Nitric oxide augments fetal pulmonary artery endothelial cell angiogenesis in vitro

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Fetal lung organogenesis and maturation are characterized by distinctive changes in structure throughout the embryonic, pseudoglandular, canalicular, and saccular phases of development (35). Lung development is critically dependent on the highly regulated and synchronized temporal and spatial expression of diverse growth and transcription factors and receptors (10, 11, 23, 28, 31). Normal lung development occurs in the relatively hypoxic environment of the fetus, but few studies have addressed the direct effects of oxygen on pulmonary vascular growth or mechanisms that modulate the response to changes in oxygen tension during early development. Low oxygen enhances epithelial branching morphogenesis and vascular development in the mouse fetal lung explant model (33). However, the direct effects of changes in oxygen tension on fetal pulmonary artery endothelial cell (PAEC) growth and function and mechanisms that regulate vasculogenesis or angiogenesis in the normal fetus remain uncertain.

Premature birth interrupts the normal sequence of intruterine lung development, resulting in the chronic lung disease known as bronchopulmonary dysplasia (BPD) (25). In addition to marked impairment of alveolarization, BPD is also characterized by dysmorphic vascular growth, which contributes to abnormal gas exchange, and the development of pulmonary hypertension (1, 8, 17, 18). Mechanisms that impair vascular growth after premature birth are uncertain, but exposure of the immature lung to marked elevations of oxygen tension may induce oxidant lung injury or directly inhibit lung growth (2, 8, 18).

In the developing fetus, the oxygen tension seen by the fetal lung is much lower than the ambient oxygen concentrations in standard tissue culture. These levels correspond to a Po2 that is 22–25 Torr, which in vitro is equivalent to an FiO2 that is 3% (16, 32). This level of fetal oxygen tension is necessary for normal embryonic (7), vascular (22, 33), and cardiac development (36). Fetal lung branching morphogenesis in vitro is enhanced by culture in low oxygen tension compared with room air (14, 33). Human fetal lung explants that were cultured at fetal oxygen tension had increased expression of vascular endothelial growth factor (VEGF) compared with explants cultured in room air (3) and inhibition of VEGF impaired lung vascular growth and airway branching (33). One of the downstream effectors of VEGF signaling, nitric oxide (NO), exhibits reciprocal regulation with VEGF (12, 13, 20, 24, 30).

Recent studies have suggested that NO may play a critical role in lung angiogenesis, especially during low oxygen tension. A recent study demonstrated abnormal lung vascular growth and abnormal spatial orientation of the pulmonary vasculature in endothelial nitric oxide synthase (eNOS) deficient fetal mice, beginning in late gestation (15). In addition, eNOS-deficient neonatal mice have an increased susceptibility to hypoxia induced inhibition of lung growth (5). In contrast to wild-type mice, mild hypoxia (16% oxygen) in the postnatal period impaired alveolarization and reduced lung parenchymal vascular volume in eNOS-deficient mice (5). These studies suggest that NO modulates or sustains lung angiogenesis and alveolarization and may play an adaptive role to preserve lung growth during fetal and neonatal life.

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Therefore, we hypothesize that low oxygen tension during fetal life enhances pulmonary endothelial cell growth and function and that NO modulates the fetal PAEC response to changes in oxygen tension. In addition, we hypothesize that the exposure of fetal PAEC to the relative hyperoxia of room air will result in abnormalities of PAEC growth and function. To test this hypothesis, we studied fetal PAEC growth, survival, migration, and tube formation under fetal (3% O₂) and postnatal (room air) oxygen tensions and the effect of an NOS inhibitor and an NO donor on PAEC exposed to these different oxygen tensions.

MATERIALS AND METHODS

Isolation and culture of fetal ovine PAEC. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Left and right main pulmonary arteries were isolated from gestation fetal lambs from mixed-breed (Columbia-Rambouillet) pregnant ewes at 135 days of gestation (term = 147 days). The ewe and fetus were anesthetized with intravenous pentobarbital sodium. The left and right main pulmonary arteries were isolated under direct visualization in a sterile manner and transferred to ice-cold Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO). The adventitia was removed from the pulmonary artery segment and opened to expose the endothelial surface. After a brief period of trypsin digestion (5 min), the endothelial surface was gently removed with a cell scraper, and the cells were transferred to a 150-mm cloning dish. Cells were cultured in DMEM with 20% fetal bovine serum (FBS), 1% l-glutamine, and 1% penicillin-streptomycin in humidified incubators (Forma Scientific, Marietta, OH) at 37°C in room air, 5% CO₂, and balance nitrogen. Pure populations of fetal PAEC were subcloned based on their morphological appearance with cloning rings (Sigma). Cells content were analyzed using Modfit LT software (Verity Software, Topsham, ME).

