Role for Endothelin-1–Induced Superoxide and Peroxynitrite Production in Rebound Pulmonary Hypertension Associated With Inhaled Nitric Oxide Therapy

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Abstract—Our previous studies have demonstrated that inhaled nitric oxide (NO) decreases nitric oxide synthase (NOS) activity in vivo and that this inhibition is associated with rebound pulmonary hypertension upon acute withdrawal of inhaled NO. We have also demonstrated that inhaled NO elevates plasma endothelin-1 (ET-1) levels and that pretreatment with PD156707, an ETA receptor antagonist, blocks the rebound hypertension. The objectives of this study were to further elucidate the role of ET-1 in the rebound pulmonary hypertension upon acute withdrawal of inhaled NO. Inhaled NO (40 ppm) delivered to thirteen 4-week-old lambs decreased NOS activity by 36.2% in control lambs (\(P<0.05\)), whereas NOS activity was preserved in PD156707-treated lambs. When primary cultures of pulmonary artery smooth muscle cells were exposed to ET-1, superoxide production increased by 33% (\(P<0.05\)). This increase was blocked by a preincubation with PD156707. Furthermore, cotreatment of cells with ET-1 and NO increased peroxynitrite levels by 26% (\(P<0.05\)), whereas preincubation of purified human endothelial nitric oxide synthase (eNOS) protein with peroxynitrite generated a nitrated enzyme with 50% activity relative to control (\(P<0.05\)). Western blot analysis of peripheral lung extracts obtained after 24 hours of inhaled NO revealed a 90% reduction in 3-nitrotyrosine residues (\(P<0.05\)) in PD156707-treated lambs. The nitration of eNOS was also reduced by 40% in PD156707-treated lambs (\(P<0.05\)). These data suggest that the reduction of NOS activity associated with inhaled NO therapy may involve ETA receptor–mediated superoxide production. ETA receptor antagonists may prevent rebound pulmonary hypertension by protecting endogenous eNOS activity during inhaled NO therapy. (Circ Res. 2001; 89:357-364.)

Key Words: nitric oxide • rebound pulmonary hypertension • receptor blockade • superoxide • peroxynitrite

Inhaled nitric oxide (NO) is a selective, potent pulmonary vasodilator that is currently used as an adjunct therapy for a number of pulmonary vasculature disease states.1 However, a number of studies have observed an acute and potentially life-threatening increase in pulmonary vascular resistance (PVR) upon acute withdrawal of inhaled NO. In children with congenital heart disease, this is manifested by an increase in pulmonary vascular resistance (PVR) upon acute withdrawal of inhaled NO. In children with congenital heart disease, this is manifested by an increase in PVR that may compromise cardiac output.2,3 In newborns with persistent pulmonary hypertension, this is manifested by a sudden decrease in systemic arterial oxygen saturation.4 This rebound pulmonary hypertension can occur after only hours of therapy and is independent of the initial response; patients with no initial pulmonary vasodilatory response can have life-threatening pulmonary vasoconstriction upon withdrawal. These manifestations of acute NO withdrawal suggest that inhaled NO may alter endogenous endothelial function, but its mechanisms are unclear.

In vitro and in vivo studies indicate that exogenous NO decreases endothelial nitric oxide synthase (eNOS) activity, an effect mediated, in part, by superoxide production.5,6 eNOS is responsible for the production of endothelium-derived NO, an important mediator of normal pulmonary vascular tone and vascular reactivity.7 Activation of eNOS occurs when endothelial cells are exposed to certain stimuli such as shear stress and receptor binding of specific vasoeductors, resulting in increased NO synthesis.8 The NO produced diffuses from the endothelial cell into the adjacent smooth muscle cell, where it triggers a cGMP-mediated cascade leading to smooth muscle relaxation. Conversely, the administration of nitric oxide synthase (NOS) inhibitors produces significant increases in resting PVR and augments the response to pulmonary vasoconstricting stimuli.9 Furthermore, recent data suggest that decreased endogenous NO production contributes to the development of pulmonary hypertensive disorders.10,11 Our recent studies have also demonstrated increased levels of plasma endothelin-1 (ET-1) during inhaled NO.12 ET-1, a 21 amino acid polypeptide synthesized by vascular endothel-
lial cells, has been implicated in the pathophysiology of pulmonary hypertensive disorders. Its complex vasoactive properties include both pulmonary vasoconstriction and/or vasodilation. ET-1 exerts its vasoactive effects through the activation of at least two different receptors, ETA and ETB. ET-1 acts as a potent vasoconstrictor mainly through the activation of the G protein–coupled ETA receptors found predominantly on vascular smooth muscle cells. We have recently demonstrated that blockade of the ETA receptor with the antagonist PD156707 prevents the rebound pulmonary vasoconstriction associated with NO withdrawal in the intact lamb.

