Endothelin-1 decreases endothelial NOS expression and activity through ET\textsubscript{A} receptor-mediated generation of hydrogen peroxide

Stephen Wedgwood\textsuperscript{1} and Stephen M. Black\textsuperscript{2,3}

\textsuperscript{1}Department of Pediatrics, Northwestern University, Chicago, Illinois; and \textsuperscript{2}Department of Biomedical and Pharmaceutical Sciences and \textsuperscript{3}International Heart Institute, University of Montana, Missoula, Montana

Submitted 23 July 2004; accepted in final form 4 November 2004

Normal pulmonary vascular tone is regulated by a complex interaction of vasoactive substances produced by the vascular endothelium (7–9, 28). These include nitric oxide (NO) and endothelin-1 (ET-1). NO is an endothelium-derived relaxing factor synthesized by the oxidation of l-arginine after activation of endothelial NO synthase (eNOS) (17). ET-1, a 21-amino acid polypeptide produced by vascular endothelial cells (EC), has potent vasoactive properties and is mitogenic for pulmonary vascular smooth muscle cells (SMC) (26). The complex pulmonary vasoactive effects of ET-1 are mediated by at least two receptors: endothelin A (ET\textsubscript{A}) receptors, located on vascular SMC, mediate vasoconstriction, whereas endothelin B (ET\textsubscript{B}) receptors, located on vascular EC, mediate vasodilation (2, 19, 20). Data indicate that NO and ET-1 regulate each other through an autocrine feedback loop (14).

Abnormal regulation of endothelial function is implicated in the pathophysiology of a number of pulmonary hypertensive disorders. In persistent pulmonary hypertension of the newborn (PPHN), pulmonary vascular resistance does not decrease normally at birth, resulting in pulmonary hypertension, right-to-left shunting, and hypoxemia (21). Newborns who die of PPHN exhibit an increase in the thickness of the smooth muscle layer within small pulmonary arteries and an extension of this muscle to nonmuscular arteries (10). Often, microvascular thrombi occlude these arteries, and there is proliferation of adventitial tissues (15). Surgical ligation of the ductus arteriosus in the fetal lamb generates a model of PPHN that resembles the human condition (1, 3, 16, 29). In this model, there is a decrease in eNOS mRNA and protein with a corresponding increase in ET-1 expression (3). Furthermore, ET\textsubscript{A} receptor antagonism attenuates fetal pulmonary hypertension and inhibits the SMC hypertrophy normally associated with ductal ligation (13). However, the mechanisms underlying this abnormal regulation of the NO and ET-1 signaling cascades are incompletely understood. We have shown previously that reactive oxygen species (ROS) production is increased in the smooth muscle layer of pulmonary arteries of ductal ligation lambs (4). In addition, ET-1 stimulates the proliferation of SMC isolated from the pulmonary arteries of fetal lambs via a pathway involving ET\textsubscript{A}-mediated ROS generation (26). Thus, the objective of this study was to test the hypothesis that in PPHN, the decrease in eNOS gene expression is mediated via the production of ROS within the pulmonary arterial smooth muscle cell (PASMC) layer that feeds back into the pulmonary arterial endothelial cells (PAEC).

MATERIALS AND METHODS

Cell culture. Primary cultures of ovine fetal PAEC (FPAEC) were isolated as described previously (27). EC identity was confirmed by their typical cobblestone appearance, contact inhibition, specific uptake of \(1',3',5'-\)dihydroxybenzylaminoyltyramine perchlorate-labeled acetylated low-density lipoprotein (Molecular Probes, Eugene, OR), and positive staining for von Willebrand factor (Dako, Carpenteria, CA). Primary cultures of fetal PASMC (FPASMC) from sheep were isolated as described previously (26). FPASMC identity was confirmed by immunostaining (>99% positive) with monoclonal antibodies against \(\alpha\)-smooth muscle actin and calponin (both from Sigma).