Apoptosis assay. To quantify the number of apoptotic cells under the conditions described above, cells were seeded at a density of 1 × 10⁴ cells/well in four chambered slides and incubated in DMEM with 10% FBS in the presence or absence of LNA (2 mM) in either 3% O₂-5% CO₂-balance nitrogen or in 16% O₂-5% CO₂-balance nitrogen. The cells were stained with Annexin V and propidium iodide (Annexin V/PI). The percentage of cells with Annexin V-PI-positive staining was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Western blot analysis. Cells were harvested, and equal cell numbers were harvested, and equal cell numbers were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and incubated with primary antibodies overnight at 4°C. The primary antibodies used were: rabbit anti-human Bax (1:200; Santa Cruz Biotech, SC492), diluted in 5% nonfat dry milk in TBS; rabbit anti-human Bcl-2 (1:200; Santa Cruz Biotech, SC526); goat anti-actin (1:1000; Sigma); and rabbit anti-pimonidazole antibody (1:500; Pharmacia Biotech, Buckinghamshire, UK). The membranes were then stripped and reprobed with an antibody to β-actin (Sigma, A5316) as a control. The bands were visualized by enhanced chemiluminescence (ECL+ kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). Purified protein was run as a control and the band that comigrated with the molecular size as identified by the manufacturer for the protein of interest. The blots were then stripped and reprobed with an antibody to β-actin (Sigma, A5316). Densitometry was performed using NIH Image (version 1.61). Changes in protein expression were analyzed after normalization for β-actin expression.

Statistical analysis. Data are presented as means ± SD. Statistical analysis was performed with the Prism 4 software package (GraphPad Software, San Diego, CA).
RESULTS

In vitro angiogenesis is dependent on oxygen tension and NO. Exposure of PAEC to 3% oxygen increased branch points by 114% compared with room air (16.4 ± 2 vs. 7.3 ± 3 branch points per hpf; \(P < 0.01\); Figs. 1, A vs. B, and 2). NOS inhibition did not affect the number of branch points in room air (7.3 ± 2 branch pts/hpf; Fig. 1C). In contrast, LNA treatment during exposure to 3% oxygen decreased the number of branch points (7.6 ± 2 branch pts/hpf; \(P < 0.01\); Fig. 1D) to levels observed in room air (Fig. 2). Treatment of PAEC with the NO donor SNAP increased the number of branch points of PAEC maintained in room air (Fig. 1E) (12 ± 2 branch points/hpf, \(P < 0.01\), Fig. 2). SNAP treatment did not have an effect on the number of branch points in 3% oxygen [16.6 ± 2 branch pts/hpf, \(P = \text{not significant (ns)}\), Fig. 2]. The addition of SNAP during NOS inhibition in room air increased the number of branch points (Fig. 1, E vs. B) to similar values that were observed in 3% oxygen (14.5 ± 2 branch pts/hpf, Fig. 2). The addition of SNAP to LNA treated fetal PAEC in 3% oxygen increased the number of branch points (Fig. 1F) to levels observed in the 3% control group (17 ± 1 branch pts/hpf, Fig. 2). All reported values are means ± SD for and \(n = 4\) in each group.

Fetal ovine PAEC migration is dependent on functional NOS. There was no difference in PAEC migration during exposure to room air or 3% oxygen (Fig. 3). In room air, LNA treatment decreased PAEC migration by 9.5% from baseline values (119 ± 8 vs. 108 ± 11 cells/hpf, \(P < 0.05\), Fig. 3) and

Fig. 2. In vitro branch point counting is reduced in fetal PAEC exposed to RA and NOS inhibition. There was no difference in the number of branch points/high-power field (hpf) in RA or RA with LNA. In 3% oxygen there was a significant increase in branch points (\(*P < 0.01\)) that was reduced to levels seen in RA with the addition of LNA. The addition of SNAP + LNA increased the number of branch points in RA to levels comparable to those seen in 3% oxygen. The addition of SNAP to LNA was able to restore branch point numbers to those seen with control in 3% oxygen. Error bars represent SD from means.

