activity during inhaled NO therapy may mediate these clinical result in rapid increases in PVR (1, 9). Data from our lab and noted with inhaled NO therapy, and its acute withdrawal can

Clinically, unpredictable or nonsustained responses have been this approach is clear, but increasing data suggest that exoge-

A rapidly expanding understanding of the vascular endothe-
lium and its role in regulating vascular tone has resulted in novel therapies that target endothelial function. The utility of this approach is clear, but increasing data suggest that exoge-
nous endothelial activation may alter endogenous endothelial function in ways that complicate or limit available therapies (34). Inhaled NO is an important example of this paradigm. Clinically, unpredictable or nonsustained responses have been noted with inhaled NO therapy, and its acute withdrawal can result in rapid increases in PVR (1, 9). Data from our lab and others indicate that alterations in endogenous endothelial activity during inhaled NO therapy may mediate these clinical findings (4, 21, 29, 35). More specifically, we have shown, in vivo, that prolonged NO exposure is associated with increased ET-1 levels and decreased NOS activity that is mediated by ETA receptor activation and is independent of changes in eNOS protein levels (4, 21). Furthermore, we have shown, in vitro, that superoxide-mediated peroxynitrite production and subsequent eNOS nitration during inhaled NO exposure may serve as the mechanism linking ETα-receptor activation and eNOS inhibition (35). The current study is, to our knowledge, the first in vivo demonstration of the role of superoxide in mediating the decrease in NOS activity and rebound pulmo-

DISCUSSION

Values are means ± SD. PEG-SOD, polyethylene glycol-conjugated superoxide dismutase; n = 7, *P < 0.05 vs. pre-NO; †P < 0.05 vs. previous column (ANOVA).

0.31 ± 0.13 mmHg·ml⁻¹·min⁻¹·kg⁻¹ (P < 0.05). Left pulmonary blood flow, mean systemic arterial pressure, heart rate, right and left atrial pressures, systemic arterial blood gases, and pH were all unchanged (Table 2). Upon discontinuation of inhaled NO, there was no change in pulmonary artery pressure, left PVR (from 0.24 ± 0.13 to 0.27 ± 0.13 mmHg·ml⁻¹·min⁻¹·kg⁻¹), left or right atrial pressures, mean systemic pressure, or systemic blood gas values (Table 2).

Our previous studies indicate that nitrification of eNOS may contribute to the decrease in NOS activity observed during inhaled NO exposure. To determine potential mechanisms for the NOS inhibition observed during NO exposure in vivo, nitrated eNOS protein levels were determined by immunoprecipitation and Western blot analysis. After 24 h of inhaled NO therapy, control animals displayed a 10-fold higher level of nitrated eNOS protein compared with PEG-SOD-treated ani-

Table 2. Hemodynamic changes during and after 24 h of inhaled NO in PEG-SOD-treated lambs

