Increased superoxide production contributes to the impaired angiogenesis of fetal pulmonary arteries with in utero pulmonary hypertension

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The pulmonary vascular resistance undergoes a rapid decrease at birth to facilitate gas exchange during postnatal life. Failure of this transition to occur can result in persistent pulmonary hypertension of the newborn (PPHN), which leads to an increased risk of death or disability (18). Angiogenesis and vasculogenesis are known to play important roles in the fetal lung development with angiogenesis playing a more important role after midgestation. Angiogenesis of pulmonary artery endothelial cells (PAEC) is impaired in PPHN (15), and impaired blood vessel formation in PPHN contributes to the elevated pulmonary vascular resistance (16). Impaired angiogenesis inhibits the formation of both blood vessels and alveoli in the developing lung from late gestation to adult life (23, 41). Impaired formation of blood vessels and alveoli also contributes to the altered lung growth observed in bronchopulmonary dysplasia (BPD) (2, 20, 36, 39), a complication of respiratory distress syndrome in premature babies. BPD can also be associated with pulmonary hypertension and prolonged oxygen requirement during postnatal life (11, 37). Abnormalities in angiogenesis can therefore affect lung growth in a variety of lung diseases in newborn infants.

The mechanism of impaired angiogenesis in PPHN remains unclear. Decreased availability of nitric oxide (NO) has been shown to contribute to decreased angiogenesis in the ductal ligation model of PPHN (15). PPHN was previously shown to increase the expression of $p67^{phox}$ and production of superoxide ($O_2^-$) in whole lungs. We therefore hypothesized that increased NADPH oxidase activity plays a role in the mechanisms that impair vasodilation in PPHN (6, 10). Our group also demonstrated that uncoupled endothelial NO synthase (eNOS) activity increased oxidative stress and impaired vasodilation of pulmonary arteries in fetal lamb model of PPHN (21). Although many different sources of oxidative stress are present in blood vessels, NADPH oxidase has been identified as a potential early source of $O_2^-$ after ductal ligation in lungs with PPHN (6). Accordingly, increased $O_2^-$ production may reduce NO bioavailability, which is required for angiogenesis (5, 31).

However, to date, no studies have directly linked $O_2^-$ to NADPH oxidase as a mechanism of impaired angiogenesis in PPHN. The objectives of our study are to investigate: 1) whether PAEC from lambs with PPHN (HTFL-PAEC) show decreased angiogenic potential; 2) whether reactive oxygen species, specifically $O_2^-$, are involved in this reduced angiogenic potential; 3) whether NADPH oxidase is a major source of $O_2^-$ that impairs angiogenesis in HTFL-PAEC; and 4) whether common antioxidants can be used to improve angiogenesis in HTFL-PAEC.

MATERIALS AND METHODS

Materials and supplies. DMEM, Dulbecco’s PBS (DPBS), HBSS, FCS, antibiotic-antimycotic (AB/AM; penicillin/streptomycin/Fungizone, 100×) liquid, lucigenin, and TrypLE were obtained from Invitrogen (Carlsbad, CA). Monoclonal antibody against Rac1 and $p67^{phox}$ and growth factor-reduced Matrigel were from BD Biosciences (Bedford, MA). Polyclonal anti-$p47^{phox}$ antibody was from Millipore (Billerica, MA). Polyclonal anti-Cu,Zn-SOD (SOD1) and anti-Mn-SOD (SOD2) antibodies were from Assay Designs (Ann Arbor, MI). Cell invasion assay kit and bromodeoxyuridine (BrdU) cell proliferation assay kit were obtained from Chemicon International (Temecula, CA). In Situ Cell Death Detection POD Kit and diaminobenzidine (DAB) substrate were from Roche Applied Science (Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO).