Fig. 3. Fetal PAEC migration is reduced by NOS inhibition. Shown is the number of ovine fetal PAEC per hpf that migrated through a layer of Matrigel and through a membrane toward the chemotactic stimulus of 10% FBS (Control, solid bars), 10% FBS plus LNA (2 mM, open bars), 10% FBS plus LNA (2 mM) + SNAP (5 μM) (diagonally hatched bars), or 10% FBS plus SNAP (5 μM) (vertically hatched bars). Under RA conditions LNA impaired PAEC migration \((**P < 0.01)\). Oxygen at 3% further reduced PAEC migration with LNA treatment \((**P < 0.01)\). The addition of SNAP to LNA was able to restore migration to control levels in both RA and 3% oxygen. Error bars represent SD from means.
by 10.6% under 3% oxygen conditions (119 ± 6 vs. 106 ± 8 cells/hpf, P < 0.01, Fig. 3). Treatment with the NO donor SNAP did not significantly increase cell migration in room air (122 ± 10 cells/hpf) or 3% oxygen (125 ± 12 cells/hpf) but did reverse the reduction in cell migration seen with NOS inhibition.

**Low oxygen tension and NOS inhibition blunt fetal ovine PAEC growth.** In room air, fetal PAEC number increased by 97% from baseline (3.7 ± 0.5 × 10⁴ cells) at 48 h but only by 47% in 3% O₂ (7 ± 0.8 × 10⁴ vs. 5.2 ± 0.3 × 10⁴ cells; P < 0.01, Fig. 4A). Compared with controls, LNA treatment in room air attenuated the increase in cell number at 24 (no change compared with day 0) and 48 h to only a 57% increase from day 0 (5.7 ± 0.2 × 10⁴ cells; P < 0.05 vs. control, Fig. 4B). During exposure to 3% oxygen, LNA treatment had a more striking effect, resulting in no increase in cell number at 24 and 48 h (P < 0.01, Fig. 4C). Under room air conditions, the addition of SNAP did not have an effect on PAEC number (Fig. 4B). In 3% oxygen conditions, SNAP increased PAEC number by 72% (6.4 ± 0.6 × 10⁴ cells) at 24 h and by 94% (7.2 ± 0.7 × 10⁴ cells) at 48 h compared with day 0 (P < 0.001, Fig. 4C), which was similar to changes in cell number seen in room air. In room air, the addition of SNAP to LNA increased PAEC number from baseline by 77% at 24 h (6.5 ± 1.3 × 10⁴ cells; P < 0.01 compared with control and LNA alone, Fig. 4B) with no further increase in cell number at 48 h (Fig. 4B). The addition of SNAP to LNA in 3% oxygen increased PAEC number from baseline by 57% at 24 h (5.8 ± 0.3 × 10⁴ cells, P < 0.01 compared with control and LNA alone, Fig. 4C) with no further increase at 48 h (Fig. 4C). The number of apoptotic nuclei per hpf was not different in fetal PAEC exposed to 3% oxygen or room air (2.1 ± 0.7 vs. 2.7 ± 0.7 nuclei/hpf, P = ns). In addition, NOS inhibition did not change the number of apoptotic nuclei in either 3% oxygen (1.7 ± 0.8 nuclei/hpf) or room air (2.6 ± 0.3 nuclei/hpf). There was no change in the ratio of Bax to Bcl-2 in PAEC in room air or 3% oxygen. There was also no change in the Bax to Bcl-2 ratio with NOS inhibition in either room air or 3% oxygen.