The above data suggest a role for impaired endogenous NO production and increased ET1–mediated vasoconstriction in rebound pulmonary hypertension, although the specific roles played by NO, ET-1, and superoxide remain unclear. Thus, the objectives of this study were to investigate the effects of ET-1 on eNOS activity during inhaled NO therapy in vivo and elucidate the mechanisms of their interactions in vitro. To facilitate this, eNOS protein levels and NO activities were determined from lung biopsies of PD156707- and vehicle-infused lambs treated with 24 hours of inhaled NO. The effects of ET-1 and NO on reactive oxygen species (ROS) production by primary cultures of ovine pulmonary artery smooth muscle cells were quantified by fluorescence microscopy, and the effect of nitration on eNOS activity was examined in vitro.

It is hoped that a better understanding of the effects of exogenous NO on endogenous NO production, and the development of potential treatment strategies to protect endogenous function, may have profound clinical implications for an increasing number of patients being treated with inhaled NO and NO donor compounds.

### Materials and Methods

#### Surgical Preparation and Experimental Protocol

The surgical preparation used was as previously described. After a 30-minute recovery, an intravenous infusion of normal saline (n = 7, vehicle control) or PD156707 (n = 6, a selective ETA receptor antagonist, 1.0 mg · kg⁻¹ · h⁻¹) was begun and continued throughout the study period. The dose of PD156707 was chosen after several previous studies showed that a 30-minute infusion completely blocked the vasoconstricting effects of exogenous ET-1 and resulted in steady-state plasma concentrations that blocked ETA 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. A peripheral lung biopsy was obtained for NO activity and eNOS protein determination as previously described.

Inhaled NO (40 ppm) was then delivered intermittently to maintain stable atrial pressures and/or vasodilation. ET-1 exerts its vasoactive effects through the activation of the G protein–coupled ETA receptors found predominantly on vascular smooth muscle cells. We have recently demonstrated that blockade of the ETA receptor with the antagonist PD156707 prevents the rebound pulmonary vasoconstriction associated with NO withdrawal in the intact lamb.

At the end of the protocol, all lambs were killed with a lethal injection of sodium pentobarbital 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 at the University of California, San Francisco.

#### 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 as previously described. 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.

#### Expression and Purification of Human eNOS Protein

The poly-His-pCWenOS vector (obtained from P.R. Ortiz de Montellano, University of California San Francisco) was transformed into the Escherichia coli strain BL21 (DE3) phsysS for expression of recombinant human eNOS. Typically, LB cultures of poly-HispCWenOS, containing 100 μg/mL of ampicillin, were grown until an OD₆₀₀ of 0.8 was reached. These cultures were then used to inoculate 6 L of LB (prechilled to <15°C) split into four 2.8-L Fernbach flasks. Induction of the bacterial promoter was then carried out by the addition of IPTG to a final concentration of 2 mmol/L. Cultures were then incubated at 18°C for 18 hours with a rotation rate of 220 rpm before harvest by centrifugation.

Purification of recombinant human eNOS was carried out using Calmodulin-Sepharose as we have described previously for neuronal NOS. The only significant difference was that DTT was added to all purification buffers at a concentration of 5 mmol/L.

#### Assay for NOS Activity

This was performed by measuring the formation of ³H-citrulline from L-arginine by methods originally described by Bush et al, using a modification previously published by our laboratory.

