rhVEGF treatment preserves pulmonary vascular reactivity and structure in an experimental model of pulmonary hypertension in fetal sheep

Theresa R. Grover, Thomas A. Parker, Neil E. Markham, and Steven H. Abman. rhVEGF treatment preserves pulmonary vascular reactivity and structure in an experimental model of pulmonary hypertension in fetal sheep. *Am J Physiol Lung Cell Mol Physiol* 289: L315–L321, 2005. First published April 15, 2005; doi:10.1152/ajplung.0038.2005.—We have previously shown that lung VEGF expression is decreased in a fetal lamb model of PPHN and that VEGF165 inhibition causes severe pulmonary hypertension in fetal lambs. Therefore, we hypothesized that treatment with rhVEGF165 would preserve endothelium-dependent vasodilation and reduce the severity of pulmonary vascular remodeling in an experimental model of PPHN. We studied the effects of daily intrapulmonary infusions of rhVEGF after partial ligation of the ductus arteriosus (DA). We performed surgery in 24 late-gestation fetal lambs and placed catheters in the main pulmonary artery, left atrium, and aorta for pressure measurements and in the left pulmonary artery for drug infusions. A pressure transducer was placed around the LPA to measure blood flow to the left lung (Qp), and the DA was surgically constricted to induce pulmonary hypertension. rhVEGF165 or vehicle was infused for 7 or 14 days. ACh or 8-BrcGMP was infused on days 2 and 13 to assess endothelium-dependent and -independent vasodilatation, respectively. ACh-induced vasodilation was reduced in PPHN lambs after 14 days (change in Qp from baseline, 106% vs. 11%). In contrast, the response to ACh was preserved in lambs treated with rhVEGF (change in Qp, 94% vs. 90%). Pulmonary vasodilation to 8-BrcGMP was not altered in PPHN lambs or enhanced by VEGF treatment. rhVEGF treatment increased expression of lung eNOS protein and decreased pulmonary artery wall thickness by 34% vs. PPHN lambs. We conclude that VEGF165 preserves endothelium-dependent vasodilation, upregulates eNOS expression, and reduces the severity of pulmonary vascular remodeling in experimental PPHN.

PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN (PPHN) is a clinical syndrome characterized by failure of pulmonary vascular resistance (PVR) to fall at birth, resulting in right to left shunting across the ductus arteriosus (DA) and foramen ovale, leading to severe hypoxemia (2, 17, 22, 27, 27). The pulmonary circulation in PPHN is characterized by endothelial dysfunction, which results in hemodynamic changes, such as elevated PVR and impaired endothelium-dependent pulmonary vasodilation (26, 27), in addition to impaired lung endothelial nitric oxide synthase (eNOS) expression and smooth muscle cell hyperplasia (2, 17, 22, 27, 33). Experimental models of PPHN have shown that altered expression of vasoactive mediators such as eNOS, endothelin-1, and vascular endothelial growth factor (VEGF) may disrupt normal vascular growth and structure, impair vascular tone and reactivity, and contribute to the pathogenesis of PPHN (1, 10, 33, 35).

VEGF is a potent endothelial cell mitogen with angiogenic and vasodilator properties (6, 21, 29). VEGF exists as five isoforms (121, 145, 165, 189, and 206 amino acid isoforms) generated by alternate splicing of a single gene (7, 21). VEGF165, the most abundant form in most human tissues, is the most potent of the isoforms (16). VEGF is critical to early vascular development since gene ablation of a single allele of VEGF in the mouse prevents normal vascular development and is lethal in the early embryonic period (5, 8). In addition to its role in vascular growth, VEGF signaling also modulates endothelial cell survival and function (12, 25). In particular, VEGF treatment upregulates eNOS in endothelial cells in vitro (18) and improves blood flow in diverse in vivo circulations (3). We have previously described that recombinant human (rh)VEGF causes potent nitric oxide (NO)-dependent vasodilation in the perinatal pulmonary circulation (10), although the role of VEGF-mediated vasodilation in pulmonary hypertension has not been studied.

