Intrauterine hypertension decreases lung VEGF expression and VEGF inhibition causes pulmonary hypertension in the ovine fetus

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Intrauterine hypertension decreases lung VEGF expression and VEGF inhibition causes pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 284: L508–L517, 2003. First published November 22, 2002; 10.1152/ajplung.00135.2002.—Although vascular endothelial growth factor (VEGF) plays a vital role in lung vascular growth in the embryo, its role in maintaining endothelial function and modulating vascular structure during late fetal life has not been studied. We hypothesized that impaired lung VEGF signaling causes pulmonary hypertension, endothelial dysfunction, and structural remodeling before birth. To determine whether lung VEGF expression is decreased in an experimental model of persistent pulmonary hypertension of the newborn (PPHN), we measured lung VEGF and VEGF receptor protein content from fetal lambs 7–10 days after ductus arteriosus ligation (132–140 days gestation; term = 147 days). In contrast with the surge in lung VEGF expression during late gestation in controls, chronic intrauterine pulmonary hypertension reduced lung VEGF expression by 78%. To determine whether VEGF inhibition during late gestation causes pulmonary hypertension, we treated fetal lambs with EYE001, an aptamer that specifically inhibits VEGF165. Compared with vehicle controls, EYE001 treatment elevated pulmonary artery pressure and pulmonary vascular resistance by 22 and 50%, respectively, caused right ventricular hypertrophy, and increased wall thickness of small pulmonary arteries. EYE001 treatment reduced lung endothelial nitric oxide synthase protein content by 50% and preferentially impaired the pulmonary vasodilator response to ACh, an endothelium-dependent agent. We conclude that chronic intrauterine pulmonary hypertension markedly decreases lung VEGF expression and that selective inhibition of VEGF165 mimics the structural and physiological changes of experimental PPHN. We speculate that hypertension downregulates VEGF expression in the developing lung and that impaired VEGF signaling may contribute to the pathogenesis of PPHN.

Persistent pulmonary hypertension of the newborn; lung development; nitric oxide; angiogenesis; vascular endothelial growth factor

PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN (PPHN) is a clinical syndrome characterized by abnormal pulmonary vascular tone, reactivity, and structure, leading to sustained elevation of pulmonary vascular resistance (PVR) and severe hypoxemia at birth (22, 28, 34). Mechanisms that cause PPHN are uncertain, but clinical and pathological studies suggest that chronic intrauterine stress alters the pulmonary circulation during late fetal life, causing failure of PVR to fall at birth (22, 28, 34). Chronic intrauterine hemodynamic stress may disrupt critical signaling pathways responsible for promoting normal vascular growth and structure and for maintaining normal vascular tone and reactivity.

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen with angiogenic and vascular permeability properties (13, 27, 39). VEGF exists as at least five isoforms (121-, 145-, 165-, 189-, and 206-amino acid isoforms) generated by alternate splicing of a single gene (15, 27). VEGF165, the most abundant form in most human tissues, is the most potent of the isoforms (21). VEGF is critical to early vascular development because gene ablation of a single allele of VEGF in the mouse prevents normal vascular development and causes early embryonic lethality (6, 12). Although VEGF is clearly essential for early vessel formation, the role of VEGF in pulmonary vascular development during late gestation is unclear. In addition to its role in vascular growth, VEGF signaling also modulates endothelial cell survival and function (19, 30). In particular, VEGF treatment upregulates endothelial nitric oxide synthase (eNOS) in endothelial cells (23) and improves blood flow in vivo (4). Thus disruption of VEGF signaling could potentially impair vascular function as well as growth and structure in the developing lung, but this has not been studied.

