Inhaled nitric oxide attenuates pulmonary hypertension and improves lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor


Inhaled nitric oxide attenuates pulmonary hypertension and improves lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor. Am J Physiol Lung Cell Mol Physiol 287: L344–L351, 2004. First published April 2, 2004; 10.1152/ajplung.00291.2003.—VEGF plays a critical role during lung development and is decreased in human infants with bronchopulmonary dysplasia. Inhibition of VEGF receptors in the newborn rat decreases vascular growth and alveolarization and causes pulmonary hypertension (PH). Nitric oxide (NO) is a downstream mediator of VEGF, but whether the effects of impaired VEGF signaling are due to decreased NO production is unknown. Therefore, we sought to determine whether impaired VEGF signaling downregulates endothelial NO synthase (eNOS) expression in the developing lung and whether inhaled NO (iNO) decreases PH and improves lung growth after VEGF inhibition. Newborn rats received a single dose of SU-5416 (a VEGF receptor inhibitor) or vehicle by subcutaneous injection and were killed up to 3 wk of age for assessments of right ventricular hypertrophy (RVH), radial alveolar counts (RAC), lung eNOS protein, and NOx production in isolated perfused lungs (IPL). Neonatal treatment with SU-5416 increased RVH in infant rats and reduced RAC. Compared with controls, SU-5416 reduced lung eNOS protein expression by 89% at 5 days (P < 0.01). IPL studies from day 14 rats demonstrated increased baseline pulmonary artery pressure and lower perfusate NOx concentration after SU-5416 treatment. Importantly, iNO treatment prevented the increase in RVH and improved RAC after SU-5416 treatment. We conclude that treatment of neonatal rats with SU-5416 downregulates lung eNOS expression and that iNO therapy decreases PH and improves lung growth after SU-5416 treatment. We speculate that decreased NO production contributes to PH and decreases distal lung growth caused by impaired VEGF signaling.

vascular endothelial growth factor; bronchopulmonary dysplasia; lung development; alveolarization

BRONCHOPULMONARY DYSPLASIA (BPD) is the chronic lung disease that follows ventilator and oxygen therapy for acute respiratory failure after prematurity birth (10, 38). Despite improved perinatal care over the past decade, the incidence of BPD remains high, with nearly 60% of surviving premature infants developing chronic lung disease (24). BPD is characterized by decreased alveolarization, impaired vascular growth, and, in some cases, progressive pulmonary hypertension (10, 21, 24, 25). Although hypoxia, inflammation, infection, and ventilator-induced lung injury are associated with the development of BPD, the exact mechanisms that disrupt lung growth in premature infants and cause persistent abnormalities in lung structure are poorly understood. Recently, experimental studies have shown that inhibition of angiogenesis decreases alveolarization in infant rats, suggesting that normal angiogenesis is necessary for alveolarization and that disruption of the developing pulmonary vasculature can lead to the features of BPD (23, 30). However, mechanisms that regulate and link vascular and alveolar growth in the developing lung are still poorly understood.

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and survival factor that stimulates angiogenesis (43) and protects endothelial cells against injury (14). Expression of VEGF mRNA and protein is localized to distal airway epithelial cells in the midgestation human fetal lung, suggesting that VEGF plays a critical role in the regulating epithelial-endothelial interactions early during lung development (3, 46). Experimental studies have previously demonstrated decreased lung VEGF expression in the rabbit and primate models of BPD (34, 35). More recently, clinical studies have shown that VEGF mRNA and protein and VEGF receptors are decreased in the lungs of infants dying with BPD, further suggesting that decreased VEGF activity may contribute to the development of BPD (9). Furthermore, experimental studies demonstrate that inhibition of VEGF receptors impairs alveolarization, decreases vascular growth, and causes pulmonary hypertension in infant rats (23, 30). However, mechanisms that impair lung growth and cause pulmonary hypertension following disruption of VEGF signaling are unclear.