Because VEGF is critical for maintenance of pulmonary vascular tone, impaired VEGF signaling has been implicated as a contributor to the pathophysiological changes seen in PPHN. We have previously shown that lung VEGF protein expression is decreased by 75% in an experimental model of PPHN in fetal lambs caused by chronic constriction of the DA (10). In addition, we treated fetal lambs with a specific VEGF inhibitor (EYE001, a VEGF aptamer) and demonstrated that VEGF inhibition impaired endothelium-dependent vasodilation, decreased eNOS expression, caused right ventricular hypertrophy (RVH), and increased muscularization of small pulmonary arteries (10). Although impaired VEGF expression contributes to the pathophysiological changes seen in PPHN, whether preservation of VEGF signaling may preserve endothelial function and attenuate these structural and functional abnormalities in PPHN has not been studied.

We hypothesized that treatment with intrapulmonary VEGF will preserve endothelium-dependent vasodilation, upregulate eNOS expression, and reduce the severity of pulmonary vascular remodeling in an experimental model of PPHN. To test this hypothesis, we studied the effects of treatment with rhVEGF165 on pulmonary vasodilation and vascular remodeling in an experimental model of PPHN in fetal lambs. We report that treatment with rhVEGF165 decreased pulmonary artery pressure (PAP), restored endothelium-dependent vasodilation, increased lung eNOS protein expression, and attenuated pulmonary vascular remodeling in fetal lambs. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Pulmonary hemodynamic measurements

**Table 1.** Pulmonary hemodynamic measurements

<table>
<thead>
<tr>
<th></th>
<th>PPHN</th>
<th>PPHN + VEGF</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td><strong>Qpa, ml/min</strong></td>
<td>day 1: 72 ± 1</td>
<td>day 13: 89 ± 5</td>
<td>day 1: 76 ± 20</td>
</tr>
<tr>
<td><strong>PAP, mmHg</strong></td>
<td>day 1: 59 ± 11</td>
<td>day 13: 84 ± 11*</td>
<td>day 1: 48 ± 9</td>
</tr>
<tr>
<td><strong>AoP, mmHg</strong></td>
<td>day 1: 47 ± 3</td>
<td>day 13: 53 ± 6</td>
<td>day 1: 37 ± 5</td>
</tr>
<tr>
<td><strong>LAP, mmHg</strong></td>
<td>day 1: 3.9 ± 1.0</td>
<td>day 13: 4.4 ± 0.4</td>
<td>day 1: 1.9 ± 0.4</td>
</tr>
<tr>
<td><strong>PVR, mmHg/ml/min</strong></td>
<td>day 1: 0.80 ± 0.1</td>
<td>day 13: 0.97 ± 0.2†</td>
<td>day 1: 0.60 ± 0.1</td>
</tr>
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</table>

Values are means ± SE. Qpa, blood flow to left lung; PAP, pulmonary artery pressure; AoP, aortic pressure; LAP, left atrial pressure; PVR, pulmonary vascular resistance; PPHN, persistent pulmonary hypertension of the newborn. *P < 0.05 PPHN day 13 vs. PPHN + VEGF day 13 and vs. control day 13. †P < 0.05 PPHN day 13 vs. PPHN + VEGF day 13. ‡P < 0.05 PPHN day 13 vs. PPHN + VEGF day 13 vs. control day 13.

This study consisted of two treatment protocols: protocol 1 (effect of rhVEGF treatment on fetal pulmonary hemodynamics), and protocol 2 (effect of rhVEGF treatment on pulmonary vascular remodeling).

Before surgery, animals were randomized to one of three treatment groups: DA ligation with rhVEGF treatment (PPHN + VEGF treatment) or vehicle control (PPHN) or sham surgery with vehicle control (control). Under inhalational isoflurane anesthesia (2–3%), a hysterotomy was performed, the fetal forelimb was exposed, and a left thoracotomy was performed. Polyvinyl catheters were placed in the left axillary artery and vein and advanced into the ascending aorta (Ao) and superior vena cava, respectively. Catheters were then placed in the left pulmonary artery (LPA), main pulmonary artery (MPA), and left atrium (LA) by direct puncture and secured by a purse-string suture. In 18 animals, a cotton umbilical tie was placed around the DA and tightened around a right angle surgical instrument to partially constrict the DA in a uniform manner. Six age-matched control animals underwent the same surgery, including isolation of the DA, but in these control animals, the DA was not ligated. An ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the LPA to measure blood flow to the left lung. An amniotic catheter was placed as a pressure referent for hemodynamic studies.