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<th>Inhaled NO, 40 ppm</th>
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<tr>
<td></td>
<td>10 min off</td>
<td>30 min Off</td>
<td>60 min Off</td>
</tr>
<tr>
<td>Pulmonary arterial pressure, mmHg</td>
<td>15.70±3.32</td>
<td>13.34±3.46*</td>
<td>12.11±2.37*</td>
</tr>
<tr>
<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>35.82±12.01</td>
<td>33.94±12.19</td>
<td>34.88±8.94</td>
</tr>
<tr>
<td>Left pulmonary vascular resistance, mmHg·ml⁻¹·min⁻¹·kg⁻¹</td>
<td>0.40±0.15</td>
<td>0.31±0.13*</td>
<td>0.22±0.08*</td>
</tr>
<tr>
<td>Systemic arterial pressure, mmHg</td>
<td>69.70±6.62</td>
<td>66.34±15.39</td>
<td>61.07±9.23</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>128.14±13.51</td>
<td>123.20±13.01</td>
<td>126.14±9.98</td>
</tr>
<tr>
<td>Left atrial pressure, mmHg</td>
<td>2.87±1.37</td>
<td>3.92±1.63</td>
<td>4.74±1.26*</td>
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<tr>
<td>Right atrial pressure, mmHg</td>
<td>3.01±1.29</td>
<td>2.84±1.37</td>
<td>3.60±0.95</td>
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<td>pH, units</td>
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<td>7.41±0.01</td>
<td>7.42±0.04</td>
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<td>PaCO₂, Torr</td>
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<td>PaO₂, Torr</td>
<td>81.75±14.68</td>
<td>74.75±14.80</td>
<td>69.50±22.09</td>
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Fig. 5. Treatment with PEG-SOD reduces eNOS protein nitration during inhaled NO therapy. Nitrated eNOS protein levels were determined by immunoprecipitation using specific antiserum raised against eNOS in tissue extracts prepared from 6 1-mo-old lambs. Determinations of nitrated eNOS protein were made in lung tissue from 3 vehicle-treated (control) lambs and 3 PEG-SOD-treated lambs following 24 h of inhaled NO. Also, as a positive control fetal pulmonary artery endothelial cells (EC) were exposed or not to nitric oxide (NO) for 1 h. Immunoprecipitated extracts were separated on a 4–20% SDS-polyacrylamide gradient gel, electrophoretically transferred to Hybond membranes, and analyzed using antisera against either eNOS or 3-NT residues. A: representative Western blot; B: densitometric analysis was carried out using NIH Image 1.6. The levels of nitrated eNOS protein relative to total eNOS protein were calculated. The levels of nitrated eNOS protein were less in lambs treated with PEG-SOD compared with vehicle-treated controls, following 24 h of inhaled NO therapy. Values are means ± SD. *P < 0.05 vs. vehicle control.
during inhaled NO therapy preserved NOS activity and blocked the increase in PVR upon the withdrawal of therapy. Furthermore, PEG-SOD-treated lambs did not display changes in superoxide or peroxynitrite. In addition, eNOS nitration was lower in PEG-SOD-treated lambs than in control lambs, following 24 h of inhaled NO therapy.

Numerous studies implicate oxidative stress in the pathogenesis and pathophysiology of a number of cardiovascular disorders (7). Superoxide is a relatively weak oxidant but reacts rapidly with NO to form peroxynitrite, a strong oxidizing agent, which reacts readily with biological molecules and is capable of nitrating free or protein-associated tyrosines. The present study was not designed to determine the source of superoxide and peroxynitrite production resulting from inhaled NO exposure. Pulmonary vessels contain many sources of superoxide including, lipoxygenase, cyclooxygenase, xanthine oxidase, NOS, and NADPH oxidase. Our previous in vitro studies suggest that ET\textsubscript{\alpha}-receptor activation is associated with superoxide production. Further studies are necessary to confirm the role of ET-1 and ET\textsubscript{\alpha} receptor activation in superoxide production during inhaled NO therapy in vivo. A number of studies suggest that NADPH oxidase is a predominant source of ROS in the vasculature, but certainly NOS itself may contribute as well (6). We presume that the reaction between NO and superoxide is a significant source of peroxynitrite production in our model, but other sources cannot be excluded. Further studies will be needed to identify the source(s) of increased ROS in response to exogenous inhaled NO.

A number of studies demonstrate that antioxidant therapies (e.g., xanthine oxidase inhibitors, vitamin C, SOD) can prevent or reverse the endothelial dysfunction associated with a wide array of systemic vascular disorders, such as hypercholesterolemia and diabetes (6, 7). The present study demonstrates a potential role for ROS in mediating changes in endogenous NO and ET-1 signaling associated with prolonged NO exposure in the pulmonary circulation and provides preliminary evidence for ROS scavenging as a potential therapeutic strategy. Further studies are warranted to determine the efficacy of other therapies, such as catalase (the enzyme that converts H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O) and urate (a peroxynitrite scavenger), and to more specifically identify the role of various ROS in the aberrant NO/ET-1 signaling associated with chronic inhaled NO therapy.