PPHN model. PPHN was induced by fetal ductal constriction from 127 ± 2 to 135 ± 2 days gestation as previously described (22). Control fetal lambs (NFL) received sham operation without ligation of the ductus arteriosus (DA). The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Medical College of Wisconsin. After 8 days of DA constriction, ewes were euthanized, and fetal lungs were removed en bloc.
Isolation of PAEC. Pulmonary arteries of fetal lambs were dissected into lung parenchyma up to the 3rd generation branches. PAEC were isolated from pulmonary arteries with the use of 0.25% collagenase type A (Roche), and cells were grown in DMEM with 20% FCS. Endothelial cell identity was verified by the presence of factor VIII antigen and acetylated LDL uptake. PAEC isolated from more than 6 fetal lambs in each group were used between the 3rd and 6th passages. PAEC from NFL (NFL-PAEC) and HTFL-PAEC from same passage number were cultured at the same time in parallel for each experiment for appropriate comparison. Confluent PAEC were detached with TrypLE reagent (Invitrogen) at 37°C and mixed with DMEM containing 5% FCS and 1% AB/AM to adjust the cell count to 10^6 per milliliter for tube formation assay or mixed with serum-free DMEM for cell invasion assay. Cell number was counted by hemocytometer, and viable cells were evaluated by trypan blue stain. Only batches with >95% viable cells were used for experiments.

In vitro tube formation assay. After overnight thaw at 4°C, 50 μl of Matrigel was gently pipetted into each well of the 96-well plate. Matrigel was allowed to solidify in the incubator for 2 h at 37°C. PAEC (2 × 10^4) were added on top of the Matrigel and incubated at 37°C at an oxygen concentration of either 5 or 21% supplemented with 5% FCS. Total tube length and branching points in each well were evaluated in triplicate using an inverted microscope (Olympus, Melville, NY) with a digital camera and analysis software (Olympus). Only tubes with more than 10 terminal points were counted. Total tube length and branching points were normalized to 100% of control for each condition:

\[
\text{Total tube length (percent of control)} = \frac{\text{Total tube length in experimental condition}}{\text{Total tube length in control condition}} \times 100
\]

Table 1. Sequence of primers used for real-time RT-PCR analyses of mRNA for NADPH oxidase (Nox) pathway, SOD, and β-actin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox2</td>
<td>5'-GCT TGT GGC TGT GAT AAG CA-3'</td>
<td>5'-GAG ATT TGG GGC TTT ATT GC-3'</td>
</tr>
<tr>
<td>p47phox</td>
<td>5'-GCT GGG TCA TCA GGA AAG AA-3'</td>
<td>5'-GTT GAC TGT GTC AGC GTT G-3'</td>
</tr>
<tr>
<td>Rac1</td>
<td>5'-CTC TCT GGC TGA TGA AAG CA-3'</td>
<td>5'-CCG ACG AGG TCC AAT AAA AA-3'</td>
</tr>
<tr>
<td>SOD1</td>
<td>5'-CGC GGC AAA GGC AGA TAC AG-3'</td>
<td>5'-TGC ACG GAG GAT TAA ACT GAG G-3'</td>
</tr>
<tr>
<td>SOD2</td>
<td>5'-ATT GCT GGC AGC GCA TCA AAC-3'</td>
<td>5'-AGC AGG GGG ATA AGA CCT GT-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GGG GGA AAT CGT GCG TGA CAT T-3'</td>
<td>5'-GAT GGA GTT GAA GGT AGT TTC GTG-3'</td>
</tr>
</tbody>
</table>

**Fig. 1.** Tube formation by pulmonary artery endothelial cells (PAEC) from fetal lambs with pulmonary hypertension of newborn (PPHN) (HTFL-PAEC) or control lambs (NFL-PAEC). PAEC were cultured under oxygen tension of 21% for 14 h. A: representative picture of NFL-PAEC showing both tubular structures and branching points. B: representative picture of HTFL-PAEC showing essentially no tubular structures or branching point and markedly increased clumping of cells. C: total tubular structure was significantly different between HTFL-PAEC (0.3 ± 0.3%) and NFL-PAEC (100.0 ± 6.0%). D: similar findings were also observed for total branching points per high-power field (HPF) (0.4 ± 0.1 vs. 6.5 ± 0.5). NFL, normotensive fetal lung; HTFL, hypertensive fetal lung. *P < 0.01 between NFL and HTFL (n = 8). The length of the white bar (A and B) equals 200 μm.
with 5% CO₂ for 8 or 14 h (n = 5–8). Our preliminary studies showed regression of tube formation in Matrigel after 14 h, especially in HTFL-PAEC cultures, as previously reported by Gien et al. (15). In some experiments, NADPH oxidase inhibitor, apocynin (Apo; 50 μM), or antioxidant, N-acetyl-cysteine (NAC; 500 μM), were added to evaluate the role of reactive oxygen species in tube formation. One representative picture was taken per well using an Olympus IX50 inverted microscope at ×20 magnification. Total tube length of HTFL-PAEC, with or without treatment, was normalized against total tube length of NFL-PAEC performed at the same time as one of the parameters of tube formation assay. The number of branching points per high-power field (HPF) was used as another parameter to quantify tube formation. Only tubular structures connecting 2 cell clusters were considered for measurements, whereas cell clusters with at least 3 tubular structures emanating out were considered to be a branching point.