Animals were killed 7 or 14 days after surgery, and the left upper lobe was rapidly frozen in liquid nitrogen and stored at −70°C for molecular studies. Body weight, lung weight, and heart weights were obtained. The right lung was removed for wet-dry weight measurements. The remaining left lung was inflated through the trachea with 10% buffered formalin at a constant pressure of 35 cmH2O and stored in formalin. The degree of RVH was determined as a ratio of the weights of the right ventricle and left ventricle plus septum.

**Methods**

**Surgical Preparation**

All procedures and protocols were previously reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Surgery was performed on 24 mixed breed (Columbia-Rambouillet) pregnant ewes between 123 and 127 days gestation according to previously published methods (2).

Pulmonary artery pressure (PAP, A) and pulmonary vascular resistance (PVR, B) on day 1 and day 13 of treatment for persistent pulmonary hypertension of the newborn (PPHN), PPHN + VEGF, and control lambs. Recombinant human (rh)VEGF treatment prevented the progressive rise in PAP compared with untreated PPHN lambs. Values are means ± SE; *P < 0.05.

VEGF signaling may attenuate the pathophysiological changes seen in neonatal pulmonary hypertension by maintaining normal endothelial function.

Study Design

**Protocol 1:** effect of rhVEGF treatment on fetal pulmonary hemodynamics. Surgery was performed on 11 fetal lambs (123–128 days gestation) as described above, with animals randomized to one of three treatment groups (PPHN, PPHN + VEGF, or control). Beginning on the day of surgery, fetal lambs were treated with daily infusions of either rhVEGF (50 μg/day, delivered over 30 min into the LPA) or vehicle control (saline, 2 ml into the LPA) for 14 days. Baseline hemodynamic measurements (pulmonary blood flow (Qp), PAP, aortic pressure (AoP), left atrial pressure (LAP), and PVR) were obtained on days 1 and 13 after surgery, and arterial blood gas tensions (pH, PaO2, PaCO2, hemoglobin, and oxygen saturation) were recorded daily. Response to endothelium-dependent [acetylcholine (ACh), 15 μg] and endothelium-independent [8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP), 1.5 mg] vasodilator stimuli were measured on days 1 and 13 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 NO production in the fetal lamb (1, 26). Baseline hemodynamic measurements were obtained before ACh or 8-BrcGMP infusion and recorded every 10 min for 30 min after infusion or until hemodynamics returned to baseline.
Protocol 2: effect of rhVEGF treatment on pulmonary vascular remodeling. Surgery was performed on 13 fetal lambs (123–127 days gestation; term = 147 days) as described above. Beginning on the day of surgery, lambs were treated with daily infusions of either rhVEGF (5 or 50 μg/day, delivered over 30 min into the LPA) or vehicle control (saline, 2 ml into the LPA) for 7 days. At the conclusion of the treatment period, tissues were obtained for histological and molecular analysis. Hematoxylin and eosin (H&E) staining was performed on distal lung sections for histological examination and morphometric analysis of small pulmonary artery wall thickness. Medial wall thickness of pulmonary arteries was assessed on lung sections from both left and right lungs to determine whether drug delivery was selective to the left lung. Lung wet-to-dry ratios were measured to determine whether rhVEGF treatment increased pulmonary edema. Western blot analysis was performed for lung eNOS protein.

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 (23). PVR in the left lung was calculated by the following equation: 

\[
PVR (\text{mmHg} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}) = (\text{mean MPA pressure} - \text{mean LA pressure}) \div \text{LPA flow}.
\]

Arterial blood gas tensions, pH, hemoglobin, 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).

Measurements of RVH. At death, hearts from fetal lambs were removed and dissected to isolate the free wall of the right ventricle from the left ventricle and septum. The ratio of right ventricle weight to left ventricle plus septum weight (RV/LV+S) was used as an index of RVH.

Wet-to-dry ratios. Lung wet-to-dry ratios were measured using the right lung, which was blotted dry, weighted (wet weight), desiccated at 50°C, and weighed daily until three consecutive weights were unchanged (dry weight).

Histological preparation. 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.