Whether decreased VEGF production contributes to the abnormal vasculature in PPHN is uncertain. A recent clinical study demonstrated decreased circulating VEGF levels in human newborns with PPHN (25).
Past studies in models of pulmonary hypertension in adult rats have demonstrated that lung VEGF mRNA is increased in chronic hypoxia-induced pulmonary hypertension (7, 43), but lung VEGF is decreased in pulmonary hypertension due to monocrotaline (36). It is unclear whether changes in VEGF contribute to the development of pulmonary hypertension in adult models or represent an adaptive mechanism. Additionally, non-hypoxia-dependent mechanisms that may regulate lung VEGF expression, such as altered hemodynamic stress, are poorly understood. Changes in lung expression of VEGF or its receptors during the development of perinatal pulmonary hypertension and the effects of VEGF inhibition on the developing pulmonary circulation during late fetal life have not been studied.

To better understand the pathophysiology of PPHN, our laboratory and others have studied the effects of chronic intrauterine compression of the ductus arteriosus (DA) in late gestation fetal lambs (2, 32). In this model, chronic DA compression leads to elevated pulmonary artery (PA) pressure (32), impaired vasodilation to endothelium-dependent agents (31, 44, 40), hypertensive structural remodeling of small pulmonary arteries, and sustained elevations in PVR at birth (2, 32). Mechanisms by which pulmonary hypertension impairs endothelial cell function, increases muscularization of small pulmonary arteries, and leads to failure of pulmonary vasodilation after birth are unknown.

We hypothesized that hemodynamic forces, such as hypertension, may decrease VEGF expression and that impaired lung VEGF activity can contribute to the pathophysiology of PPHN. To test this hypothesis, we first measured lung VEGF and VEGF receptor (VEGFR) expression in fetal and postnatal lambs to determine the normal ontogeny of VEGF expression. Next, we defined the effects of hypertension on VEGF expression in an experimental lamb model of PPHN caused by chronic fetal constriction of the DA (2, 32). Finally, we determined whether chronic inhibition of VEGF could induce changes in pulmonary vascular structure, reactivity, and tone that mimic abnormalities of the lung circulation in PPHN and whether these changes were due to decreased eNOS expression and activity. We report that lung VEGF expression markedly increases during late gestation in the normal fetus but is dramatically decreased by chronic intrauterine pulmonary hypertension. We also found that treatment with EYE001, an aptamer shown to specifically inhibit VEGF (previously known as NX1838 (24–26)), causes structural and functional abnormalities of the fetal pulmonary circulation that are similar to PPHN and that VEGF inhibition decreases eNOS protein expression and activity. These findings support the hypothesis that hypertension downregulates VEGF expression in the developing lung and that impaired VEGF signaling may contribute to the pathogenesis and pathophysiology of PPHN.

**METHODS**

**Study protocols.** All procedures and protocols were previously reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. This study included three protocols. First, we defined normal maturational changes in lung VEGF expression during early, middle, and late fetal life and in postnatal lambs. (Protocol 1. Maturational changes in lung VEGF and VEGFR expression in ovine fetus). Second, we measured lung VEGF expression in an experimental model of PPHN caused by partial compression of the DA in the late-gestation fetus (Protocol 2. Effect of chronic intrauterine pulmonary hypertension on lung VEGF expression). Finally, we determined whether inhibition of VEGF with a selective aptamer altered pulmonary vascular structure and tone and caused pulmonary hypertension (Protocol 3. Effects of VEGF inhibition on pulmonary vascular structure and function).

**Protocol 1. Maturational changes in lung VEGF and VEGFR expression in ovine fetus.** Lung tissue was obtained from 19 fetal and neonatal Columbia-Rambouillet sheep at 113–115 days gestation (“early,” canalicular stage; n = 5; term = 147 days); 123–126 days (“middle,” saccular stage; n = 5); 132–140 days (“late,” alveolar stage; n = 5); and 3–5 days postnatal (n = 4). All animals were euthanized with a rapid injection of intravenous pentobarbital sodium. The lungs were exposed through a midline sternotomy, and the left lung was removed, frozen rapidly in liquid nitrogen, and stored at −70°C until assay. The trachea was cannulated for fixation, and the right lung was inflated with 10% buffered formalin at 30 cmH2O pressure. Tissues were stored in formalin until histological analysis and immunohistochemistry studies were performed (using methods described below).