**NO is required for progression of fetal PAEC through cell cycle.** Cell cycle analysis demonstrated differences between PAEC grown in room air vs. 3% oxygen conditions. Under 3% oxygen conditions, a greater percentage of PAEC were in S-phase of the cell cycle at 48 h compared with room air PAEC (20 ± 5% vs. 10 ± 1%; P < 0.05, Fig. 5A). NOS inhibition in room air decreased the percentage of PAEC in S-phase at 24 and 48 h (to 9 ± 0.6% and 6.4 ± 1.6%; P < 0.05, Fig. 5B). NOS inhibition during 3% oxygen exposure decreased the number of S-phase cells at 24 and 48 h (to 8 ± 0.6% and 11.4 ± 3.7%; P < 0.02, Fig. 5C). The addition of SNAP with LNA increased the percentage of PAEC in S-phase at 24 and 48 h in room air compared with LNA alone (to 11.8 ± 0.8% and 10 ± 0.5%; P < 0.05, Fig. 5B). However, SNAP did not increase the percentage of cells in S-phase during exposure to 3% oxygen and LNA (Fig. 5C).

**DISCUSSION**

We report that exposure of fetal PAEC to fetal oxygen tension of 3% oxygen markedly augments tube formation, has little effect on cell migration, and blunts the rate of fetal PAEC proliferation in vitro compared with room air. Interestingly, blockade of NOS by LNA in room air impaired PAEC growth and did not further reduce PAEC tube formation, but the addition of the NO donor SNAP increased fetal PAEC growth rate and improved tube formation to levels seen in the fetal oxygen tension. This suggests that the impairment of tube formation in room air conditions may be due to decreased NO production in fetal PAEC in room air. We further observed that in low oxygen tension the growth and function of fetal PAEC are dependent on functional NOS. Inhibition of NOS with LNA in 3% oxygen impairs fetal PAEC growth, migration, and tube formation. NOS inhibition in 3% oxygen impairs PAEC tube formation to levels seen in room air. The effect of pharmacologic NOS inhibition in a low oxygen tension envi-

![Fig. 4. Fetal PAEC growth in RA and 3% oxygen. Ovine PAEC were quiesced in low serum (0.5% FBS) for 48 h and then stimulated with 10% FBS (control, ○), 10% FBS plus LNA (2 mM, ◦), and LNA plus SNAP (5 μM, ●). PAEC were grown in RA or in 3% oxygen. A: the rate of increase in cell number in RA was not matched by PAEC exposed to 3% oxygen. B: LNA treatment reduced cell growth RA that was rescued by the addition of SNAP (P < 0.01). C: in 3% oxygen LNA reduced cell growth, which was also reversible by the addition of SNAP (P < 0.01). SNAP treatment alone in 3% oxygen increases PAEC growth to levels seen in RA. Error bars represent SD from means.
Oxygen and NO in Fetal PAEC Angiogenesis

The lungs of infants with BPD have been described as having a dysmorphic vascular structure with alveolar simplification and impaired angiogenesis (8, 17). In the premature baboon model of BPD, exposure to mild increases in oxygen tension required to maintain normal oxygenation resulted in abnormalities of alveolar growth and impaired angiogenesis, with a reduction in vascular density and a dysmorphic vascular structure (9). In this same model and in the premature sheep model of BPD, there is a reduction of lung eNOS expression (4, 21). Angiogenesis is a process that requires endothelial cells to proliferate, migrate, and form vascular tubes. In this present study, fetal PAEC incubated in low oxygen tension demonstrate a marked improvement in one of these three functions (tube formation). In contrast, room air exposure results in a reduction in number and stability of vascular tubes. Impairment of endogenous NO production during low oxygen tension exposure impairs in vitro angiogenesis by decreasing cell proliferation, impairing PAEC migration, and a reducing vascular tube formation. Exposure of fetal PAEC to the “relative hypoxia” of room air reduces tube formation to levels observed after NOS inhibition in fetal oxygen tension. This suggests that room air exposure impairs PAEC function in a similar fashion to the effects of NOS inhibition in 3% oxygen. In addition, preliminary experiments (unpublished data) from our lab suggest that NO production during exposure to 3% oxygen is necessary for PAEC progression through the cell cycle, and inhibition of NOS results in G0/G1 arrest. The addition of an exogenous NO donor (SNAP) was able to rescue fetal PAEC from the effects of NOS inhibition under low oxygen conditions. Interestingly, the use of an NO donor also improved fetal PAEC growth and vascular tube formation during exposure to room air. These findings suggest that NO augments cell proliferation, migration, and vascular tube formation in fetal PAEC during culture in 3% oxygen. In addition, the normal low oxygen environment of the fetus is critical for normal PAEC growth and function and that with exposure to relative hypoxia of room air fetal PAEC proliferation and function is abnormal. Furthermore, during exposure to increased oxygen tension of room air, NO plays a critical role in preserving and promoting cell proliferation and vascular tube formation and migration, the essential elements required for normal blood vessel growth.