Cells were maintained in DMEM containing phenol red supplemented with 10% fetal calf serum (Hyclone), antibiotics (MediaTech), and 100 U/mL penicillin/streptomycin (Hyclone). Cells were passaged when 90% confluent.
and antimycotics (MediaTech) at 37°C in a humidified atmosphere with 5% CO₂-95% air. Cells were between passages 3 and 10, seeded at ~25% confluence, and utilized when fully confluent.

Detection of ET-1-induced H₂O₂ production. For monolulture experiments, FPAEC and FPASMC were seeded onto six-well plates and allowed to adhere for at least 18 h. For coculture experiments, FPAEC and FPASMC were seeded onto six-well Transwell plates (Corning) containing inserts with optically clear polyester membranes and a 0.4-μm pore size. Transwell plates separate the cell monolayers by ~1 mm while allowing intercellular signaling via a microporous membrane. Cells were then washed in PBS and incubated in DMEM containing 0 or 100 nM ET-1 (Sigma) for 18 h at 37°C. Cells were then incubated with dichlorodihydrofluorescein diacetate (H₂DCF-DA; 5 μM, Molecular Probes) with or without 250 μM of polyethylene glycol (PEG)-catalase (Sigma) for 30 min at 37°C. In addition, cells were incubated in DMEM containing 100 nM ET-1 plus 10 μM BQ-123 (Sigma) or 10 μM Res-701-3 (American Peptide) for 18 h at 37°C before being exposed to 5 μM H₂DCF-DA. BQ-123 is a competitive antagonist for ET-1 binding to the ET₁ receptor. Res-701-3 is a cyclic peptide ET₁ antagonist that selectively inhibits ET-1 binding to the ET₂ receptor. Cells were washed with PBS and observed using a Nikon Eclipse TE-300 fluorescent microscope with excitation at 485 nm and emission at 530 nm. Fluorescent images were captured using a CoolSnap digital camera, and the average fluorescent intensities (to correct for differences in pixel number) were quantified using Metamorph imaging software (Fryer).

Detection of H₂O₂ release. H₂O₂ levels in the culture medium were determined using the Amplex Red H₂O₂ assay kit (Molecular Probes) according to the manufacturer’s instructions. This is a fluorescence assay in which H₂O₂ reacts with the Amplex Red in a 1:1 stoichiometry to generate a fluorescent resorufin. The assay is linear in the range from 0 to 5 μM H₂O₂. The level of resorufin produced in the presence and absence of ET-1 (100 nM) was quantified in a fluorescent plate reader (Spectra Max Gemini XS, Molecular Devices) using excitation at 565 nm with emission detection at 587 nm.

Cell transfection and promoter assays. eNOS promoter activity was determined using a 1,600-bp promoter construct as described previously (27). FPAEC or bovine aortic endothelial cells (BAEC) were transfected with 10 μg of plasmid DNA on a 10-cm² tissue culture plate at 90% confluence using Lipofectamine (GIBCO BRL) according to the manufacturer’s instructions. After 24 h, the cells were split onto six-well plates to correct for different transfection efficiencies and allowed to adhere for a further 24 h. The culture medium was replaced with DMEM containing 0–100 μM H₂O₂, such that untreated and H₂O₂-treated cells came from the same transfection reaction. In coculture experiments, transfected cells were exposed to FPASMC or with or without 100 nM ET-1 (Sigma) in the presence or absence of 250 μM catalase (Sigma) for 18 h. The luciferase activity of 10 μl of protein extracts was determined using the Dual-Luciferase Reporter Assay System (Promega) and a Femtomaster FB12 luminometer (Zylux).

TdT-mediated dUTP nick end labeling analysis. FPAEC were incubated in DMEM containing 100 μM or 1 mM H₂O₂ for 18 h. Then, TdT-mediated dUTP nick end labeling (TUNEL) analysis was performed as we have described (24). TUNEL-positive cells were visualized by indirect immunofluorescence with excitation at 485 nm and emission at 530 nm.