**Protocol 2. Effect of chronic intrauterine pulmonary hypertension on lung VEGF expression.** Surgery was performed on 10 mixed-breed pregnant ewes between 125 and 133 days gestation, according to previously published methods (2). A polyvinyl catheter was placed in the left axillary artery and advanced into the ascending aorta (Ao). In five animals, a cotton umbilical tie was placed around the DA and was tightened around a metal probe to partially constrict the DA in a uniform manner. Five age-matched control animals (125–133 days gestation) underwent the same surgery, including isolation of the DA, but in these control animals, the DA was not ligated. Animals were euthanized 7 days after surgery. The left lung was rapidly frozen in liquid nitrogen and stored at −70°C for molecular studies. The right lung was inflated through the trachea with 10% buffered formalin at 30–40 cmH2O and stored in formalin. Western blot analysis was performed for VEGF, VEGFR-1, and VEGFR-2 protein expression, and immunohistochemistry was performed for localization of VEGF. Wall thickness of small pulmonary arteries associated with terminal and respiratory bronchioles was measured to quantitate the degree of pulmonary hypertension after chronic DA constriction. Because VEGF is produced by epithelial cells, we performed Western blot analysis for an epithelial cell protein, prosurfactant protein C (proSP-C), from normal and hypertensive lung tissue.

**Protocol 3. Effects of VEGF inhibition on pulmonary vascular structure and function.** Sixteen mixed-breed pregnant ewes between 125 and 130 days gestation (term = 147 days) had surgery performed, and animals were randomized to either control (n = 8) or EYE001 treatment (n = 8) groups. Catheters were placed in the Ao, left pulmonary artery (LPA), main pulmonary artery (MPA), and left atrium (LA) by direct puncture and secured by a purse string suture. The DA was not manipulated during surgery. An ultrasonic flow
probe (Transonic Systems, Ithaca, NY) was placed around the LPA to measure blood flow to the left lung.

Baseline hemodynamic measurements (left lung blood flow (Q_LPA), pulmonary artery pressure (PAP), aortic blood pressure (AoP), left atrial pressure (LAP), PVR) and arterial blood gas tensions (pH, PaO_2, PaCO_2, oxygen saturation) were recorded daily. Response to endothelium-dependent (acetylcholine; ACh) and endothelium-independent (8-bromo-cGMP) vasodilator stimuli was measured on days 1 and 7 of the treatment protocol. These drugs were selected for study based on previous studies that have shown that ACh-induced pulmonary vasodilation is dependent on endogenous nitric oxide production in the fetal lamb (1, 31). Baseline hemodynamic measurements were obtained before ACh or 8-Br-cGMP infusion and recorded every 10 min for 30 min after infusion.

At the conclusion of EYE001 treatment, tissues were obtained for histological and molecular analysis. Hematoxylin and eosin (H&E) staining was performed for histological examination and morphometric analysis of small PA wall thickness, pulmonary vessel density, and radial alveolar count of right ventricle (RVH) and left ventricle plus septum. After the pulmonary arteries were perfused with a barium sulfate mixture, angiograms were performed on control and treated lambs to define the branching pattern of the pulmonary arterial bed. Western blot analysis was performed for lung eNOS protein to determine whether endothelial cell function was altered by EYE001 treatment. Finally, to determine whether chronic VEGF inhibition decreased expression of other endothelial cell proteins, we assayed lung tissue for platelet/endothelial cell adhesion molecule (PECAM) by Western blot analysis.