Recent studies have demonstrated that endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) play an important role in VEGF-induced angiogenesis in the systemic circulation and tumors (37, 40, 50). VEGF can upregulate eNOS mRNA, protein, and activity and increase NO production in human and bovine vascular endothelial cells in vitro (19, 40). In addition, inhibition of NO activity blocks VEGF-induced angiogenesis in vitro (5, 36, 39) and in vivo (19, 40). Moreover, VEGF-induced angiogenesis is impaired in mice with a genetic deficiency in eNOS (44). More recent studies have shown that eNOS-deficient neonatal mice are more susceptible to impaired alveolarization and lung vascular growth following exposure to mild hypoxia (6). Similarly, inhibition of NO activity decreases branching morphogenesis in fetal rat lungs and postpneumonectomy lung growth in adults (32, 49). Overall, these studies suggest that VEGF induces angiogenesis through increased expression of NO and that NO production may play a critical role in the development of BPD.
an important role in lung growth during development. However, whether impaired VEGF signaling downregulates eNOS expression and decreases NO production in the developing lung is unknown.

Therefore, we hypothesize that VEGF regulates pulmonary angiogenesis and lung growth through NO-dependent mechanisms and that disruption of VEGF signaling decreases eNOS expression and NO production in the developing lung, which may contribute to impaired lung growth and pulmonary hypertension. To test this hypothesis, we first determined whether neonatal treatment with a VEGF receptor inhibitor decreases eNOS expression and NO production. To further determine the potential role of impaired VEGF-NO signaling on alveolar and vascular growth in the developing lung, we determined whether prolonged treatment with inhaled NO improves lung growth and decreases pulmonary hypertension in infant rats after VEGF receptor inhibition. We report that early disruption of VEGF receptor activity downregulates lung eNOS expression and that treatment with low doses of inhaled NO attenuates pulmonary hypertension and improves lung growth in this experimental model of BPD.

MATERIALS AND METHODS

Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver’s altitude (1,600 m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 wk before giving birth. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 h. Rats were killed with an intraperitoneal injection of pentobarbital sodium (0.3 mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA).

Study Design

This study includes two basic protocols. In protocol 1, we first determined whether treatment of neonatal rats with a VEGF receptor inhibitor impaired lung eNOS expression during lung development, and whether this reduction in NO production was sustained throughout infancy. In the second protocol, we studied the effects of low-dose inhaled NO therapy on pulmonary hypertension and lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor. The study protocols are described below.

Protocol 1: to determine whether neonatal treatment with a VEGF receptor inhibitor reduces lung eNOS expression and NO production in the developing lung. The VEGF receptor inhibitor SU-5416 was obtained from Calbiochem (San Diego, CA). Our selection of a treatment dose for SU-5416 of 20 mg/kg was based on our previous study in which a single dose of SU-5416 in newborn rats caused pulmonary hypertension and impaired lung growth in infant rats (30). In this study, 3-day-old rats received a single dose of SU-5416 (20 mg/kg) or its vehicle [carboxymethyl cellulose (CMC)] by subcutaneous injection. Litters were divided equally between SU-5416-treated and vehicle controls, and rats were raised in room air at Denver’s altitude. Rats from each group were killed for studies at 5 days, 6 days, 7 days, and 2 wk of age. We studied four to eight rats at each time point for morphometric studies and Western blot analysis. Studies included assessment of pulmonary hypertension [by measuring right ventricular hypertrophy (RVH)] and Western blot analysis to assay lung protein expression for eNOS, VEGF receptor-2 [VEGFR-2 or kinase insert domain-containing receptor (KDR/fetal liver kinase-1 (Flk))], and platelet endothelial cell adhesion molecule-1 (PECAM). In addition, NOx was measured in isolated perfused lungs (IPL) in 2-wk-old rats after SU-5416 (n = 5 animals) and vehicle treatment (n = 6 animals) to determine NO production. In addition, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay was performed at 2 wk to detect cells undergoing apoptosis from control and SU-5416-treated rats.

Protocol 2: to determine the effects of inhaled NO on pulmonary hypertension and lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor. In this protocol, 1-day-old rats were treated with a single subcutaneous injection of SU-5416 (20 mg/kg) or its vehicle (CMC). Litters were divided equally between SU-5416-treated and vehicle controls. One day after injection, animals were either maintained in room air or placed in a Plexiglas chamber and exposed to a mix of room air and inhaled NO (10 ppm). O2, NO, and NO2 levels were monitored with an INO Vent controller (INO Therapeutics, Clinton, NJ) with the exhaust directed into an outside air vent at 10 l/min. Eight to twelve animals were studied within each of the four study groups. At 3 wk of age, animals were killed for studies of pulmonary hypertension (by measuring RVH), pulmonary vascular structure and growth (by measuring pulmonary artery wall thickness and pulmonary artery density), and alveolarization [by measuring radial alveolar count (RAC)].

