

Vascular Endothelial Growth Factor Causes Pulmonary Vasodilation through Activation of the Phosphatidylinositol-3-Kinase–Nitric Oxide Pathway in the Late-Gestation Ovine Fetus

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ABSTRACT

Vascular endothelial growth factor (VEGF) causes vasodilation in adult models of peripheral vascular disease and myocardial ischemia through the acute release of nitric oxide (NO). However, the hemodynamic effects of VEGF and its effects on NO production have not been studied in the developing lung circulation. We hypothesized that VEGF causes fetal pulmonary vasodilation, and that its actions are mediated through the release of endogenous NO. We performed surgery in 16 fetal lambs (125–135 d gestation; term = 147 d), and placed catheters in the main pulmonary artery, aorta, and left atrium to measure pressures. An ultrasonic flow transducer was placed on the left pulmonary artery (LPA) to measure blood flow, and a catheter was placed in the LPA for local drug infusion. Pulmonary vascular resistance in the left lung was calculated as pulmonary artery pressure minus left atrial pressure divided by LPA flow. Fetal lambs were treated with brief infusions of recombinant human VEGF (dose, 0.5–2.0 μg) into the LPA. Recombinant human VEGF infusions acutely increased LPA flow by up to 3-fold ($p < 0.02$) and decreased pulmonary vascular resistance by 65% ($p < 0.05$) in a dose-related fashion, without affecting aortic pressure or heart rate. To determine the mechanism of VEGF-induced vasodilation, we studied the effects of nitro-L-arginine, an NO synthase inhibitor, and LY294002, a phosphatidylinositol-3-kinase inhibitor, on the response to VEGF. We found that pretreatment with either nitro-L-arginine or LY294002

completely inhibited the vasodilator response to recombinant human VEGF ($p < 0.005$). These findings suggest that recombinant human VEGF causes fetal pulmonary vasodilation, and that this response is likely mediated by the release of NO through activation of phosphatidylinositol-3-kinase. (*Pediatr Res* 52: 907–912, 2002)

Abbreviations

VEGF, vascular endothelial growth factor
NO, nitric oxide
NOS, NO synthase
MPA, main pulmonary artery
Ao, aorta
LA, left atrium
LPA, left pulmonary artery
PVR, pulmonary vascular resistance
MPAP, MPA pressure
AoP, Ao pressure
LAP, LA pressure
L-NA, nitro-L-arginine
PI-3-K, phosphatidylinositol-3-kinase
eNOS, endothelial NOS
Qp, pulmonary blood flow
BL, baseline
HR, heart rate

VEGF is a potent endothelial cell mitogen with angiogenic and vascular permeability properties (1–3). VEGF is critical to early vascular development inasmuch as gene ablation of a single allele of VEGF in the mouse prevents normal vascular development and causes early embryonic lethality (4, 5).

VEGF signaling not only stimulates vascular growth, but also modulates endothelial cell survival and function (6, 7). In particular, VEGF treatment up-regulates eNOS in endothelial cells *in vitro* (8) and improves systemic blood flow *in vivo* (9). Although VEGF is clearly essential for early lung vascular structure and growth, the effects of VEGF on pulmonary vascular tone and reactivity during late fetal life are unclear.

Exogenous VEGF administration causes vasodilation in both local and systemic adult vascular beds (10–12). Several novel and exciting therapeutic interventions have recently examined the role of VEGF in manipulation of angiogenesis and maintenance of endothelial function in adult models of vascu-

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lar disease, including its use in ischemic limb (13, 14) and myocardial disease (15, 16). These studies have demonstrated improved collateral blood vessel development and endothelium-dependent blood flow after local administration of VEGF. Hemodynamic studies in adult models have also shown that VEGF is a potent vasodilator (10–12), causing a rapid fall in vascular resistance and increased blood flow. Whether VEGF also causes vasodilation in the developing pulmonary circulation is unknown.