Protein preparation and Western blotting. FPAEC were incubated with 0–100 μM H₂O₂ in DMEM for 18 h at 37°C. Protein extracts were prepared using M-PER protein extraction reagent (Pierce). Protein extracts (20 μg) were separated on 7.5% denaturing polyacrylamide gels (Bio-Rad) at 150 V for 60 min using a PowerPac Basic (Bio-Rad). All gels were electrophoretically transferred to Hybond-PVDF membranes (Amersham, Arlington Heights, IL) at 100 mA for 90 min using a PowerPac Basic. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween for 16 h at 4°C. After being blocked, the membranes were washed three times in TBS containing 0.1% Tween for 5 min at room temperature and incubated for 1 h at room temperature with the appropriate dilution of a specific eNOS monoclonal antibody (1: 2,500, cat. no. 610297, Transduction Laboratories). After being washed three times in TBS containing 0.1% Tween for 5 min at room temperature, the membranes were incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (1:1,000, cat. no. 1858413, Pierce) for 1 h at room temperature. Membranes were washed three times in TBS containing 0.1% Tween for 5 min at room temperature. The protein bands were visualized by chemiluminescent procedures (3) using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), and band intensities were determined using Kodak ImageStation software.

Detection of NO production. FPAEC were incubated in DMEM containing 0 or 1 mM Nω-nitro-l-arginine methyl ester (l-NAME; Sigma), 0 or 100 μM H₂O₂ (Fishcer), or 0 or 250 μM of PEG-catalase (Sigma) in monolculture or in DMEM containing 0 or 100 nM ET-1 (Sigma) in coculture with FPASMC for 18 h at 37°C. Cells were loaded with 10 μM 4,5-diaminofluorescein diacetate (DAF2-DA) and incubated at 37°C for 30 min. Cells were washed three times in PBS and visualized and quantified by fluorescence microscopy using excitation at 485 nm and emission at 530 nm.

Determination of catalase activity. Catalase activities were measured as we have previously described (24).

Statistical analysis. The relative fluorescent intensity was calculated for H₂DCF-DA and DAF2-DA and expressed as fold change (vs. untreated cells ± SD). Chemiluminescence intensities were determined for secondary antibodies and expressed ± SD. Relative luminescent intensity was determined for the eNOS promoter-luciferase construct in ET-1- and H₂O₂-treated FPAEC or BAEC and expressed as fold change (vs. untreated cells) ± SD. Comparisons between treatment groups were made by the unpaired t-test using the GSTAT software program. P < 0.05 was considered statistically significant.

RESULTS

Initially, we used the fluorescent dye H₂DCF-DA to determine whether H₂O₂ is elevated in FPASMC exposed to ET-1. Our results indicated that ET-1 exposure (100 nM) produced a 2.1-fold increase in H₂O₂ in FPASMC after 18 h of exposure (P < 0.05 vs. untreated, Fig. 1, A and B). This increase was ablated when FPASMC were coincubated with cell-permeable PEG-catalase indicating the specificity of the H₂DCF-DA signal for H₂O₂ (Fig. 1, A and B). Conversely, ET-1 significantly decreased H₂DCF-DA fluorescence in FPAEC (P < 0.05 vs. untreated, Fig. 1, A and B). We then used ET-1 receptor antagonists to investigate the role of the ET receptors in regulating H₂O₂ production. BQ-123, an ET₁ receptor antagonist, significantly attenuated the ET-1-mediated increase in H₂DCF-DA fluorescence in FPASMC (P < 0.05 vs. ET-1 alone, Fig. 1, A and B) but did not prevent the decrease in FPAEC (Fig. 1, A and B). In contrast, Res-701-3, an ET₂ receptor antagonist, prevented the ET-1-mediated decrease in H₂DCF-DA fluorescence in FPAEC but had no significant effect on the increase in FPASMC (P < 0.05 vs. ET-1 alone, Fig. 1, A and B). The above data are in agreement with our previous studies demonstrating an increase in ET₁ receptor-mediated superoxide production (as detected by dihydroethidium fluorescence) by FPASMC (26). Using the same technique, we also found that ET-1 decreased superoxide production in FPAEC, which was prevented by Res-701-3 (data not shown). In coventure experiments, ET-1 treatment increased H₂DCF-DA fluorescence by 2.7-fold in both FPASMC and FPAEC, which was reduced in the presence of
PEG-catalase (Fig. 2, A and B). Overall, these data suggest that ET_A receptor stimulation on FPASMC increases H_2O_2 production that can be released and taken up by FPAEC. We confirmed the ET-1-mediated increase in H_2O_2 in FPASMC and FPAEC/FPASMC cocultures using a separate fluorescent method to detect H_2O_2 released into the medium (Fig. 3). ET-1 significantly increased H_2O_2 release into the medium in FPASMC monocultures and FPASMC/FPAEC cocultures, and
this increase was abolished by cotreatment with non-cell-permeable catalase (Fig. 3). Again, ET-1 significantly decreased H_2O_2 release into the medium in FPAEC monocultures (Fig. 3).