**Study methods: physiological measurements.** Ao, MPA, and LA catheters were connected to a computer-driven pressure transducer and recorder (Biopac Systems, Santa Barbara, CA). The flow transducer cable was attached to an internally calibrated flowmeter (Transonic Systems) for continuous measurements of LPA flow. Absolute values of flow were determined from phasic blood flow signals, as previously described (29). PVR in the left lung was calculated by the following equation: PVR [mmHg·ml⁻¹·min⁻¹] = (mean PA pressure − mean LAP) / LPA flow (Q_LPA). Arterial blood gas tensions, pH, and oxygen saturations were measured from blood samples that were drawn from the Ao catheter and measured at 39.5 °C with a blood gas analyzer and hemoximeter (OSM-3; Radiometer, Copenhagen, Denmark).

**Drug preparation.** Stock solution of EYE001, a synthetic RNA oligonucleotide aptamer that binds solely to the three-dimensional conformational protein-receptor binding site of VEGF, was prepared by being dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. The drug was infused over 30 min into the LPA catheter (1.5 mg bid for 7 days). Control animals received an infusion of normal saline (1.5 cc bid for 7 days). The EYE001 aptamer was modified with a 40-kDa polyethylene glycol, as previously described (35), to increase its plasma half-life to 7–9 h (35, 38), and the dose chosen was based on previous animal studies utilizing this drug (35).

ACh (Sigma Chemical, St. Louis, MO) was dissolved in sterile saline at a concentration of 15 µg/ml (total vol 2 ml) in the LPA catheter. 8-Br-cGMP (Sigma) was dissolved in sterile saline and infused at a dose of 1.5 mg over 10 min in the LPA.

**Western blot analysis.** Western blot analysis for lung VEGF, VEGFR-1, VEGFR-2, eNOS, PECAM, and pro-SP-C protein was performed using 25 µg of lung protein per lane, according to previously published methods (26). Immunodetection was performed with a monoclonal antibody to VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000, a rabbit polyclonal IgG secondary antibody diluted 1:10,000, and ECL Plus (Amersham, Arlington Heights, IL) detection. Luminescence was determined by exposure to X-ray film for 5–60 s. Densitometry was performed with a scanner and NIH Image software.

The samples for protocols 1 and 2 were analyzed on multiple Western blots due to the large number of samples, with a standard curve of a randomly selected control sample (125-day normal lamb) used on each blot as a reference. The control sample was loaded at three increasing amounts (15, 25, and 35 µg), and all treatment samples fell within the linear range of the standard curve. Densitometry units for each study sample were expressed as a percentage of the standard control sample (25 µg).

Immunodetection was performed using a rabbit polyclonal antibody to VEGFR-2 (KDR/FK-1; Santa Cruz), a rabbit polyclonal antibody to VEGFR-1 (Flt-1; Santa Cruz), a mouse monoclonal IgG antibody to eNOS (Transduction Laboratories, Lexington, KY), a goat polyclonal antibody to PECAM (Santa Cruz), and a polyclonal antibody to pro-SP-C (gift from Dr. Dennis Voelker, National Jewish Hospital, Denver, CO).

**Histology and immunohistochemistry.** Histology and immunohistochemistry for VEGF, VEGFR-1, and VEGFR-2 protein were performed according to previously published methods (18). Formalin-fixed lung tissue was paraffin embedded. H&E staining was performed on paraffin sections from the lungs of three to seven animals in each study group.

**Barium angiograms.** Lungs were infused with barium sulfate according to previously established methods (9). Immediately after death, PBS was infused through the MPA catheter to flush the pulmonary circulation free of blood. A barium sulfate-gelatin mixture was heated to 70°C and infused into the MPA at 74 mmHg. Pressure was maintained for at least 5 min to ensure penetration of the barium mixture. Barium-filled lungs were then exposed on X-ray film.

**Morphometric analysis.** Morphometry was performed on small PAs (20–80 µm) on H&E-stained lung sections using a Zeiss Interactive Digital Analysis System. Wall thickness and external diameter were directly measured, and percent wall thickness was calculated as (2× wall thickness/vessel diameter) × 100 to assess medial hypertrophy (2). Five animals from each study group (control and EYE001-treated lambs) were examined, and 10 vessels were measured for each animal by a blinded observer. Alveolarization was measured by the radial alveolar count (RAC) methods of Cooney and Thurlbeck (8) and Emery and Mithal (10) according to previously published methods.