VEGF-induced vasodilation is mediated at least in part through acute release of NO. VEGF stimulates eNOS and inducible NOS mRNA and protein expression under a variety of conditions (8, 17). *In vivo* studies have shown that VEGF dramatically decreases systemic blood pressure and resistance and improves blood flow, and these changes are inhibited by pretreatment with NOS antagonists (10, 12, 18). Furthermore, VEGF stimulates NO release through phosphorylation of Akt (protein kinase B) by PI-3-K activation in both *in vitro* and *in vivo* models of adult systemic vascular disease (19–22). Whether VEGF causes NO-mediated vasodilation through this signal transduction pathway in the developing pulmonary circulation has not been studied.

Therefore, we hypothesized that VEGF is a vasodilator of the fetal pulmonary circulation, and that its actions are mediated through the release of endogenous NO. To address this question, we examined the pulmonary hemodynamic response to VEGF infusions and determined its mechanism of action in the fetal lung.

METHODS

Surgical Preparation

All procedures were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center (Denver, CO, U.S.A.). Sixteen mixed-breed (Columbia-Rambouillet) pregnant ewes between 125 and 135 d gestation were fasted for 48 h before surgery. Ewes were sedated with intramuscular Buprenex (0.6 mg) and i.v. ketamine (60 mg) and diazepam (10 mg), and intratracheally intubated. Ewes were anesthetized using inhaled isoflurane (2% to 3%), and remained sedated but breathed spontaneously throughout surgery. Under sterile conditions, the left forelimb of the fetal lamb was delivered through a uterine incision. A skin incision was made under the left forelimb after local infiltration with 1% lidocaine. Polyvinyl catheters were inserted into the axillary artery and advanced into the ascending Ao. A left axillary to sternal thoracotomy exposed the heart and great arteries. Polyvinyl catheters were inserted into the LPA, MPA, and LA by direct puncture and secured into position with purse-string sutures. A 6-mm ultrasonic flow transducer (Transonic Systems, Ithaca, NY, U.S.A.) was placed around the LPA to measure blood flow to the left lung (Qp). A catheter was placed in the amniotic cavity to serve as a pressure referent. The thoracotomy incision was closed in layers. The uteroplacental circulation was kept intact, and the fetus was gently replaced in the uterus. Ampicillin (500 mg) was added to the amniotic cavity before closure of the hyste-

rotomy. The ewe was allowed to recover from surgery for 48 h before fetal drug administration and hemodynamic studies.

Physiologic Measurements

The Ao, MPA, and LA catheters were connected to a computer-monitored pressure transducer and recorder (Biopac Systems, Santa Barbara, CA, U.S.A.). Pressures were referenced to amniotic pressures, and the pressure transducer was calibrated with a mercury manometer. The flow transducer cable was attached to an internally calibrated flowmeter (Transonic Systems) for continuous measurements of LPA flow. The absolute values of flow were determined from phasic blood flow signals as previously described (23). PVR in the left lung was calculated with the following equation: $PVR \text{ (mm Hg}\cdot\text{mL}^{-1}\cdot\text{min)} = (\text{mean MPAP} - \text{mean LAP})/Q_{LPA}$. Arterial blood gas tensions, pH, Hb, oxygen saturation, and methemoglobin 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 (model OSM-3, Radiometer, Copenhagen, Denmark).

Study Drugs

rhVEGF (R&D Systems, Inc., Minneapolis, MN, U.S.A.) was dissolved in PBS (1 mg/mL stock solution) and stored at 4°C. The required amount of solution was drawn up in a sterile syringe at room temperature at the time of study. The drug was infused into the LPA catheter for 1 min, and followed by infusion of 1 mL of saline.

L-NA (10 mg, Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in 2 mL of normal saline plus 2–3 drops HCl, and titrated with NaOH to achieve a pH of 7.4. The drug was infused into the LPA by an infusion pump set to deliver 10 mg for a 10-min period. L-NA was prepared immediately before drug administration, and the dose chosen was based on previous studies from our laboratory (24).

LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), a specific PI-3-K inhibitor (21) (Calbiochem, San Diego, CA, U.S.A.) was dissolved in DMSO (5 mg/mL stock solution), then diluted with normal saline for a final concentration of 0.5 mg/mL. The drug was infused into the LPA catheter for 1 min, and followed by 1 mL of saline infusion.