**Monolayer scratch recovery assay.** PAEC (3 × 10⁵) were seeded into each well of a 6-well plate and grown to confluence in DMEM with 20% FCS and 1% AB/AM at an oxygen concentration of 5 or 21%. For the study, culture medium was changed to DMEM with 0.5% FCS + 1% AB/AM to serum-starve the cells for 2 h, and 2 scratches were created by a 1-ml pipette tip per well. The detached PAEC were washed away with HBSS. The medium was then changed to DMEM with 5% FCS, NAC (500 μM) or Apo (20 μM) was added to some wells to evaluate the role of reactive oxygen species in monolayer scratch recovery. After 24 h, 4 randomly selected fields per scratch line were imaged under an inverted microscope (Olympus IX50). The number of PAEC that migrated outside the margin of scratch per square millimeter and the distance of the recovery frontline (in micrometers) were measured for comparisons.

**Invasion assay.** PAEC (2 × 10⁵) in serum-free DMEM were placed into the insert of a modified Boyden chamber (Chemicon International) made of polycarbonate membrane with 8-μm pores and coated with basement membrane matrix as previously described (38). The outer chamber was filled with 500 μl of DMEM containing 1% AB/AM and 5% FCS as the chemoattractant. The chambers were then placed in an incubator at 37°C with 21% O₂ and 5% CO₂ for 24 h. The inserts were removed, and the noninvading cells were removed by cotton-tipped swabs. The inserts were then stained and imaged under an inverted microscope (Olympus IX50). Three randomly selected
fields were imaged per each insert, and invading cells were counted in each picture. The results were normalized to NFL-PAEC values. Apo (20 μM) was added into the system in some experiments to evaluate its effect on cell invasion.

BrdU proliferation assay. BrdU incorporation was used to estimate PAEC proliferation. PAEC (2 × 10^5) were seeded in each well of the 96-well plate and incubated in DMEM containing 20% FCS + 1% AB/AM at 37°C and at 5 or 21% O_2. Cells were initially incubated for 8 h to allow them to attach. The medium was then changed to DMEM containing 0.5% FCS + 1% AB/AM for overnight incubation. The medium was again changed to DMEM supplemented with 5% FCS, BrdU, and 1% AB/AM for 24 h in 37°C at 5 or 21% O_2. Then the plate was washed and fixed before addition of anti-BrdU antibody and conjugated secondary antibody. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate was added, and absorbance at 450 nm (Abs450) was measured. Proliferation was assessed as being proportional to the increase in absorbance.

Apoptosis assay. We used the method described by Sgonc et al. (35) with some modifications. PAEC (3 × 10^5) were seeded into each well of the Lab-Tek II four-well chamber slide and incubated in DMEM with 20% FCS and 1% AB/AM at 37°C and 21% O_2/5% CO_2 for 16 h. The cells were fixed by freshly prepared 4% formalin in DPBS at pH 7.4 for 1 h at 15–25°C followed by 3% H_2O_2 in methanol for 10 min at 15–25°C to block the endogenous peroxidase activity. After washing with HBSS, the cells were permeabilized with 0.1% Triton X-100 for 2 min on ice and then treated with labeling mixture and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Before staining with DAB, the cells were pretreated with peroxidase converter solution (Roche) for 30 min at 37°C. After hematoxylin-eosin (H&E) counter stain, the brownish apoptotic nuclei were counted under microscope and expressed as percent of all nuclei.

Measurements of O_2 production by DHE epifluorescence. PAEC (1 × 10^5) in DMEM supplemented with 5% FCS and 1% AB/AM were seeded into each well of a Lab-Tek II four-well chamber slide and grown at 37°C with 21% O_2/5% CO_2 to near confluence. The PAEC were then treated with HBSS and dihydroethidium (DHE; 10 μM) to detect the intracellular O_2 production (32). Fluorescence was imaged under a Nikon Eclipse TE200 fluorescence microscope with excitation and emission at 510 and 590 nm, respectively.