Next, we determined whether ET-1 altered eNOS promoter activity in FPAEC alone or in coculture with FPASMC utilizing an eNOS promoter fragment fused to a luciferase reporter gene as we have described (27). ET-1 (100 nM, 18 h) had no effect on eNOS promoter activity in FPAEC monoculture (Fig. 4). However, eNOS promoter activity was significantly decreased in FPAEC in coculture with FPASMC (Fig. 4). eNOS promoter activity was restored in the coculture when cells were coincubated with 250 U/ml of cell-impermeable catalase to remove FPASMC-derived H_2O_2 from the coculture medium (Fig. 4).

To confirm that the inhibitory mechanism of ET-1 on eNOS expression involved H_2O_2, we next examined the effects of exogenous H_2O_2 on eNOS gene expression in FPAEC. We found that 50 and 100 μM H_2O_2 decreased eNOS promoter activity to 40 and 20% of untreated cells after 18 h (Fig. 5A). We then performed TUNEL assays to exclude the possibility that these doses were inducing apo-
tosis in FPAEC, thereby giving an apparent reduction in promoter activity. Treatment with 100 μM H₂O₂ gave no detectable TUNEL-positive FPAEC after 18 h, although programmed cell death was evident at a dose of 1 mM (Fig. 5B). Furthermore, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based viability assays detected no changes in the number of FPAEC treated with 100 μM H₂O₂ relative to untreated cells after 3 days (data not shown).

We next determined the effects of exogenous H₂O₂ on eNOS protein levels in FPAEC. Low concentrations of H₂O₂ (≤12 μM) elevated the levels of eNOS protein in a concentration-dependent manner, with an increase of 50% at 12 μM (Fig. 6, A and B). However, concentrations of 50 and 100 μM H₂O₂ decreased eNOS protein to 50% of untreated cells (Fig. 6, A and B). As a marker for eNOS activity, we looked at changes in DAF2-DA fluorescence, a probe we have used previously to detect increases in NO production in FPAEC exposed to laminar shear stress (27). In monoculture, the eNOS inhibitor l-NAME (1 mM) and H₂O₂ (100 μM) decreased DAF2-DA fluorescence in FPAEC to 60 and 65%, respectively, of control levels after 18 h of incubation, although PEG-catalase (250 U/ml) gave no significant

![Fig. 6. Effects of H₂O₂ on eNOS protein levels and nitric oxide (NO) production in FPAEC.](image)