Experimental Design

Protocol 1: Hemodynamic effects of acute rhVEGF administration. The purpose of this protocol was to determine the effects of acute intrapulmonary administration of rhVEGF on fetal pulmonary hemodynamics. After a 48-h recovery period after surgery, BL hemodynamic measurements were recorded for Q_{LPA} , MPAP, AoP, LAP, and HR ($n = 6$). After BL measurements were stable for a 30-min period, rhVEGF (1 μg) was infused into the LPA for 1 min. Hemodynamic measurements were recorded every 10 min for an additional 30 min after drug administration. Arterial blood gas tensions were obtained before and approximately 5 min after drug administration.

A dose-response study was performed to determine the dosage needed for optimal response. After a stable BL period, rhVEGF was infused at one of several doses (0.5, 1, and 2 μg ; $n = 6$). Each dose was administered no more frequently than every 24 h because of concerns for tachyphylaxis. Each dose was infused for 1 min, and hemodynamic measurements recorded every 10 min for 30 min after drug administration. Dosage of drug administration was randomly assigned.

To determine whether lambs develop tachyphylaxis after repeat rhVEGF infusions, lambs were treated with serial doses of rhVEGF (1 μg ; $n = 4$). After BL measurements were obtained, lambs were treated with rhVEGF (1 μg), and hemodynamics were measured every 10 min for 30 min. A second and third dose (each 1 μg) were administered 30 min apart in the same manner after allowing pulmonary blood flow to return to BL values.

Protocol 2: Effects of NO inhibition on VEGF-induced pulmonary vasodilation. The purpose of this protocol was to determine whether VEGF-induced pulmonary vasodilation was mediated through NO production. After a stable BL period, rhVEGF (1 μg) was infused into the LPA ($n = 7$). Hemodynamic measurements were recorded every 10 min for 40 min. The following day, fetal lambs were treated with LNA (10 mg), infused for 10 min into the LPA catheter, followed by rhVEGF (1 μg). Hemodynamic measurements were recorded every 10 min for an additional 40 min.

Protocol 3: Effects of PI-3-K inhibition on VEGF-induced pulmonary vasodilation. The purpose of this protocol was to determine whether VEGF causes pulmonary vasodilation by activating PI-3-K. After BL measurements were recorded for 30 min, fetal lambs ($n = 4$) were treated with VEGF (1 μg) in the LPA, and hemodynamic measurements were recorded for 40 min. The following day, a specific PI-3-K inhibitor, LY294002 (2 mg) (21) was infused in the LPA, followed by rhVEGF (1 μg). Hemodynamic measurements were recorded every 10 min for an additional 40 min. Arterial blood gas tensions were obtained before and after drug administration. The dosage for LY294002 was based on previous *in vivo* studies (20, 21, 25).

Statistical Analysis

Statistical analysis of hemodynamic data was performed using one-way ANOVA. When significant differences were identified, *post hoc* analysis was performed with Student-Newman-Keuls test. All statistical measurements were performed using a commercially available statistics package (GraphPad Prism, GraphPad Software, Inc., San Diego, CA, U.S.A.). The level of significance was set at $p < 0.05$. The results are reported as mean \pm SEM.

RESULTS

Protocol 1: Hemodynamic Effects of Acute rhVEGF Administration

Intrapulmonary administration of rhVEGF (1 μg) caused a nearly 3-fold rise in rise pulmonary blood flow (Qp; 186 ± 39 mL/min after treatment *versus* 67 ± 13 mL/min at BL; $p <$

0.02; Fig. 1A) and a 65% fall in PVR (0.30 ± 0.1 mm Hg $\cdot\text{mL}^{-1}\cdot\text{min}$ *versus* 0.84 ± 0.2 mm Hg $\cdot\text{mL}^{-1}\cdot\text{min}$; $p < 0.05$; Fig. 1B). This response was maximal 3–5 min after infusion was completed, and was sustained for 10 min. There was no effect of rhVEGF on MPAP, AoP, HR, or arterial blood gas tensions (Table 1).

Dose-response study. The dose-response study demonstrated a consistent rise in Qp and fall in PVR at each of the three treatment doses (0.5, 1, and 2 μg) when compared with BL values. In this study, the higher doses (1 and 2 μg) increased Qp and reduced PVR more than the lowest dose of rhVEGF (0.5 μg ; Fig. 2). There was no difference in vasodilator response between the 1- μg and 2- μg doses.