Pulmonary vessel density was performed after factor VIII staining of distal lung sections. The number of pulmonary vessels was counted per high-powered field (hpf; ×200 magnification) by a blinded observer. Five animals from each study group were examined, and five sections per animal were counted. Fields containing large airways or major vessels were avoided.

**Data analysis.** Statistical analysis of protein content and hemodynamic measurements was performed by one-way analysis of variance or unpaired t-tests. Where significant differences were identified, post hoc analysis was performed using Student-Newman-Keuls test. All statistical measurements were performed using a commercially available statistics package (GraphPad Prism, GraphPad Software). The
in lung samples from control and hypertensive lambs. Chronic intrauterine pulmonary hypertension decreased lung VEGF protein content by 79% compared with age-matched control animals (7,111 ± 1,794 densitometry units vs. 2,128 ± 786; control vs. pulmonary hypertension; P < 0.05; Fig. 2A). Lung VEGFR-2 protein content increased twofold after chronic intrauterine pulmonary hypertension (103 ± 10% control [pulmonary hypertension] vs. 44 ± 27% control; P < 0.05; Fig. 2B), but VEGFR-1 was not different between the study groups [35 ± 5% control (pulmonary hypertension) vs. 50 ± 18% (control); P = not significant (ns)]. Western blot analysis revealed no change in expression of pro-SP-C, an epithelial cell protein, after chronic intrauterine pulmonary hypertension compared with control lambs (91 ± 13% control vs. 99 ± 6% control; pulmonary hypertension vs. control; P = ns). Immunohistochemistry for VEGF revealed intense staining of the epithelium of medium- and large-sized airways for both control and hypertensive animals (Fig. 3). Staining for VEGF was also seen in the PA endothelial and smooth muscle cells of control animals but appeared diminished after chronic intrauterine hypertension, particularly in smooth muscle cells.

Protocol 3. Effects of VEGF165 inhibition on pulmonary vascular structure and function. Inhibition of VEGF165 with EYE001 caused RVH and severe structural remodeling of the pulmonary vasculature. RVH, as expressed by the right ventricle to left ventricle plus septum weight ratio, was increased by 26% in the
EYE001-treated animals compared with control lambs (0.70 ± 0.03 vs. 0.55 ± 0.01, EYE001 vs. control; \( P < 0.005 \); Fig. 4). Lung histology revealed smooth muscle cell hyperplasia of distal PAs (Fig. 5A) and a 35% increase in wall thickness after treatment with EYE001 (69 ± 2 vs. 51 ± 2, EYE001 vs. control; \( P < 0.005 \); Fig. 4). In addition, barium arteriograms revealed decreased filling of small distal PAs and narrowing of conduit vessels after 7 days of EYE001 treatment (Fig. 5B). RACs were not different after EYE001 treatment (8.78 ± 0.9 vs. 6.75 ± 0.1; EYE001 treatment vs. control; \( P = 0.14 \)), and pulmonary vessel density was also unchanged (26 ± 1 vessels per hpf vs. 28 ± 2; EYE001 treatment vs. control; \( P = 0.39 \)).

**Hemodynamic effects.** Fetal Q_LPA, PAP, AoP, PVR, pH, Pa CO₂, and Pa O₂ were not different between EYE001 treatment and control groups at baseline (Table 1). After 7 days of treatment, EYE001 caused a 22% elevation in PAP (50 ± 2 vs. 41 ± 1; \( P < 0.005 \)) and a 50% increase in PVR (0.92 ± 0.07 vs. 0.61 ± 0.07; \( P < 0.005 \)) when compared with control animals (Fig. 6). Compared with baseline values, PAP and PVR were elevated at day 7 in EYE001-treated lambs, but not controls. Other baseline hemodynamic values, including Q_LPA, AoP, and arterial blood gas tensions were not different between groups at day 1 or day 7 (Table 1).