Mounting evidence indicates that eNOS is regulated at the transcriptional, posttranscriptional, and posttranslational levels (14, 23). For example, laminar shear stress increases eNOS transcription. In addition, factors such as intracellular location, protein-protein interactions (e.g. calmodulin, caveolin, and heat shock protein 90), phosphorylation, and substrate and cofactor availability can all participate in the regulation of eNOS (11, 24, 28). Furthermore, recent evidence indicates that ROS may participate in the regulation of eNOS (15, 30, 33). Our previous in vitro data indicate that increased superoxide and peroxynitrite levels are associated with increased eNOS nitration and that preincubation of purified human eNOS with peroxynitrite results in an increase in eNOS nitration and a 50% decrease in activity (35). The nitration of essential tyrosine residues has been shown to alter protein structure and function, but whether eNOS can be regulated in this manner is not known (17). The in vivo data presented here suggest that ROS-mediated eNOS nitration may, at least in part, participate in the decrease in eNOS activity associated with inhaled NO exposure in the lamb.

Several limitations of this study are noteworthy. Lung tissue was utilized for the determination of NOS protein levels and activity, and ROS generation. Although distal lung segments were obtained to sample areas containing pulmonary resistance vessels, lung biopsies represent a number of cell types. Further experiments are needed to elucidate the contributions of specific pulmonary vessels (e.g., arteries and veins) of various sizes to the NO/ET-1/ROS interactions described in the current study. In addition, only one dose (40 ppm) of inhaled NO was utilized and only one treatment duration was studied (24 h). The effects of inhaled NO on endogenous pulmonary function are likely dose and time dependent. Furthermore, experiments were carried out in room air (F\textsubscript{\text{IO}_2}, of 0.21). Clinically, inhaled NO is often administered in combination with higher oxygen concentrations, which could be expected to increase the overall oxidative stress. In addition, intact lambs with a normal pulmonary circulation were utilized for these studies. Inhaled NO is normally administered in the setting of increased pulmonary vascular tone. The signaling described in the intact lamb may differ in lambs with preexisting pulmonary hypertension, for example (25). In fact, H\textsubscript{2}O\textsubscript{2} may be a pulmonary vasoconstrictor in certain disease states (18, 38); under these conditions PEG-SOD administration may increase H\textsubscript{2}O\textsubscript{2} production and thereby worsen rebound pulmonary hypertension. Finally, juvenile lambs (4 – 6 wk) were utilized for this study. Increasing data suggest that developmental changes occur in both the NO/cGMP cascade and the ET-1 cascade(31, 37). Furthermore, the ability to generate and/or scavenge ROS may be developmentally regulated. Future studies will be needed to compare these novel NO/ET-1/ROS interactions between differing developmental stages (i.e., newborn, juvenile, adult).

Endothelial dysfunction is a final common pathway for a wide array of vascular pathology. In this study, we have confirmed previous findings that implicate changes in NO and ET-1 signaling in the rebound pulmonary hypertension associated with inhaled NO therapy. Moreover, we have demonstrated that ROS, at least in part, mediate these alterations. More specifically, to our knowledge this is the first in vivo demonstration that inhaled NO therapy leads to significant increases in lung tissue superoxide and peroxynitrite and that superoxide scavenging, during inhaled NO therapy, preserves NOS activity, decreases eNOS nitration, and prevents rebound pulmonary hypertension upon the acute withdrawal of inhaled NO. As the utilization of targeted endothelial therapies increases, so too must our understanding of the effects of these therapies on endogenous vascular function. The novel NO/ET-1/ROS interactions described in the present study advance this understanding and thus may have important implications for a number of systemic as well as pulmonary vascular disorders.

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