Tachyphylaxis. Repeated administration of rhVEGF (1 μg) attenuated pulmonary vasodilation (Fig. 3). The initial dose of rhVEGF increased pulmonary blood flow by $184 \pm 25\%$, whereas the subsequent doses (doses 2 and 3) given 30 min apart resulted in a smaller rise in Qp ($75 \pm 4\%$ increase in Qp for dose 2, and $64 \pm 28\%$ increase for dose 3; $p < 0.05$ *versus* dose 1 for both).

Protocol 2: Effects of NO Inhibition on VEGF-Induced Pulmonary Vasodilation

Pretreatment of fetal lambs with the NO antagonist, L-NA, completely blocked VEGF-induced vasodilation. VEGF caused pulmonary blood flow to increase 140%, from 54 ± 11 mL/min at BL to 130 ± 20 mL/min (VEGF *versus* BL; $p < 0.05$; Fig. 4A) and caused a 57% decrease in PVR (0.34 ± 0.07

Table 1. Hemodynamic measurements at baseline and after rhVEGF, L-NA, and LY294002 infusion

	rhVEGF BL	rhVEGF (0.5 μg)	rhVEGF (1 μg)	rhVEGF (2 μg)	L-NA + rhVEGF	LY294002 + rhVEGF
PAP (mm Hg)	40 ± 2	42 ± 2	41 ± 2	42 ± 2	44 ± 2	44 ± 3
AoP (mm Hg)	40 ± 3	42 ± 2	40 ± 3	40 ± 2	43 ± 2	42 ± 1
Pao ₂ (mm Hg)	21 ± 2	20 ± 2	21 ± 2	21 ± 2	21 ± 2	20 ± 2

Values are mean \pm SEM.

Pao₂, arterial Po₂.

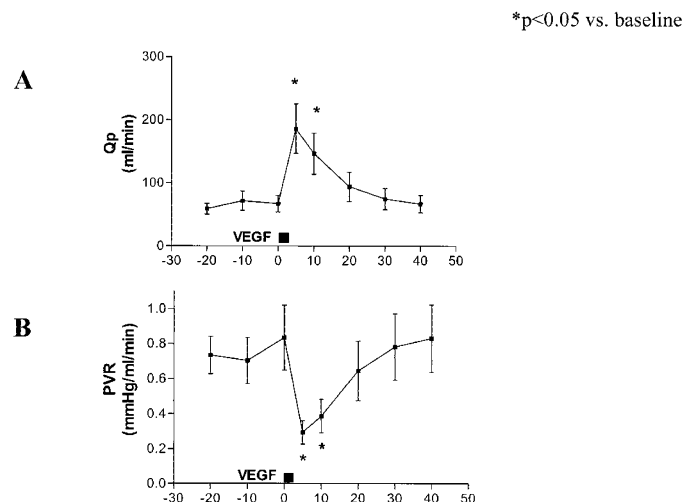


Figure 1. Effect of rhVEGF infusion (1 μg) on pulmonary blood flow (A) and PVR (B). * $p < 0.05$ vs BL.

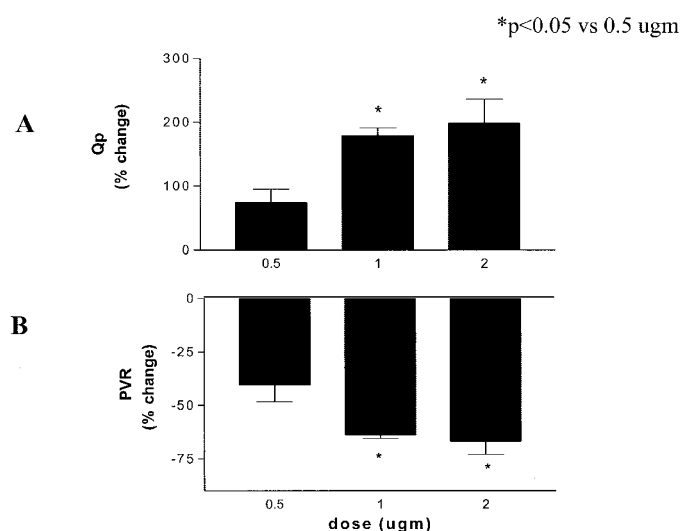


Figure 2. Dose-response effect of rhVEGF infusion on fetal pulmonary blood flow (A) and PVR (B). rhVEGF doses are 0.5, 1.0, and 2.0 μg, delivered into the LPA. * $p < 0.05$ vs 0.5-μg dose.