At baseline, ACh, an endothelium-dependent agonist, caused a similar fall in PVR for the two study groups, reducing PVR by 50 ± 10% and 59 ± 4% in the EYE001 and control groups, respectively (\( P = \text{ns} \); Fig. 7). After 7 days, the vasodilator response to ACh was absent in the EYE001 group but persisted in the control animals (−4 ± 4% change in PVR on day 7 EYE001 vs. −48 ± 8% change in control on day 7; \( P < 0.01 \)). In contrast, the vasodilator response to 8-Br-cGMP, an endothelium-independent agonist, remained intact after EYE001 treatment (−44 ± 11% change in PVR on day 7 in EYE001 vs. −31 ± 10% change in PVR on day 7 in control; Fig. 7).

**Effects of EYE001 on lung eNOS protein content.** Western blot analysis revealed a 48% reduction in lung eNOS protein content after treatment with EYE001 (\( P < 0.005 \); Fig. 8). In contrast, lung PECAM expression, an endothelial cell marker, was not different after treatment with EYE001 (19,750 ± 1,495 densitometry units vs. 17,220 ± 2,565 densitometry units; control vs. EYE001; \( P = 0.40 \)).
Fig. 5. Effects of EYE001 on lung vascular structure in the late-gestation fetus. A: histology demonstrates marked increase in muscularization of small pulmonary arteries (arrows) in EYE001-treated fetal lambs compared with control lambs (×64 magnification). B: angiograms show reduced filling of distal arteries and narrowing of conduit arteries after EYE001 treatment.

Table 1. Hemodynamic and arterial blood gas parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 1</th>
<th>Day 7</th>
<th>EYE001</th>
<th>Day 1</th>
<th>Day 7</th>
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<tr>
<td>Q_{LPA}, ml/min</td>
<td>78 ± 25</td>
<td>58 ± 8</td>
<td>67 ± 10</td>
<td>66 ± 7</td>
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<td>PAP, mmHg</td>
<td>42 ± 2</td>
<td>44 ± 2</td>
<td>41 ± 1</td>
<td>50 ± 2*</td>
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<td>AOP, mmHg</td>
<td>41 ± 2</td>
<td>44 ± 2</td>
<td>40 ± 1</td>
<td>44 ± 2</td>
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<tr>
<td>PVR, mmHg·ml⁻¹·min⁻¹</td>
<td>0.45 ± 0.06</td>
<td>0.62 ± 0.13</td>
<td>0.61 ± 0.07</td>
<td>0.92 ± 0.07#</td>
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<tr>
<td>pH</td>
<td>7.39 ± 0.01</td>
<td>7.37 ± 0.02</td>
<td>7.36 ± 0.01</td>
<td>7.35 ± 0.01</td>
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<tr>
<td>PaO₂, mmHg</td>
<td>20.5 ± 1.0</td>
<td>19.3 ± 2.1</td>
<td>19.7 ± 1.1</td>
<td>17.2 ± 1.3</td>
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<td>PaCO₂, mmHg</td>
<td>43 ± 2</td>
<td>41 ± 3</td>
<td>45 ± 3</td>
<td>45 ± 2</td>
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Values are means ± SE. Hemodynamic and arterial blood gas parameters at baseline and after EYE001 treatment. Q_{LPA}, left lung blood flow; PAP, pulmonary artery pressure; AOP, aortic blood pressure; PVR, pulmonary vascular resistance; PaO₂, arterial PO₂; *#P < 0.005 vs. control day 7 and vs. EYE001 treatment day 1.
DISCUSSION