Morphometric analysis. Morphometry was performed on small pulmonary arteries associated with terminal bronchioles (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 percentage wall thickness was calculated as (2\times \text{wall thickness})/\text{external diameter}.

Table 2. Arterial blood gas, hemoglobin concentration, and oxygen saturation measurements

<table>
<thead>
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<th></th>
<th>PPHN</th>
<th>PPHN + VEGF</th>
<th>Control</th>
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<tbody>
<tr>
<td><strong>pH</strong></td>
<td>7.39±0.01</td>
<td>7.33±0.01</td>
<td>7.36±0.01</td>
</tr>
<tr>
<td><strong>PaO₂, mmHg</strong></td>
<td>21±1</td>
<td>20±2</td>
<td>22±1</td>
</tr>
<tr>
<td><strong>PaCO₂, mmHg</strong></td>
<td>45±1</td>
<td>49±3</td>
<td>49±2</td>
</tr>
<tr>
<td><strong>Hb, g/dL</strong></td>
<td>7.2±1</td>
<td>6.7±0.2</td>
<td>7.1±1</td>
</tr>
<tr>
<td><strong>% Oxygen saturation</strong></td>
<td>60±6</td>
<td>55±5</td>
<td>54±5</td>
</tr>
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Values are means ± SE. PaO₂, arterial PO₂; PaCO₂, arterial PCO₂; Hb, hemoglobin concentration. *P < 0.05 PPHN + VEGF day 13 vs. control day 13.

Fig. 2. Hemodynamic response to endothelium-dependent stimulus (ACH, 15 μg) measured on day 1 and day 13 in PPHN, PPHN+VEGF, and control lambs. Change in pulmonary blood flow (Qp; A) and PVR (B) after ACh infusion. *P < 0.05, #P < 0.01, †P < 0.005.

Fig. 3. Hemodynamic response to endothelium-independent stimulus [8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP), 1.5 mg] measured on day 1 and day 13 in PPHN, PPHN+VEGF, and control lambs. Change in Qp (A) and PVR (B) after 8-BrcGMP infusion. P = not significant.
Protein assay was performed using the Bradford method and 25% wall thickness was determined as described above. Four to seven animals from each study group were examined, and 10 vessels were measured for each animal by a blinded observer. Additional sections from both left and right lungs were examined, and percentage wall thickness was determined as described above.

Western blot analysis. Western blot analysis for lung eNOS protein was performed according to previously published methods (20). Protein assay was performed using the Bradford method and 25 μg of lung protein loaded per lane. Samples were run on a Bis-Tris 4–12% polyacrylamide gel, using Rainbow (Amersham Biosciences, Piscataway, NJ) marker to determine molecular weight. After transfer, the blot was stained with Ponceau S to ensure consistent loading and transfer. Immunodetection was performed with a mouse monoclonal IgG antibody to eNOS (Transduction Laboratories, Lexington, KY) and a rabbit polyclonal IgG secondary antibody. ECL Plus (Amer sham, Arlington Heights, IL) detection was performed, and luminescence was determined by exposure to X-ray film for 20 s. Blots were also probed for β-actin (Sigma, St. Louis, MO), and densitometry values for eNOS are presented as normalized to β-actin. Densitometry was performed with a scanner and NIH ImageJ software.

Drug preparation. Stock solution of rhVEGF,50 (generously supplied by Genentech, San Francisco, CA) was prepared by diluting in sterile saline at a concentration of 50 μg/ml. The drug was diluted in normal saline for a total volume of 2 ml and infused over 30 min into the LPA catheter (5 or 50 μg daily for 7 or 14 days). Control animals received an infusion of normal saline (2 ml daily for 7 or 14 days). The dose of rhVEGF was chosen based on preliminary data demonstrating selective pulmonary vasodilation without adverse systemic hemodynamic effects (50 μg).

ACh (Sigma Chemicals) was dissolved in sterile saline and infused at a dose of 15 μg over 10 min (total vol 2 ml) in the LPA catheter. 8-BrGMP (Sigma Chemicals) was dissolved in sterile saline and infused at a dose of 1.5 mg over 10 min in the LPA.