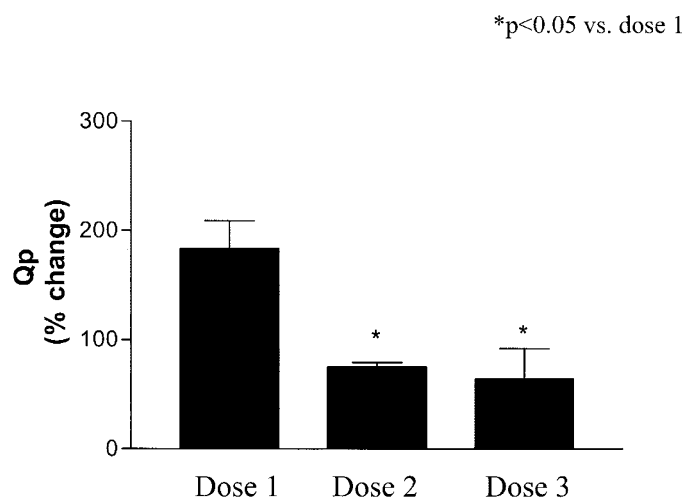


Figure 3. Effect of repeated doses of rhVEGF (1 μg) on fetal pulmonary blood flow (Qp). As shown, repeated infusions of VEGF resulted in progressive blunting of the vasodilator response (* $p < 0.05$ vs dose 1).

mm Hg·mL⁻¹·min versus 0.80 ± 0.18 mm Hg·mL⁻¹·min; VEGF versus BL; Fig. 4B). L-NA infusion 10 min before VEGF treatment reversed the vasodilation response to VEGF, resulting in a 7% fall in pulmonary blood flow (63 ± 9 mL/min versus 69 ± 11 mL/min; $p < 0.0001$ versus percent change in Qp after VEGF alone) and a $16 \pm 13\%$ increase in PVR (0.8 ± 0.16 mm Hg·mL⁻¹·min versus 0.72 ± 0.14 mm Hg·mL⁻¹·min; $p < 0.0005$ versus percent change in PVR after VEGF alone). There was no effect of L-NA or VEGF infusion on MPAP, AoP, HR, or arterial blood gas tensions compared with BL values (Table 1).

Protocol 3: Effects of PI-3-K Inhibition on VEGF-Induced Pulmonary Vasodilation

Pretreatment with the PI-3-K inhibitor, LY294002, markedly blunted the vasodilator response to rhVEGF, resulting in only a 9% increase in pulmonary blood flow (65 ± 7 mL/min

versus 60 ± 6 mL/min; LY294002 + VEGF versus BL) compared with a 140% increase in Qp after VEGF alone ($p < 0.0005$; percent change in Qp following LY294002 + VEGF versus VEGF alone; Fig. 4A). LY294002 + VEGF decreased PVR by 11% (0.71 ± 0.19 mm Hg·mL⁻¹·min versus 0.78 ± 0.08 mm Hg·mL⁻¹·min) compared with a 57% decrease after VEGF alone ($p < 0.005$; Fig. 4B). There was no effect of LY294002 or VEGF infusion on MPAP, AoP, HR, or arterial blood gas tensions compared with BL values (Table 1).

DISCUSSION

We found that rhVEGF causes NO-mediated pulmonary vasodilation during late fetal life. VEGF acutely increased pulmonary blood flow nearly 3-fold and reduced PVR by 65%, without affecting systemic blood pressure. rhVEGF exhibited both dose-dependent vasodilation and evidence of tachyphylaxis. In addition, we found that pretreatment with L-NA, an NOS inhibitor, and LY294002, a PI-3-K inhibitor, blocked pulmonary vasodilation to rhVEGF, suggesting that the vasodilator response is mediated by the release of endogenous NO through activation of PI-3-K. Although the critical role of VEGF in vascular development and endothelial function during early fetal life has been well described, this is the first report describing the hemodynamic response to exogenous VEGF in the developing pulmonary circulation.