Measurement of intracellular O_2 production by HPLC. PAEC were seeded in 60-mm plates in DMEM supplemented with 5% FCS and 1% AB/AM to confluence. DHE (10 μM) was added into the...
cultures for 30 min before harvest. Intracellular \( \text{O}_2 \) production was then measured by HPLC according to the method described by Zhao et al. (42).

**NADPH oxidase activity.** NFL-PAEC and HTFL-PAEC were grown in 100-mm plates to confluence. Cells were washed twice with ice-cold PBS and scraped into 1 ml of 20 mM potassium phosphate buffer (pH 7.0) containing protease inhibitor cocktail (1:100; Sigma) and 1 mM EGTA. The cell suspension was freeze-thawed twice in liquid nitrogen and then sonicated with 15% power output for 30 s on ice. Then 0.1 ml of lysate was mixed with 0.9 ml of working solution (50 mM potassium phosphate buffer at pH 7.0, 1 mM EGTA, 150 mM sucrose, 100 \( \mu \)M NADPH, and 5 \( \mu \)M lucigenin) at room temperature. The chemiluminescence generated was measured by a luminometer (AutoLumat LB 953; Berthold) every 60 s for 12 min and expressed as relative light units (RLU). Protein contents were assayed by bicinchoninic acid (BCA) method. Data are expressed as RLU per milligram protein.

**Quantification of mRNA abundance.** PAEC were plated \( (n=4) \) and cultured at 21% \( \text{O}_2 \)-5% \( \text{CO}_2 \) and 100% humidity to confluence. RNA was extracted via TRIZol (Sigma) method, and the contaminated DNA was removed by TURBO DNA-free Kit (Ambion) in 37°C water bath for 60 min. Complementary DNA was synthesized from the extracted RNA using iScript cDNA synthesis kit (Bio-Rad). The PCR primers were designed using Primer3 as previously described (33) and are shown in Table 1. Real-time RT-PCR was performed by iQ5 multicolor real-time PCR detection system (Bio-Rad). The PCR cycle started at 95.0°C for 3 min followed by 40 cycles of 95.0°C for 10 s and then 58.0°C for 1 min. Melting temperatures were monitored for each pair of primers, and single peak melting temperature was observed for all of the primer pairs. Number of the threshold cycle \( (Ct) \) for each target mRNA was corrected against the corresponding \( Ct \) of \( \beta \)-actin mRNA to obtain the \( \Delta Ct \), and then \( 2^{-\Delta Ct} \) was calculated against the corresponding NFL-PAEC as the surrogate of mRNA abundance (26).

**Western blot analysis.** PAEC were cultured until confluent and then washed twice with ice-cold HBSS. The cells were lysed in modified RIPA buffer (12). The cell lysate was sonicated twice on ice with 15% power output, and cell debris was removed by centrifugation at 13,000 rpm, 4°C, for 10 min. Protein content of the lysate was determined by BCA assay. An aliquot of protein was used for immunoblotting with monoclonal antibodies for p67phox (1:500), Rac1 (1:1,000), \( \beta \)-actin (1:5,000) and polyclonal antibodies for p47 phox (1:1,000), SOD1 (1:1,000), and SOD2 (1:2,000) to determine the levels of these proteins. The membranes were blotted with horseradish peroxidase...
(HRP)-conjugated anti-mouse IgG antibody (1:9,000; Bio-Rad) or HRP-conjugated anti-rabbit IgG antibody (1:8,000; Bio-Rad) and exposed to CL-XPosure films (Pierce) after treatment with SuperSignal West Pico (Pierce). The signals were analyzed with ImageJ and normalized against the expression of the internal control, β-actin.

Ex vivo sprouting angiogenesis of resistant pulmonary arteries. Growth factor-reduced Matrigel (50 μl) was added into each well of a 96-well plate as described for the in vitro tube formation assay. Fetal lungs and heart were removed en bloc and kept on ice before dissection. The pulmonary arteries were dissected from 5 to 7 generations to obtain resistant pulmonary arteries (RPA) under the microscope. Segments of RPA with a length of ~0.5 mm were obtained after removal of the soft tissue and transferred onto the Matrigel. Another 50 μl of Matrigel was gently added onto the RPA segment. After returning from the 37°C incubator for 30 min, 100 μl of DMEM with 20% FCS and AB/AM was added with or without Apo (20 μM) or NAC (500 μM) and was changed daily. The tube-like structure was viewed under inverted microscope after 6 days at ×20 magnification. The distal-most tip of the tube away from the edge of the segment was measured for comparison.