#### Detection of Nitrated Protein

To determine total lung protein nitration levels, peripheral lung protein extracts (25 μg) prepared from lambs exposed to 24 hours of inhaled NO in the presence or absence of PD156707 or purified eNOS protein (2 μg) were precipitated with peroxynitrite (100 μmol/L, Upstate Biotechnology) were separated on 7.5% denaturing polyacrylamide gels and electrophoretically transferred to Hybond PVDF membranes.

To determine purified eNOS levels, peripheral lung biopsies were homogenized in immunoprecipitation (IP) buffer (3×wt/vol of 25 mmol/L HEPES [pH 7.5], 150 mmol/L NaCl, 1% Igepal CA-630, 10 mmol/L MgCl₂, 1 mmol/L EDTA, and 2% glycerol) containing a protease inhibitor cocktail (Calbiochem). Tissue homogenates (400 μg) were precipitated with a mouse monoclonal antibody against 3-nitrotyrosine (1 μg, Upstate Biotechnology) in IP buffer (final volume of 1 mL) at 4°C overnight. A rabbit anti-mouse IgG (5 μg) was added and incubated at 4°C for 30 minutes. Protein A-Sepharose (10 μL, Zymed) was added and incubated at 4°C for a further 30 minutes. The precipitated proteins were washed 3 times in 50 volumes of IP buffer, the pellet resuspended in Laemelli buffer (20 μL), boiled, and separated on a 4% to 20% SDS-PAGE gel. eNOS protein levels were then detected by Western blot analysis.

The nitration of purified human eNOS was determined by Western analysis using 2 μg/mL of the anti-nitrotyrosine polyclonal antibody followed by incubation with goat anti-rabbit IgG HRP.

#### Cell Culture and Fluorescence Analysis

Primary cultures of pulmonary arterial smooth muscle cells, prepared from 1-month-old lambs, were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum.
Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) at 37°C in a humidified atmosphere with 5% CO₂. Cells were seeded onto 8-well Permanox slides (Nalge Nunc International Corp) and allowed to adhere for at least 18 hours. Cells were then washed in PBS and incubated in serum-free DMEM, with PD156707 included at 1 μmol/L where required, for 6 hours. Dihydroethidium (20 μmol/L) or dichlorofluorescein (10 μmol/L, both from Molecular Probes) was added to the medium for 15 minutes; ET-1 (1 μmol/L) and Spermine/NO complex (1 mmol/L, both from Sigma) were then added where appropriate, and incubation continued for a further 15 minutes. Cells were washed with PBS and imaged using an Olympus BX40 fluorescent microscope. Dihydroethidium-stained cells were observed using excitation at 518 nm and emission at 530 nm; dichlorofluorescein-stained cells were observed using excitation at 485 nm and emission at 530 nm. Fluorescent images were captured using Studio Pro software (Pixera), and the average fluorescent intensities quantified using Digital Science Imaging software (Kodak). Statistical analyses between treatment groups were carried out as detailed below.

Statistical Analysis
The mean±SD was calculated for all NOS activities. Comparisons of NOS activity before and after inhaled NO were made by paired t test. Comparisons between treatment groups (PD156707 versus control or purified eNOS versus peroxynitrite treated) were made by unpaired t test.

Band intensities from Western blot analysis were analyzed densitometrically on a Macintosh computer (model G4, Apple Computer, Inc) 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 unpaired t test.

The relative fluorescent intensity was calculated for both dihydroethidium and dichlorofluorescein and expressed as mean±SD. Comparisons between treatment groups were made by unpaired t test. A value of P<0.05 was considered statistically significant.