VEGF plays a critical role in normal lung vascular growth and has been extensively studied during the embryonic period. However, little is known about its functional roles or mechanisms that regulate its expression in the fetal pulmonary circulation during late gestation, or whether impaired VEGF activity can contribute to the pathophysiology of PPHN. We report that lung VEGF protein expression peaks before birth and decreases during the early postnatal period in the normal lung. We also report that hemodynamic forces, specifically intrauterine hypertension, prevent the rise in lung VEGF expression during late gestation, resulting in a marked (79%) reduction in lung VEGF protein content. To investigate whether impaired VEGF expression could cause changes in the fetal pulmonary circulation that mimic PPHN, we studied the effects of fetal treatment with EYE001, an aptamer that selectively inhibits VEGF165 (35, 38, 45). EYE001 treatment elevated PVR and PAP and caused increased RVH with muscularization of distal PAs after 7 days. We also found that EYE001 treatment reduced lung eNOS protein content and preferentially attenuated endothelium-dependent vasodilation in the perinatal lung. Together, these findings provide new evidence that, in addition to its role in vascular growth during the embryonic period, VEGF signaling maintains endothelial cell function and normal vascular structure during late gestation.

PPHN is a clinical syndrome characterized by abnormal vascular growth, structure, and reactivity, leading to sustained elevation in PVR at birth. Because VEGF has an important role in the development and maintenance of normal vascular structure and function early in development, we considered impaired VEGF activity a likely candidate for many of the pathological and pathophysiological changes that develop late in gestation in PPHN. Our study is the first report that shows marked reduction in lung VEGF expression in an experimental model of PPHN. In support of our findings, Lassus et al. (25) have also recently described markedly decreased circulating plasma levels of VEGF in human infants with PPHN. In addition, this work further demonstrates that hemodynamic forces, such as hypertension, independent of changes in oxygen tension or other stimuli, can downregulate VEGF expression.
pression in the developing lung. Furthermore, we have shown that selective inhibition of the VEGF165 isoform causes structural and functional evidence of pulmonary hypertension.

Past studies with models of adult pulmonary hypertension induced by chronic hypoxia or monocrotaline (7, 36, 43) have demonstrated conflicting effects on lung VEGF expression. Both in vivo and ex vivo experiments have demonstrated increased lung VEGF expression after chronic hypoxia-induced pulmonary hypertension in the adult rat (7, 43). In contrast, monocrotaline-induced pulmonary hypertension, which is characterized by intense lung inflammation, causes a marked and sustained fall in lung VEGF mRNA content (36). In our model of fetal pulmonary hypertension caused by chronic DA compression, PA02 is not different from the low PA02 of the normal fetus, and histological signs of inflammation are not present. In this model of intrauterine pulmonary hypertension, hemodynamic stress caused by partial ligation of the DA increases PAP without sustained increases in flow (2). Although the exact mechanism by which hypertension decreases lung VEGF expression is unclear, this study shows that high pressure, independent of hypoxia, can impair VEGF expression. Recent studies have shown that cyclic stretch can modulate VEGF gene expression in isolated vascular smooth muscle cells in vitro (42). Because VEGF protein is found within the vasculature as well as airway epithelium, intravascular hemodynamic stress may have a more profound effect on VEGF expression and activity than previously presumed. Alternatively, hypertension may alter the production of mediators that affect VEGF expression, such as nitric oxide (NO) (40, 44). NO is a positive regulator of VEGF expression in smooth muscle cells (16), and since lung eNOS expression is reduced in this model of PPHN (40, 44), we speculate that reduced NO production may contribute to impaired VEGF expression caused by hypertension.

Inhibition of VEGF165 may cause pulmonary hypertension by impaired endothelial cell function. In addition to its role in stimulating endothelial cell growth and proliferation, VEGF also acts as an endothelial cell survival factor by inhibiting apoptosis (19, 20, 30) and by acting as a maintenance, or “trophic factor,” to preserve endothelial cell function (15). Furthermore, VEGF upregulates eNOS and prostacyclin expression in vitro and in the postnatal systemic circulation (23, 33), which are critical for the regulation of normal vascular tone and reactivity. In this study, we show that inhibition of VEGF decreased eNOS protein expression and impaired endothelium-dependent vasodilation in the late fetal pulmonary circulation. These findings suggest that VEGF maintains eNOS function in the fetal lung before birth and that impaired VEGF expression contributes to sustained increases in PVR after birth in PPHN.