Statistical analyses. Data are shown as means ± 1 SE. Comparison of data between two groups was done by Student’s t-test if the data were normally distributed, and Mann-Whitney U test was used for data that did not pass the normality test. One-way ANOVA with Student-Newman-Keuls post hoc test was used when more than two groups were analyzed. Data were analyzed with MedCalc, which was designed by Frank Schoonjans. A P value of <0.05 was considered as significant.

RESULTS

In vitro tube formation. Tube formation is an integrated angiogenic activity involving proliferation, migration, invasion, and apoptosis. We used Matrigel to simulate extracellular matrix for PAEC to study mechanisms of tube formation. Total tube lengths and branching points number per HPF were significantly less in HTFL-PAEC (Fig. 1). Changing O2 concentration from 21 to 5% to simulate fetal arterial Po2 (PaO2; Fig. 2) or time allowed for tube formation (8 vs. 14 h; Fig. 3) did not alter the impaired tube formation in HTFL-PAEC. NAC and Apo increased total tube length and branching points in HTFL-PAEC cells (Fig. 4) at both 8- and 14-h time points.

Monolayer scratch recovery assay. When scratched, cell monolayers respond to disruption of the monolayer and intercellular contacts at the margin by an increase in migration and...
proliferation. The number of cells that migrated out from the scratch margin after 24 h was significantly lower for HTFL-PAEC compared with NFL-PAEC (Fig. 5). This impaired response in HTFL-PAEC is not affected by ambient O2 concentration (Fig. 5, A–E). Addition of NAC (500 μM) or Apo (20 μM) into the media after scratch improved migration and proliferation in HTFL-PAEC but not in NFL-PAEC (Fig. 5, G and H).

Invasion assay. After 24 h of culture, the number of cells that migrated through the modified Boyden chamber was significantly (P < 0.0001) higher for NFL-PAEC compared with HTFL-PAEC (Fig. 6). Addition of Apo significantly increased the migration/invasion of HTFL-PAEC cells. The cell invasion for HTFL-PAEC was significantly increased (P < 0.001; n = 6) after addition of Apo, whereas no increases were observed in NFL-PAEC after addition of Apo (100.0 ± 1.0 vs. 98.2 ± 7.8%).

BrdU proliferation assay. In the BrdU proliferation assay, an increase in Abs450 occurs in proportion to BrdU incorporation and, therefore, proliferation. Abs450 in NFL-PAEC (1.37 ± 0.14, n = 6) was increased to a greater extent (P < 0.01) than in HTFL-PAEC (0.79 ± 0.11, n = 6) when the assay was conducted at 21% O2. However, there was no significant differences in Abs450 (P = 0.44; n = 8) between HTFL-PAEC (0.55 ± 0.07) and HTFL-PAEC (0.45 ± 0.11) when the assay was conducted at 5% O2. Our observations suggest that ambient O2 concentrations affect proliferation rates of fetal PAEC and that rates of proliferation for HTFL-PAEC are impaired at 5% O2. The decreased Abs450 at 5% O2 may due to PAEC proliferation. The number of apoptotic cells in HTFL-PAEC cultures (P < 0.001; n = 8). These data suggest that reactive oxygen species play an important role in the mechanisms mediating increased apoptosis in HTFL-PAEC.

Measurements of reactive oxygen species production. Superoxide anion production, assessed by DHE epifluorescence, increased in HTFL-PAEC compared with NFL-PAEC (Fig. 8). Addition of Apo (100.0 ± 0.07) and HTFL-PAEC (0.45 ± 0.07) was increased to a greater extent (P < 0.001; n = 6) when the assay was conducted at 5% O2. However, there was no significant differences in Abs450 (P = 0.44; n = 8) between NFL-PAEC (0.55 ± 0.07) and HTFL-PAEC (0.45 ± 0.11) when the assay was conducted at 5% O2. Our observations suggest that ambient O2 concentrations affect proliferation rates of fetal PAEC and that rates of proliferation for HTFL-PAEC are impaired at 21% O2. The decreased Abs450 at 5% O2 may due to PAEC adaptation to our regular culture environment at 21% O2.