Results
Seven control and 6 PD156707 (1 mg · kg⁻¹ · h⁻¹) treated lambs were exposed to inhaled NO (40 ppm) for 24 hours. There were no differences in age, weight, sex distribution, or baseline hemodynamic variables between control and PD156707-treated lambs (data not shown). To determine the effects of inhaled NO and PD156707 infusion on endogenous NO production, we evaluated NOS activity and eNOS protein levels from peripheral lung biopsies. After 24 hours of inhaled NO, there was a 36.2% decrease of NOS activity in control animals but no change in NOS activity in PD156707-infused lambs (Figure 1). Western blot analysis indicated no significant change in eNOS protein levels after 24 hours in both control and PD156707-infused animals (Figure 2). Western blot analysis using specific antisera to inducible NOS and neuronal NOS indicated that these proteins did not appear to be present in the wedge biopsy samples (data not shown).

The effects of ET-1 and NO on ROS production by ovine pulmonary artery smooth muscle cells (PASMCs) were determined using ROS-sensitive dyes and fluorescent microscopy. ET-1 increased fluorescence intensity by 33% over a 15-minute period in dihydroethidium (DHE)-loaded cells but had no effect on cells preincubated with PD156707 (Figure 3). PASMCs coincubated with ET-1 and PEG-SOD did not display an increase in DHE fluorescence, suggesting that ET-1 treatment induced superoxide in these cells. Moreover, PD156707 treatment did not decrease DHE fluorescence in cells treated with the Cu/Zn SOD inhibitor DETC, suggesting that PD156707 was not merely scavenging superoxide. NO contains an unpaired electron and is paramagnetic and can react rapidly with superoxide to form peroxynitrite, which is undetected by DHE. Therefore, we carried out a series of experiments to determine whether NO and ET-1 could be increasing the level of peroxynitrite as a mechanism responsible for the inhibition of NOS activity during inhaled NO therapy. Initially, cells were exposed to either ET-1 or 1 mmol/L spermine/NO complex alone or ET-1 and 1 mmol/L spermine/NO complex in combination. This concentration of NO was used because we have previously demonstrated a 50% decrease in eNOS enzyme activity, comparable to the decrease in activity seen in lung tissue from NO-ventilated lambs, in PASMCs treated with 1 mmol/L spermine/NO complex.6 Cells were then fixed and subjected to immunohistochemical analysis using an antibody specific to 3-nitrotyrosine as a marker for peroxynitrite. The results obtained (Figure 4A) indicated that peroxynitrite

![Figure 1. Relative NOS activity (normalized to time 0) of lung tissue from 7 control and 6 PD156707-infused lambs before and after 24 hours of inhaled NO therapy. Values are mean±SD. *P<0.05 vs vehicle alone.](image)

![Figure 2. Western blot analysis of eNOS protein in lung tissue before and after 24 hours of inhaled NO therapy. A, Representative Western blot with 25 μg of protein extract prepared from control lambs and PD156707-infused lambs. B, Densitometric values for relative eNOS protein (normalized to time 0) from 7 control and 6 PD156707-infused lambs. Values are mean±SD.](image)
levels were increased only in the presence of both ET-1 and NO. Cells treated with either ET-1 alone, PD156707 alone, NO alone, or untreated did not show increased 3-nitrotyrosine signal (data not shown). Quantitation of the 3-nitrotyrosine signal was not possible. Therefore, we used another technique in which cells were preloaded with the peroxynitrite-sensitive dye dichlorofluorescein (DCF) in order to study the effects of ET-1 and NO cotreatment. Incubation with ET-1 alone generated an 11% increase in DCF fluorescence, which rose to 26% in the presence of NO. PD156707 blocked these increases (Figures 4B and 4C).

We further determined whether peroxynitrite-induced nitration of eNOS produced enzyme inhibition. This was determined in vitro by preincubating purified recombinant human eNOS enzyme with peroxynitrite. Western blot analysis confirmed tyrosine nitration of eNOS after preincubation with peroxynitrite but not with the NO-donating compound SPERNO (Figure 5). NOS assays revealed the nitrated
enzyme possessed ≈50% activity relative to the untreated enzyme after 20 minutes (Figure 6). Western blot analysis demonstrated lower levels of nitrated proteins in extracts prepared from peripheral lungs of PD156707-treated lambs during inhaled NO, compared with vehicle-treated lambs (Figure 7). However, PD156707 did not appear to alter the levels of nitrated protein detected in peroxynitrite-treated PASMCs, suggesting that it was not directly affecting protein nitration (data not shown). We then determined whether PD156707 treatment would reduce the nitration of eNOS during inhaled NO therapy. Tissue extracts from PD156707- and vehicle-treated lungs were subjected to immunoprecipitation using an antibody specific for 3-nitrotyrosine followed by Western blot analysis using an eNOS specific antibody. The results obtained (Figure 8) demonstrated that PD156707 reduced the levels of nitrated eNOS during inhaled NO exposure. This suggests that PD156707 may protect NOS activity during inhaled NO therapy by blocking ET-1–mediated superoxide production thereby limiting the formation of peroxynitrite during inhaled NO therapy.