Baseline PAP was not different on the day after surgery (day 1) for lambs in each study group (59 ± 1, PPHN; 48 ± 9, PPHN+VEGF; and 47 ± 3, control; Fig. 1A, Table 1). Mean PAP progressively increased in PPHN lambs by day 13 (84 ± 11), but treatment with rhVEGF prevented the increase in mean PAP (52 ± 11, PPHN+VEGF; P < 0.05 vs. PPHN, day 13). Mean PAP after rhVEGF treatment was similar to control lambs (46 ± 4, control; P < 0.05 vs. PPHN, day 13, Fig. 1A).

PVR was not different on the day after surgery but was increased on day 13 in PPHN lambs when compared with controls (0.97 ± 0.2, PPHN vs. 0.44 ± 0.1, control; P < 0.05; Fig. 1B, Table 1). PVR was not different on day 13 in rhVEGF-treated PPHN lambs when compared with controls (0.74 ± 0.2 PPHN+VEGF vs. 0.44 ± 0.1, control; Table 1) or compared with PPHN lambs (0.74 ± 0.2 PPHN+VEGF vs. 0.97 ± 0.2 PPHN).

Qp and AoP were not different on the day after surgery or after treatment for any of the study groups (Table 1). LAP was lower on day 13 of VEGF treatment compared with PPHN lambs (P < 0.05; Table 1).

There was a decrease in arterial pH after chronic intrauterine pulmonary hypertension in VEGF-treated lambs when compared with controls, although PaO2, PaCO2, hemoglobin, and oxygen saturation were not different (Table 2).

Endothelium-dependent vasodilation to ACh was reduced in PPHN lambs after 13 days (change in Qp from baseline, 92 ± 27% PPHN day 1 vs. 10 ± 7% PPHN day 13; P < 0.05, Fig. 2A). In contrast, the response to ACh was preserved in PPHN lambs treated with rhVEGF. We found no change in the vasodilation response to ACh over time in PPHN lambs treated with rhVEGF [change in Qp, 94 ± 24% PPHN+VEGF day 1 vs. 102 ± 23% PPHN+VEGF day 13; P = not significant (ns)]. Additionally, the ACh response on day 13 was higher in rhVEGF-treated PPHN lambs compared with untreated PPHN lambs (change in Qp, 102 ± 23% PPHN+VEGF vs. 10 ± 7%
**Table 3. Morphometric measurements**

<table>
<thead>
<tr>
<th></th>
<th>PPHN</th>
<th>PPHN + VEGF5</th>
<th>PPHN + VEGF50</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>3,446±310</td>
<td>3,385±266</td>
<td>3,008±218*</td>
<td>4,478±343</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>106±11</td>
<td>90±11</td>
<td>91±9</td>
<td>142±14*</td>
</tr>
<tr>
<td>Lung wt/body wt</td>
<td>0.031±0.001</td>
<td>0.028±0.005</td>
<td>0.030±0.002</td>
<td>0.032±0.002</td>
</tr>
<tr>
<td>Lung wet/dry wt</td>
<td>8.9±0.4</td>
<td>7.3±0.1*</td>
<td>8.8±0.2</td>
<td>8.8±0.2</td>
</tr>
<tr>
<td>RV/LV + S</td>
<td>0.74±0.05</td>
<td>0.74±0.02</td>
<td>0.72±0.05</td>
<td>0.57±0.03*</td>
</tr>
<tr>
<td>LV + S weight, g</td>
<td>13.8±2</td>
<td>14.7±1</td>
<td>12.3±2</td>
<td>16.8±2</td>
</tr>
<tr>
<td>Vessel density (no. per hpf)</td>
<td>22±2</td>
<td>20±1</td>
<td>22±2</td>
<td>25±1</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.01 vs. control; †P < 0.05 vs. all groups. RV/LV + S, right ventricle weight to left ventricle plus septum weight. PPHN + VEGF 5, PPHN + VEGF 5 µg/day; PPHN + VEGF 50, PPHN + VEGF 50 µg/day.

PPHN; P < 0.01). Although PPHN lambs had impaired vasodilation to ACh on day 13 when compared with controls (change in Qp, 10 ± 7% PPHN day 13 vs. 213 ± 46% control day 13; P < 0.01), the response to ACh in rhVEGF-treated PPHN lambs was not different from control animals on day 13 (change in Qp, 102 ± 23% PPHN+VEGF vs. 213 ± 46% control day 13; P = ns; Fig. 2A).