Past studies have demonstrated that VEGF is essential for normal lung vascular growth during the embryonic period (4, 5). VEGF is highly expressed in the early embryonic lung (26), and gene expression increases as gestation progresses (27). However, whether VEGF is vasoactive and can release NO in the fetal lung had not been previously studied. In addition to promoting angiogenesis, VEGF signaling can modulate endothelial cell function and survival (6) and up-regulate eNOS expression in the adult systemic circulation (8, 17). In this

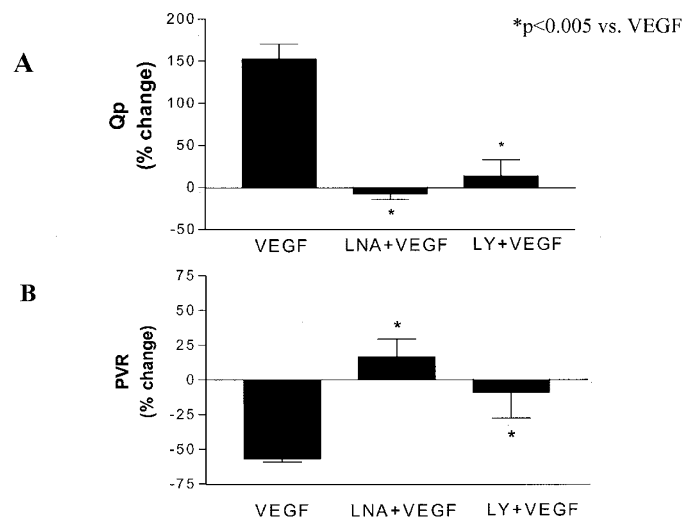


Figure 4. Effect of NOS and PI-3-K inhibition on VEGF-mediated vasodilation in the fetal lung. Percent change in pulmonary blood flow (Qp; A) and PVR (B) after rhVEGF infusion (1 μg) alone, and after pretreatment with the NOS inhibitor, L-NA, and pretreatment with the PI-3-K inhibitor, LY294002. * $p < 0.005$ vs VEGF alone.

report, we demonstrate that VEGF causes acute release of NO leading to vasodilation in the fetal lung.

VEGF-induced vasodilation has been studied in a variety of settings with a wide range of doses. *In vivo* studies have consistently demonstrated decreased mean arterial pressure, stroke volume, cardiac output, and total peripheral resistance, as well as tachycardia, after i.v (11, 18, 28). or intracoronary (12, 29, 30) VEGF administration. Significant adverse effects have been observed at high doses of VEGF (100 μg –2 mg), including refractory hypotension (12, 18) leading to death of 50% of animals in one study (12). Lopez *et al.* (12) demonstrated selective local vasodilation with minimal systemic effects after intracoronary infusion of rhVEGF protein at low doses (1 or 10 μg). In addition, chronic treatment with a low dose of VEGF (2 μg) has been shown to induce physiologically significant angiogenesis and improve blood flow in a porcine model of myocardial ischemia (30). In this study, we found that low doses of rhVEGF (0.5–2 μg) administered selectively into the left lung caused marked pulmonary vasodilation without systemic hypotension. In addition, we found that repeated doses of rhVEGF resulted in tachyphylaxis, which has been previously reported after repeated intracoronary infusion in adult pigs (12). Tachyphylaxis likely reflects diminished NO production, but the mechanisms underlying this response are unknown. Further studies are needed to fully examine the mechanisms responsible for this response.