**Discussion**

Increasing data suggest that vascular endothelial function plays an essential role in the regulation of pulmonary vascular tone and growth.²⁴ This is mediated by a complex interaction of locally produced vasoactive substances such as NO and ET-1.⁸ There is growing evidence to suggest that NO and ET-1 are involved in the regulation of each other through an autocrine feedback loop.⁵ In vascular endothelial cells, for example, ETB receptor activation stimulates eNOS activity whereas NO-cGMP inhibits ET-1 secretion and gene expression.²⁵,²⁶ Aberrations in endothelial function have been implicated in the pathophysiology of many pulmonary hypertensive disorders. For example, decreased NOS gene expression and increased ET-1 gene expression have been associated with advanced pulmonary vascular disease.¹³,²⁷ The potential effects on endogenous endothelial function during inhaled NO therapy have initiated several lines of research. In vitro data suggest that NO exposure inhibits NOS activity in various cell types.⁶,²⁸–³² These findings were supported by our previous in vivo studies, which demon-
strated a decrease in lung NOS activity to 45% of pretreatment values after 24 hours of inhaled NO. This ovine model exhibits the characteristic acute increase in PVR upon withdrawal of inhaled NO suggesting a role for decreased endogenous NOS activity in rebound pulmonary hypertension. The same model, we have also demonstrated that inhaled NO increased plasma ET-1 concentrations.12 Furthermore, pretreatment with the selective ETA receptor antagonist PD156707 completely blocked the acute increase in PVR associated with inhaled NO withdrawal. These data strongly implicate ET-1 in the mechanism leading to rebound pulmonary hypertension. ET-1–mediated vasoconstriction via ETA receptor activation is likely to play an important role in this process. However, given the increasing evidence of coregulation between NO and ET-1 within the pulmonary circulation,25,33,34 we investigated the possibility that ET-1 may also affect endogenous NOS activity via ETA receptor signaling.

Using the same model, we have also demonstrated that inhaled NO increased plasma ET-1 concentrations.12 Furthermore, pretreatment with the selective ETA receptor antagonist PD156707 completely blocked the acute increase in PVR associated with inhaled NO withdrawal. These data strongly implicate ET-1 in the mechanism leading to rebound pulmonary hypertension. ET-1–mediated vasoconstriction via ETA receptor activation is likely to play an important role in this process. However, given the increasing evidence of coregulation between NO and ET-1 within the pulmonary circulation,25,33,34 we investigated the possibility that ET-1 may also affect endogenous NOS activity via ETA receptor signaling.

In this study, we have demonstrated the preservation of NOS activity in PD156707-infused lambs. Overall, these data suggest a direct link between ETA receptor signaling, NOS activity, and rebound pulmonary hypertension. Our previous results demonstrated that NOS activity decreases after 24 hours of inhaled NO and does not return to pre-NO levels 2 hours after discontinuation of inhaled NO.5 Because the levels of eNOS protein remain unchanged throughout the period of study, these data suggest that there may be both a reversible and an irreversible component of NOS inhibition that is independent of eNOS gene expression. However, the exact mechanisms by which NO inhibits NOS activity remain unclear.