ACh decreased PVR in PPHN lambs, although the response was attenuated on day 13 (change in PVR from baseline, −44 ± 7% PPHN day 1 vs. −20 ± 8% PPHN day 13; P < 0.05; Fig. 2B). In contrast, the fall in PVR after ACh infusion was preserved in PPHN lambs treated with rhVEGF and in control animals on day 13 (Fig. 2B). Endothelium-independent vasodilation to 8-BrcGMP remained intact in all study groups, with similar changes in Qp (Fig. 3A) and PVR (Fig. 3B) on day 1 and at the conclusion of the study period (day 13).

**Protocol 2: Effect of rhVEGF Treatment on Pulmonary Vascular Remodeling**

Treatment with daily intrapulmonary infusions of rhVEGF protein decreased pulmonary vascular remodeling in this experimental model of PPHN. Histological examination revealed a marked decrease in medial wall thickening and smooth muscle cell hyperplasia in small pulmonary arteries after VEGF treatment (Fig. 4). Percent wall thickness of small pulmonary arteries was decreased in PPHN lambs after rhVEGF treatment in a dose-dependent fashion, reaching values equivalent to sham surgery controls (P < 0.001 PPHN vs. PPHN+VEGF5, vs. PPHN+VEGF50, and vs. control; P < 0.01 PPHN+VEGF5 vs. PPHN+VEGF50; Fig. 5). The percent wall thickness was similar in left and right lungs treated with rhVEGF 50 µg/day (50 ± 1% left lung vs. 48 ± 2% right lung) despite drug delivery selectively to the left lung.

RVH (RV/LV+S weight) was increased in PPHN lambs and rhVEGF-treated PPHN lambs when compared with controls (Table 3; P < 0.05). In addition, LV+S weights were unchanged after rhVEGF treatment (Table 3).

Lung weights were lower in PPHN and VEGF-treated PPHN lambs when compared with controls (P < 0.05, Table 3). PPHN lambs treated with 50 µg/day of VEGF also had reduced body weight, although lung weight:body weight ratios were not different among the groups (Table 3). Lung wet: dry ratios were decreased after rhVEGF treatment (5 µg/day; P < 0.05 vs. all groups) and were otherwise similar for all groups (Table 3), suggesting that rhVEGF treatment did not increase pulmonary edema.

Western blot analysis demonstrated a dose-dependent increase in lung eNOS protein expression after rhVEGF treatment in PPHN lambs (P < 0.05, PPHN vs. PPHN+VEGF50, control vs. PPHN+VEGF5, and control vs. PPHN; Fig. 6).

**DISCUSSION**

We report that daily intrapulmonary treatment with rhVEGF improved pulmonary hemodynamics, increased lung eNOS protein expression, and decreased pulmonary vascular remodeling in an experimental model of PPHN induced by in utero constriction of the DA. Chronic rhVEGF treatment decreased baseline PAP after DA ligation, restored endothelium-dependent vasodilation to ACh, and upregulated lung eNOS protein. Histological examination revealed decreased remodeling of the pulmonary vasculature, and medial wall thickening of small pulmonary arteries was decreased in a dose-dependent fashion following VEGF treatment. These findings support our hypothesis that impaired VEGF expression contributes to the pathophysiological changes of PPHN and that preservation of endothelial function by VEGF attenuates pulmonary hypertension in fetal lambs.

This is the first study to demonstrate a physiological benefit of VEGF treatment on the perinatal pulmonary circulation in an experimental model of PPHN. VEGF is critical for normal pulmonary vascular growth and maintenance of endothelial survival and function (5, 8, 25). During late fetal life, VEGF expression in the lung increases, with a surge in pulmonary VEGF just before birth (10). Fetal lambs with chronic pulmonary hypertension induced by DA ligation have a marked reduction in lung VEGF expression during this time (10), likely contributing to endothelial dysfunction and vascular remodeling characteristic of this condition. Our previous studies in this model have also demonstrated that inhibition of
rhVEGF IMPROVES PPHN

VEGF during late stages of pulmonary development using a VEGF aptamer (EYE001) (10) or a VEGF receptor antagonist (SU-5416) (19) causes structural remodeling of pulmonary vessels, induces RVH, and impairs endothelium-dependent pulmonary vasodilation. We report that treatment with rhVEGF preserves endothelial function, attenuates many of these pathophysiological changes, and results in less severe pulmonary hypertension.