A: protein extracts (40 μg), prepared from ovine FPAEC exposed or not to H₂O₂ (0–100 μM), were analyzed by Western blot analysis using a specific antiserum raised against eNOS protein. Shown is a representative blot indicating the detection of eNOS at around 135 kDa. No other bands were detected. B: densitometric values for eNOS protein from ovine PAEC exposed or not to H₂O₂ (0–100 μM). eNOS protein expression is increased, relative to untreated cells, at concentrations ≤12 μM H₂O₂ and decreased at concentrations ≥50 μM. Values are means ± SD; n = 3. *P < 0.05 vs. untreated cells. C: representative images of FPAEC stained with the fluorophore 4,5-diaminofluorescein diacetate (DAF2-DA) to detect NO levels. In monoculture, DAF2-DA fluorescence is decreased following an 18-h incubation with 1 mM N(G)-nitro-l-arginine methyl ester (l-NAME), an eNOS inhibitor, and with 100 μM H₂O₂, but not with 250 U/ml of PEG-catalase. DAF2-DA fluorescence is also diminished in FPAEC treated with 100 nM ET-1 for 18 h in coculture with FPASMC. D: average fluorescence intensities from images in C were determined. Values are means ± SD; n = 4. *P < 0.05 vs. untreated.
change (Fig. 6, C and D). Furthermore, ET-1 (100 nM, 18 h) decreased DAF2-DA fluorescence in FPAEC in coculture with FPASMC to 60% of control levels (Fig. 6, C and D).

Because Drummond and colleagues (6) have shown that 50–200 μM H2O2 induced an increase in eNOS expression in BAEC, we carried out experiments to try and determine the reasons for the differences in response between the two cell lines. Our data indicate that concentrations of H2O2 (100 μM) that produced a decrease in eNOS transcription in FPAEC produced an increase in eNOS transcription in BAEC following transfection with the eNOS promoter-luciferase construct (Fig. 7A). However, we also found that BAEC had approximately twofold more catalase activity compared with FPAEC (Fig. 7B). This suggests that the oxidant load per unit of exogenous H2O2 is likely to be less in BAEC than in FPAEC and may account for the different effects on eNOS transcription.

**DISCUSSION**

The ductal ligation lamb model of PPHN displays abnormal regulation of the ET-1 and NO signaling cascades such that ET-1 levels are elevated and eNOS expression is decreased (3). ET-1-mediated vasoconstriction coupled with reduced NO-mediated vasodilation is likely to make a significant contribution to pulmonary hypertension. ET-1 stimulates the proliferation of FPASMC via a pathway involving ETA-mediated superoxide synthesis (26), and superoxide production is increased in the smooth muscle layer of pulmonary arteries of ductal ligation PPHN lambs (4). Because ROS appear to play a central role in PPHN, we examined the effects of ROS on eNOS gene expression in FPAEC. We chose to look at the effects of H2O2 since this ROS is more stable than superoxide and may be a more likely intercellular signaling molecule between FPASMC and FPAEC.

ET-1 induces H2O2 production in FPASMC that then appears to traffic into the EC where it decreases both eNOS activity and expression. This decrease in eNOS expression and activity could be prevented when the cells are cultured in the presence of membrane-impermeable catalase as an H2O2 scavenger. Our data also indicate that the direct addition of H2O2 (100 μM) to FPAEC decreases eNOS transcription and protein levels. Together, our data suggest that an ET-1-mediated increase in H2O2 is at least partly responsible for the decreased eNOS expression in the ductal ligation model of PPHN (3). It should be noted that our data do not delineate the compartment in which the H2O2 either is generated in the SMC or acts in the EC. Thus further studies using either catalase or glutathione peroxidase overexpression may be useful in delineating the cellular compartment where the H2O2 is generated in SMC and the site of action in the EC.

The mechanisms by which H2O2 regulates eNOS are complex and incompletely understood. For example, Drummond et al. (6) have found a concentration- and time-dependent increase in eNOS mRNA and protein induced by H2O2 that is produced by both increases in transcription and mRNA stability. We found that H2O2 increases eNOS expression at lower concentrations but not at the higher H2O2 concentrations used in the study by Drummond et al. (6). Although these data appear to conflict with our current findings, it is possible that the differences in antioxidant capacity we have observed, where BAEC exhibited a twofold higher catalase activity than ovine FPAEC, may account for the observed responses to H2O2. In EC from the fetal pulmonary circulation, which would normally encounter blood with low-oxygen content, the reduced antioxidant activity may render the cells more sensitive to ROS. In cells from the adult aorta, which contact blood with high-oxygen content, the increased antioxidant activity may prevent oxidative damage.