To characterize further the pathways involved in NOS activity inhibition, we examined the effects of ET-1 on primary cultures of PASMCs prepared from 1-month-old lambs. Thus, these cells were prepared from lambs at the same age as those used for the in vivo studies. ET-1 treatment increased superoxide production via a PD156707-sensitive pathway, which resulted in elevated levels of 3-nitrotyrosine in the presence of NO. This was presumably as a result of the formation of peroxynitrite, because DCF fluorescence increased significantly when cells were coincubated with ET-1 and NO. DCF is also sensitive to hydrogen peroxide, although cotreatment with PEG catalase or with the hydrogen peroxide scavenger pyruvate did not affect this increase in fluorescence intensity, suggesting that hydrogen peroxide formation does not play a significant role (data not shown).

However, the limitations of DCF as a probe for ROS have been illustrated by the work of Rota et al.35,36 In fact, ET-1 alone did give a significant increase in DCF fluorescence in the absence of NO that may be H2O2 rather than peroxynitrite-mediated.

Additional studies demonstrated that peroxynitrite exposure resulted in the inhibition of eNOS activity in vitro. Furthermore, we detected significantly reduced levels of nitrated eNOS protein in biopsies from PD156707-treated lambs after inhaled NO compared with vehicle-treated lambs. It is possible that nitration decreases the ability of the antibody to detect the eNOS protein in the protein extracts prepared from inhaled NO-treated lambs. This would decrease the level of eNOS detected and reduce the differences between inhaled NO alone and in the presence of PD156707 infusion. This could potentially explain why total nitrated proteins decreased by 90% and eNOS nitration was reduced by only 40%.
From these collective data, we can infer a possible mechanism of NOS activity inhibition during inhaled NO. First, exogenous NO causes an elevation in plasma ET-1 concentrations. This is probably due to increased ET-1 release because the expression of several proteins in the ET-1 cascade remains constant during inhaled NO. Binding of ET-1 to ETA receptors on smooth muscle cells induces superoxide production, generating peroxynitrite from the reaction with exogenous NO. The peroxynitrite diffuses into the adjacent endothelial cells resulting in the nitration and irreversible inhibition of the eNOS enzyme. After withdrawal of inhaled NO, the activity of the endogenous eNOS is reduced to the extent that it cannot synthesize sufficient levels of NO to maintain vasodilation, resulting in increased PVR. This effect is likely to be compounded by ET-1–induced vasoconstriction mediated via ETA receptor signaling. Alternative nitrination pathways may also occur in lung tissue, for example, the tyrosine nitration catalyzed by myeloperoxidase and eosinophil peroxidase. However, it is likely that peroxynitrite formation resulting from the rapid reaction between exogenous NO and ET-1–induced superoxide makes a considerable contribution to the increases in nitrated proteins seen in the lungs of NO-ventilated animals. The mechanism by which peroxynitrite inhibits eNOS activity remains to be elucidated. Nitrination of essential tyrosine residues can affect protein structure and function, but it is not known whether this effect is specific for the ET-1 pathway. Furthermore, the nitration of other critical proteins may also contribute to rebound pulmonary hypertension.

One limitation of the present in vivo study lies with the use of lambs having a normal pulmonary circulation. Patients with pulmonary hypertension often have preexisting aberrations in the NO-cGMP and ET-1 cascades. In addition, the lambs in this study were ventilated with 21% O2. Most neonates receiving inhaled NO therapy are exposed to much higher concentrations of inspired O2. The potential increases in ROS induced by inhaled NO may be exacerbated by high concentrations of O2. Therefore, further studies are required to investigate the effects of inhaled NO on NOS activity in the abnormal pulmonary circulation. Additional studies using varying doses and duration times of exogenous NO and inspired O2 may also be of help to further determine the mechanisms involved in rebound pulmonary hypertension.

In conclusion, we have demonstrated the importance of maintaining normal endothelial function to overcome the increased PVR associated with inhaled NO withdrawal. ETA receptor antagonists, such as PD156707, which inhibit the ET-1 signaling pathway leading to vasoconstriction as well as preserving endogenous NOS activity, represent a useful starting point in the development of strategies to prevent rebound pulmonary hypertension. Further studies to dissect the mechanisms involved in NOS inactivation during inhaled NO may reveal other potential targets for drug therapy.

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