The relatively low oxygen tension that the fetus develops (3% oxygen) is essential for normal embryonic growth, including the regression or loss of tissue allowing for normal patterns of organogenesis (7). Low oxygen tension is also involved in the maintenance and establishment of the hemangioblast, the bipotential progenitor of endothelial and hematopoietic cells (27). Low oxygen tension is critical for the stimulation and expression of several proangiogenic factors that include VEGF, platelet-derived growth factor, and hypoxia-inducible factor (HIF)-1α (22). Fetal lung epithelial cells raised in low oxygen tension have improved plating efficiency and mature rates of lipid synthesis despite preservation of immature morphology (32). The ability of fetal cells in culture to maintain an immature morphology but display mature functions under fetal oxygen tension suggests that the optimal oxygen tension for normal fetal lung growth is the low oxygen environment of the fetus. In fetal lung explants 3% oxygen increases branching of the terminal epithelium and mesenchyme and maintains epithelial differentiation compared with 21% oxygen (14, 33). Van Tuyl and colleagues (33) went further to show that during hypoxia, the disruption of two genes that are involved in hypoxic angiogenesis, HIF-1α or VEGF, results in dysmorphic lung angiogenesis and that lung vascular development is essential for lung branching morphogenesis. In the higher oxygen concentration of room air, NO has been suggested to aid in the stabilization of HIF-1α (26), thus abnormalities in NO production could impair angiogenesis via impaired HIF signaling. Our present study is the first to link the improved growth and function, in terms of endothelial tube formation, of fetal PAEC to NO modulation in low oxygen tension. Our study is also the first to suggest that NO may play an adaptive role in the fetal lung exposed to room air by enhancing angiogenesis.

The potential limitations of this study include the possibility that NOS inhibition may have resulted in cell death, although we did not observe an increase in apoptotic nuclei or a shift in the ratio of Bax/Bcl-2 to favor apoptosis. In addition, the effect of NOS inhibition may have increased oxygen radical formation, which could indirectly impair endothelial cell growth and function. Further studies will have to be performed to address the effect of NOS inhibition during hypoxia on apoptosis and

Fig. 5. S-phase of the cell cycle of quiescent ovine fetal PAEC stimulated with 10% serum (○), 10% serum plus LNA (2 mM, □), and 10% serum + LNA (2 mM) + SNAP (5 μM) (△). A: there is an increase in the percentage of cells in S-phase in 3% oxygen after 48 h. LNA treatment inhibits the progression of cells through the cell cycle in 3% oxygen (C) and RA (B) (**P < 0.02). The addition of SNAP overcomes the effect of LNA in RA (B) but not in 3% oxygen (C). Error bars represent SD from means.

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superoxide production, in addition to exploring further the role of NO in PAEC cell cycle progression and on the regulatory proteins of cell cycle progression. There exists the possibility that the fetal PAEC phenotype may be affected by isolation and expansion in room air compared with hypoxia. The fetal PAEC used in this study were from large vessels, and there may exist differences in the behavior of these cells compared with microvascular endothelial cells. These potential limitations will be addressed in future studies. Finally, we had difficulties in directly measuring the NOx levels in the cell media, due to relatively high nutrient nitrate levels in the cell culture media (data not shown).

We conclude that endothelially derived NO is essential in preserving PAEC function in vitro, especially when the fetal lung is exposed to the relative hyperoxia of room air. We speculate that exposure of the premature lung to increases in oxygen tension results in impaired angiogenesis, in terms of vascular tube formation, which causes abnormal lung structure. Furthermore, in the lungs of premature infants, reduced endothelial NO production impairs one of the adaptive mechanisms for pulmonary vascular growth in the fetal lung and thus increase the susceptibility for the development of BPD and late pulmonary vascular disease.

REFERENCES