Clinical studies in adults with ischemic limb disease and myocardial infarction have shown therapeutic benefit of VEGF treatment (3, 4, 28, 31), albeit with some serious side effects observed (14, 15, 32). Patients treated with high doses of VEGF (500–1,000 µg) for these conditions showed improved blood flow, new collateral blood vessel growth, and higher regional blood pressure (4, 28, 31). However, significant side effects included systemic hypotenion (14, 15) and edema at the treatment site (15, 32). Animal studies have shown that much lower doses of VEGF are required to improve endothelial cell function and vasodilation and have fewer side effects (11, 13, 24). Our study utilized these lower doses of VEGF and demonstrated improved endothelium-dependent vasodilation without systemic hypotenion or pulmonary edema. Local delivery of low-dose VEGF mediates protective vascular effects by improving endothelial function within the pulmonary circulation of fetal lambs with chronic pulmonary hypertension.

Although we have demonstrated that VEGF treatment attenuates pulmonary vascular remodeling and endothelial dysfunction in an experimental model of PPHN, the mechanisms underlying these findings are not entirely clear. We have previously shown that VEGF directly stimulates vasodilation in the perinatal pulmonary circulation through stimulation of phosphatidylinositol 3-kinase and subsequent NO release (11). The increase in lung eNOS protein expression after VEGF treatment in lambs with pulmonary hypertension represents improved endothelial function, as also demonstrated by preservation of endothelium-dependent vasodilation and decreased PAP and subsequently decreased pulmonary vascular remodeling. However, whether upregulation of eNOS directly improved pulmonary vasodilation and structural remodeling is uncertain. VEGF also independently acts as an endothelial cell survival factor and inhibits endothelial cell apoptosis (12) and may have acted directly on the endothelium to mediate the improved vasodilation response that we observed. In addition, VEGF inhibits smooth muscle cell growth and proliferation through upregulation of NO (9), which may have contributed to the lack of pulmonary vascular remodeling in our VEGF-treated lambs with pulmonary hypertension. Furthermore, VEGF has been shown to increase PG12 expression (34), and endothelin-1 can alter VEGF expression in vitro (30). Effects on these and other vascular mediators may have contributed to the findings of attenuated pulmonary hypertension in our study.

There are several possible limitations to this study. Despite a marked improvement in wall thickness of pulmonary arteries, PAP, and endothelium-dependent vasodilation, we did not observe an improvement in RVH in VEGF-treated lambs with PPHN. Although delivery of VEGF was directly into the left lung, we found similar reductions in remodeling of pulmonary vessels in the right lung of these animals. This suggests that the drug was delivered systemically and may have had systemic effects, although we did not see changes in aortic blood pressure after VEGF treatment. In addition, VEGF may have had indirect effects such as stimulation and mobilization of bone marrow-derived endothelial progenitor cells, which have the potential to improve pulmonary vascular function. It is also feasible that due to our dosing strategy of daily infusions rather than continuous delivery, the serum concentrations of VEGF varied between doses and therefore caused fluctuations in PAP during the treatment period. However, the lack of vascular remodeling in the VEGF-treated lungs suggests that PAP was not elevated for a prolonged period. Whether higher doses or more prolonged treatment periods would have reduced RVH is uncertain but is an area that requires further study.

We conclude that chronic treatment with rhVEGF after intrauterine pulmonary hypertension decreases basal PAP, restores endothelium-dependent vasodilation, increases lung eNOS protein expression, and decreases medial wall thickness of small pulmonary arteries. We speculate that VEGF plays a protective role in maintaining endothelial cell function in the setting of chronic pulmonary hypertension and that VEGF may be a potentially useful adjuvant therapy for neonatal pulmonary hypertension. However, further studies are needed to determine the optimum dosing and delivery strategies of VEGF and to determine whether VEGF treatment will prove useful as a therapeutic option for established pulmonary hypertension in newborns.

ACKNOWLEDGMENTS

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GRANTS

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