Persistent pulmonary hypertension of the newborn is a disorder characterized by disrupted vascular growth and function in late fetal and early neonatal life, and animal models of this disease demonstrate alterations in lung VEGF expression (31–33). Several recent studies of adult vascular diseases have shown that exogenous VEGF treatment improves angiogenesis and augments endothelium-dependent blood flow (9, 13–16), but the hemodynamic effects of VEGF have not been previously examined in the developing pulmonary circulation. We found that rhVEGF causes marked vasodilation in the developing lung circulation without adverse systemic effects, demonstrating the feasibility of using rhVEGF infusions for disorders of pulmonary vascular growth and function in the fetal lung.

rhVEGF-mediated vasodilation in the developing lung circulation is likely mediated through PI-3-K activation resulting in NO production. *In vitro* studies have shown that exogenous VEGF increases eNOS mRNA and protein levels in endothelial cells (8, 17), leading to enhanced NO production. Animal studies demonstrate that VEGF-induced vasodilation is mediated at least in part by endogenous NO release inasmuch as pretreatment with the NOS inhibitor, L-NA, blocks this response (11, 12, 18, 28, 29). Although both VEGF and eNOS protein have been detected in the early fetal lung (27, 33), the hemodynamic response to VEGF in the fetal lung has not been previously studied. We report that rhVEGF causes marked vasodilation and that in concordance with previous observations, this response was completely blocked by L-NA. Past studies have demonstrated that VEGF phosphorylation of Akt (protein kinase B) by PI-3-K activation leads to NO release (19, 20) and *in vivo* contributes to acetylcholine-mediated vasodilation (20), but is blocked by the specific inhibitor of

PI-3-K, LY294002 (21). We also found that VEGF-mediated vasodilation in the fetal lung is markedly attenuated by pretreatment with LY294002, suggesting that the mechanism by which rhVEGF stimulates endogenous NO release is through activation of the PI-3-K pathway.

VEGF stimulates pulmonary vasodilation in a temporal pattern similar to that seen after stimulation with several other vasodilator agents. Prolonged exposure of the fetal pulmonary circulation to oxygen, acetylcholine, histamine, or tolazoline causes an abrupt increase in pulmonary blood flow and fall in PVR, peaking at 30–40 min, and returning to BL values by 90 min (34–36). Interestingly, tolazoline mediates pulmonary vasodilation through the release of histamine, a known PI-3-K agonist (37, 38). Furthermore, tolazoline exhibits a tachyphylaxis response similar to that described in this study for rhVEGF, and this tachyphylaxis response is thought to be a calcium-dependent process mediated by α -adrenergic mechanisms (36). Inasmuch as acute infusions of rhVEGF cause pulmonary vasodilation in a pattern similar to these other vasodilators and demonstrate tachyphylaxis, it is possible that rhVEGF causes vasodilation, in part, through histamine release and subsequent PI-3-K activation, but this requires further investigation.

This study does have several potential limitations. First, although LY294002 has been shown to cause selective inhibition of PI-3-K (21), other growth factors such as platelet-derived growth factor, IGF-1, and epidermal growth factor also mediate many of their functions through this signal transduction pathway (22). It is, however, unlikely that the acute effects of VEGF are caused by abrupt release of these other growth factors. Moreover, recent studies have determined that in addition to its effects on endothelial cell function, PI-3-K may also have direct effects on proliferation and migration of human vascular smooth muscle cells (39). It is feasible that LY294002 may block both endothelial cell release of NO and pulmonary artery smooth muscle cell NO-mediated vasodilation. Furthermore, this study examined only the acute effects of exogenous VEGF on the fetal pulmonary circulation, and although the vasodilator effect of VEGF appears short-lived, whether VEGF treatment can alter pulmonary vascular tone or growth in the developing lung remains unknown.

CONCLUSIONS

We conclude that low-dose rhVEGF causes potent vasodilation in the fetal lung with no adverse systemic effects. This vasodilation is dose-dependent and demonstrates tachyphylaxis with repeated doses. The mechanism by which VEGF causes pulmonary vasodilation is by the release of endogenous NO through activation of PI-3-K because this response is blocked by both the NOS inhibitor, L-NA, and a selective PI-3-K inhibitor, LY294002. We speculate that VEGF may be an important modulator of vascular tone in the developing pulmonary circulation, and that its effects are mediated through NO production. Whether VEGF treatment can enhance endothelial function and sustain NO production in models of persistent pulmonary hypertension of the newborn is unknown.

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