The increase in eNOS protein, but not promoter activity, in FPAEC treated with 12 μM H2O2 suggests that a posttranscriptional mechanism is active under these conditions. This may be similar to the Janus kinase 2-mediated increase in BAEC eNOS mRNA stability at higher doses of H2O2 (5). Additional studies will be required to elucidate these mechanisms in FPAEC. ROS-mediated regulation of eNOS transcription may involve mechanisms that are developmentally and spatially regulated within the vasculature. However, little is known about the transcription factors involved in the regula-
tery mechanisms of eNOS gene expression. The eNOS promoter region contains potential *cis*-elements for several redox-sensitive transcription factors, including activator protein-1 (AP-1), Sp1, and antioxidant-responsive elements (18, 22, 23). We have recently shown that AP-1 is involved in the developmentally regulated transcription of eNOS in FPAEC exposed to shear stress (27), but the involvement of ROS in AP-1-mediated eNOS transcription in these cells has yet to be established. Although 100 mM H2O2 decreased NO levels in FPAEC, incubation with PEG-catalase failed to stimulate NO production, suggesting that the mechanisms by which ROS regulate the activation and repression of eNOS expression are complex and warrant further investigation. However, the identification of specific transcription factors regulated by H2O2 in FPAEC will help to determine ROS-induced pathways in the fetal pulmonary circulation.

In the ductal ligation lamb, ETA receptor blockade attenuates pulmonary hypertension and SMC growth (13). Conversely, prolonged infusion of fetal lambs with an ETB receptor antagonist generates increases in pulmonary arterial pressure, pulmonary vascular resistance, and muscularization of small pulmonary arteries (12). Our data complement these studies by demonstrating that ETA blockade inhibits ET-1-mediated increases in H2O2 production by FPASMC, whereas ETB antagonism prevents ET-1-mediated decreases in H2O2 levels in FPAEC. These data suggest a central role for ET-1 and ROS in producing an abnormal intercellular signaling cascade between FPAEC and FPASMC in PPHN. In our proposed pathway, ET-1 released by FPAEC activates ETA receptors on FPASMC, resulting in vasosconstruction and the stimulation of NADPH oxidase activity. The subsequent increase in ROS induces FPASMC proliferation (26) and vascular remodeling. In addition, H2O2 released by FPASMC elevates the levels of this ROS in FPAEC, resulting in decreased eNOS promoter activity. Less NO can then diffuse back into the FPASMC to stimulate vasodilation and inhibit SMC growth. Indeed, we have recently shown that exogenous NO attenuates FPASMC proliferation by stimulating Gs/Gi1 growth (25). Furthermore, activation of NO production in rat aortic EC inhibits the proliferation of rat aortic SMC in coculture (11), suggesting that endothelium-derived NO can influence the growth of adjacent SMC. Overall, the predicted effects of these events are reduced NO-mediated vasodilation, elevated ET-1-mediated vasoconstriction, and increased ROS-mediated vascular remodeling. In support of this hypothesis, our previous studies have demonstrated that fifth-generation pulmonary arteries isolated from PPHN lambs had significantly decreased relaxations to the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) compared with control lambs (4). However, the addition of PEG-superoxide dismutase (SOD) to pulmonary arteries isolated from PPHN lambs significantly enhanced their relaxations to SNAP. Because these scavengers had no effect in control pulmonary arteries, this enhancement appears to be unique to PPHN. Although additional studies will be required to determine the effects of NO, superoxide, and PEG-SOD on eNOS expression in FPAEC, these data suggest that excess ROS inhibit relaxations in PPHN.

In conclusion, our data establish a link between increased ET-1 and increased ROS generation in the development of PPHN. Our data suggest that antioxidants could be a potential therapy to reduce or prevent the pulmonary vascular remodel-