Apoptosis assay. Apoptosis is an important component of blood vessel formation and maintenance. Too much apoptosis can impair angiogenesis. The percent of apoptotic cells was significantly greater in HTFL-PAEC cultures (P < 0.001; n = 8) compared with NFL-PAEC (Fig. 7) when the experiment was performed at 21% O2. Interestingly, apoptosis in NAC- or Apo-treated HTFL-PAEC was significantly less compared with the number of apoptotic cells in HTFL-PAEC cultures (P < 0.001; n = 8). These data suggest that reactive oxygen species play an important role in the mechanisms mediating increased apoptosis in HTFL-PAEC.

Quantification of Nox2, p47phox, Rac1, Nox4, SOD1, and SOD2 mRNA abundance. There was no difference in Ct of β-actin between NFL-PAEC and HTFL-PAEC (P > 0.2; n = 4). Relative mRNA abundance of Nox2, p47phox, Rac1, and Nox4 were greater in HTFL-PAEC (P < 0.05; n = 4; Fig. 9A). There was, however, no difference in mRNA abundance for either SOD1 or SOD2. NAC or Apo treatments did not significantly change mRNA levels for Nox2, p47phox, Rac1, Nox4, SOD1, and SOD2 in HTFL-PAEC (Fig. 9B).

Western blot analyses. Protein levels for p67phox were significantly greater in HTFL-PAEC compared with the levels in NFL-PAEC (P < 0.05; n = 4). Similarly, Rac1 levels were significantly greater in HTFL-PAEC than in NFL-PAEC (P < 0.01; Fig. 10A). However, no differences were detected in the levels of p47phox, SOD1, or SOD2 between NFL-PAEC and HTFL-PAEC. Treating HTFL-PAEC with NAC or Apo did not affect protein expression of Nox2, Nox4, Rac1, p67phox, SOD1, or SOD2 in HTFL-PAEC, but, interestingly, expression levels for p47phox actually increased (P < 0.05; n = 4; Fig. 10B).

Ex vivo sprouting angiogenesis of RPA. Sprouting from HTFL pulmonary artery segments was less (P < 0.05; n = 3)
compared with sprouting from NFL pulmonary artery segments. Sprouting from HTFL pulmonary artery segments increased when treated with NAC (Fig. 11A). Apo also improved sprouting from HTFL pulmonary artery segments (Fig. 11B). These findings are consistent with the in vitro tube formation results in PAEC.

**DISCUSSION**

Although the incidence of PPHN is estimated to be 2–5 per 1,000 live births, it accounts for nearly 10% of all neonatal intensive care unit (NICU) admissions. Affected infants have increased mortality and risk of long term sequelae (18). PPHN decreases the number of blood vessels and impairs angiogenesis in the lungs, which together contribute to the increase in pulmonary vascular resistance (14). The mechanisms of impaired vascular growth are unclear, but impaired responses to VEGF or reduced NO availability have both been suggested as possible causes (17).

Fetal lambs with partial compression (1) or ligation (28) of DA in utero are commonly used to study mechanisms of PPHN. Recent studies in fetal lambs with prenatal ductal constriction demonstrated impaired angiogenesis and growth of the lungs (16). Our previous studies in this model revealed that PPHN impaired relaxation responses of pulmonary artery rings to ATP, decreased eNOS association with hsp90 (22), and increased eNOS uncoupling (21). Uncoupled eNOS activity results in the generation of O$_2^-$, which further reduces NO bioavailability (21). In addition to uncoupled eNOS activity, other oxidative enzymes may be involved in producing O$_2^-$ such as Nox, xanthine oxidase/dehydrogenase, cytochrome P-450, and mitochondria (29, 35). Brennan et al. (6) demonstrated an increase in O$_2^-$ production with increased expression of p67$^{phox}$, a critical subunit of Nox in the pulmonary arteries from fetal lambs with in utero ductal constriction. Increased p67$^{phox}$ expression developed as early as 48 h after ductal constriction, suggesting that NADPH oxidase is an important early enzymatic source of O$_2^-$ in PPHN (6). Our data support the observations of Brennan et al. (6) by showing that NADPH oxidase expression was increased at the transcriptional level even 8 days after ductal constriction. Our findings extend the time frame for the role of NADPH oxidase to 8 days, and, along with our observation of increased NADPH oxidase activity, we propose that NADPH oxidase plays a critical role in the impaired angiogenesis in this PPHN model.