VEGF inhibition may also cause pulmonary hypertension by directly impairing distal pulmonary vessel growth, as suggested by barium angiograms, thereby interrupting normal ligand-receptor signaling. However, Western blot analysis revealed no change in lung PECAM expression, a marker of endothelial cells. PECAM is, however, independently regulated, and the direct or indirect effects of VEGF inhibition on PECAM expression are unknown. Another possible mechanism by which VEGF inhibition causes vascular remodeling is through its direct effects on smooth muscle cell growth. Both eNOS and VEGF have been shown to inhibit smooth muscle cell proliferation (17, 24). Because chronic intrauterine hypertension impairs production of both of these mediators, the loss of the “braking effect” that eNOS and VEGF have on smooth muscle cell proliferation ultimately could lead to many of the structural and functional changes seen in PPHN.

Past studies of fetal and neonatal mouse lung have shown that VEGF expression increases with maturation and is upregulated just before birth (5, 11). Human lung explant studies also reveal an increase in VEGF mRNA and protein levels in distal airway epithelium as development progresses (3). Few studies have examined the developmental expression of VEGFRs during gestation; however, one study demonstrated a decline in mouse VEGF-2 (Flik-1) mRNA expression as gestation progressed (5). These maturational studies are consistent with our findings, demonstrating an increase in lung VEGF expression during the alveolar stage of lung development just before birth, at a time when the vascular bed has already predominantly been established. This suggests that VEGF not only plays a critical role in promoting normal vascular growth but also preserves vascular function during late gestation.

There are several possible limitations of this study. First, the antibody for VEGF used in this study may react with several VEGF isoforms. The results of our Western blot analysis, however, revealed a single band for VEGF at ~17 kDa, which has been previously described for VEGF165 in sheep (14, 37). We believe that the 17-kDa band represents the VEGF165 isoform based on previous studies in our laboratory demonstrating that the 17-kDa band comigrates with a control sample of recombinant human VEGF165 and that addition of a blocking peptide to the VEGF antibody resulted in the loss of expression of the 17-kDa band. In addition, although our study does not directly demonstrate the selective effects of the aptamer EYE001 on VEGF expression, several studies have clearly demonstrated that its effects are selective for the VEGF165 isoform (35, 38, 45). Because aptamers bind to the specific three-dimensional arrangement of complementary contact sites between protein and receptor, and because these specific binding sites are not expressed by other proteins, these compounds are very specific to their targets (45). Finally, hypertension decreased lung VEGF protein expression but increased lung VEGF-2 protein content. Although the exact mechanisms that caused opposing changes in VEGF and VEGF-2 expression are unknown, this effect may be caused by impaired NO production since NO negatively regulates VEGF-2 expression (41).
Past studies of the developing lung have primarily focused on the role of VEGF during the embryonic period of lung development. Our studies demonstrate that VEGF undergoes maturation-related changes in expression during normal pulmonary development, peaking just before birth and decreasing rapidly in the early postnatal period. Chronic pulmonary hypertension decreases lung VEGF protein expression in late gestation, and this effect is not dependent on changes in oxygen tension. Furthermore, inhibition of the VEGF165 isoform causes perinatal pulmonary hypertension and this effect is not dependent on changes in oxygen tension. Additionally, inhibition of the VEGF165 isoform causes perinatal pulmonary hypertension, manifested by structural vascular remodeling, elevated PVR and PAP, and impaired endothelial cell vasodilation. These findings suggest that VEGF plays an important role in fetal pulmonary vascular development under both normal and pathological conditions and that VEGF is critical for maintaining normal pulmonary vascular function during late gestation. We speculate that VEGF may modulate pulmonary vascular changes contributing to the pathogenesis of PPHN.

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