Reactive oxygen species were previously shown to be important mediators of angiogenic signaling for endothelial cells...
from the systemic vasculature (8, 40). VEGF-induced endothelial migration is promoted by the activation of Rac1 (13). Rac1 is a cytoplasmic subunit for NADPH oxidase, and its activation leads to increased O$_2^-$ production. Arnal et al. (3) showed that proliferating endothelial cells generate increased levels of O$_2^-$ by a NADPH oxidase-dependent mechanism. Interestingly, eNOS expression and NO production are also increased by an increase in eNOS association with hsp90, which is essential for maintaining coupled eNOS activity in proliferating endothelial cells (4, 30). In support of this idea, studies by Balasubramaniam et al. (5) demonstrated that NO is an important mediator of angiogenesis in PAEC. Since O$_2^-$ reacts with NO at a diffusion-limited rate (19), the availability of NO will decrease with increased O$_2^-$ production. We therefore investigated whether increased O$_2^-$ production impairs angiogenic activity in HTFL-PAEC. We studied O$_2^-$ production and angiogenic activity of HTFL-PAEC at O$_2$ concentrations that simulated fetal and postnatal conditions to determine if ambient O$_2$ tension alters the effect of O$_2^-$ on angiogenesis. We observed that the decreased angiogenesis in HTFL-PAEC was a consistent feature and retained even at different O$_2$ concentrations.

Our studies demonstrated impairment of both in vitro and ex vivo angiogenesis and increased O$_2^-$ production in HTFL-PAEC. The increase in apoptosis and suppression of proliferation observed in HTFL-PAEC in our studies is different from previous studies in adults with idiopathic pulmonary artery hypertension (27). Although some apoptosis is necessary to complete the process of angiogenesis, too much apoptosis impairs angiogenesis (5, 9). PAEC from adult patients show an initial apoptosis followed by increased proliferation of apoptosis-resistant PAEC in artificial perfusion systems (34). The possible explanations are differences in maturity and duration of pulmonary hypertension; PAEC in our lamb model of PPHN.
were exposed to increased pressures for 8 days compared with months or years in adults with idiopathic pulmonary arterial hypertension (IPAH). It is possible that phenotypical selection of the endothelial cells in our model remains in the early apoptosis-prone stage.

We also observed that NAC or the NADPH oxidase inhibitor, Apo, improved angiogenic activity of HTFL-PAEC and pulmonary artery segments. These findings suggest that increased production of reactive oxygen species, possibly through increased activity of NADPH oxidase, contributes to impaired angiogenesis in HTFL-PAEC. We observed greater NADPH oxidase expression in HTFL-PAEC than in NFL-PAEC. The increased expression in HTFL-PAEC occurs at both the transcriptional (increased abundance of mRNA for
Nox2, Nox4, p47phox, and Rac1) and the translational level (increased p67phox and Rac1 protein expression). A potential role for NADPH oxidase in impairing angiogenesis is further supported by partial restoration of angiogenesis in HTFL-PAEC by NAC and Apo. Although the increased p47phox mRNA was not accompanied by an increase in protein expression, the increase p67phox expression in our HTFL-PAEC cultures is similar to increased p67phox in lungs of fetal lambs with PPHN (6). The increased angiogenic activity of HTFL-PAEC in response to antioxidant treatments suggests a potential therapeutic strategy in neonates with PPHN. Antioxidants PAEC in response to antioxidant treatments suggests a potential therapeutic strategy in neonates with PPHN. Antioxidants with PPHN (6). The increased angiogenic activity of HTFL-PAEC is due to the ability of these cells to generate rather than scavange O$_2^-$.

In conclusion, our study demonstrated that in utero pulmonary hypertension is associated with increased O$_2^-$ production and impaired angiogenesis in PAEC. Our data demonstrate that this phenotype change in HTFL-PAEC is the result of increased expression of NADPH oxidase, which becomes a source of O$_2^-$ production.

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