Study Measurements

RVH. At autopsy, the heart was resected through a midline sternotomy. The right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed, and the ratio of RV to LV+S weight was determined as an index of RVH.

Western blot analysis for eNOS, KDR, and PECAM. Western blot analysis was performed with the following primary antibodies: a monoclonal antibody to eNOS (Transduction Laboratories, Lexington, KY) diluted 1:500, a rabbit polyclonal antibody to KDR/Flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200, and a goat polyclonal antibody to PECAM (Santa Cruz) diluted 1:200, as previously published (eNOS/KDR/PECAM, Ref. 15). Lung tissue was homogenized on ice in 25 mM Tris-HCl buffer containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin A. The homogenate was centrifuged at 1,500 g at 4°C for 10 min to remove cell debris. Lung homogenates (25 μg) were separated by SDS-PAGE and then transferred to nitrocellulose membranes by electroblotting. Blots were blocked overnight at 4°C in 5% of nonfat dried milk (for eNOS and VEGFR-2) or 5% BSA (for PECAM). Immunodetection was performed with the primary antibody diluted in blocking buffer for 1 h at room temperature. After the blots were washed to remove unbound antibody, a secondary antibody [horse-radish peroxidase-conjugated anti-mouse (eNOS), anti-rabbit (VEGFR-2), or anti-goat (PECAM)] was diluted in blocking buffer (1:10,000) and applied for 30–60 min. After three washes, ECL Plus detection (Amersham, Piscataway, NJ) was performed. We initially determined the accuracy and consistency of the protein loads for each gel by Ponceau S staining before applying the different antibodies. In addition, each gel was stripped and reprobed with β-actin for use as a housekeeping protein to compare expression between samples. The experiments were performed with at least five animals per study group, and data represent at least three different experiments.

[NOx] in lung perfusate. After anesthesia with pentobarbital sodium, a thoracotomy was performed, and 100 units of heparin were injected into the RV. The lung was ventilated with humidified gas mixture (21% O2, 5% CO2, and balance N2) at 60 breaths/min, an inspiratory pressure of 9 cmH2O, and an end-expiratory pressure of 2 cmH2O with a Harvard rodent ventilator. The RV was opened, and an 18-gauge blunt-tipped catheter was placed in the main pulmonary artery. After placement of a similar catheter in the LV, the lung was perfused by a peristaltic pump at 0.04 ml·g body wt−1·min−1 with physiological salt solution (Earle’s balanced salt solution) containing
(in mmol/l) 116.3 NaCl, 5.4 KCl, 0.83 MgSO4, 19.0 NaHCO3, 1.04 NaH2PO4, 1.8 CaCl2·H2O, and 5.5 d-glucose at pH 7.35–7.45. Ficoll (4 g/100 ml, type 70; Sigma) was included as a colloid, and 3.1 μmol/l sodium meclofenamate (Sigma) was added to inhibit prostaglandin synthesis. Lung and perfusate temperatures were maintained at 37°C by use of a heated table and warming light. Samples (0.5 ml) of effluent perfusate were collected from the LV cannula 5 min after the circulation was established. Perfusate samples were stored at −70°C for up to 4 wk before measurement of NOx with an NO chemiluminescence analyzer (Sievers Research). Aliquots of perfusate (10 μl) were added to 2 ml of 0.1 M vanadium chloride (type III, Aldrich) dissolved in 1 N HCl and heated to 90°C in the purge vessel of the NO analyzer to reduce all NOx to NO. We drove the liberated NO into the chemiluminescence chamber by bubbling the reaction mixture with argon. We generated linear calibration curves by measuring the NO produced by 10-, 20-, 30-, 50-, and 100-pM sodium nitrate (Mallinkrodt) solutions.

**Barium-gelatin infusion and fixation of lung tissue.** Rat lungs were prepared and fixed in situ at the end of the 3-wk study period in protocol 2. PBS was infused through a main pulmonary catheter to flush the pulmonary circulation free of blood. A barium sulfate-gelatin mixture was heated to 70°C and infused through a pulmonary artery catheter at 73 mmHg of pressure, as previously described (12). Pressure was maintained for 5 min to ensure penetration of the barium mixture, and the lungs were fixed by tracheal instillation of 10% buffered formalin at constant pressure (20 cmH2O). The trachea was ligated to maintain fixation pressure, and the lungs were removed and submersed in fixative for 24 h at 4°C. To obtain arteriograms, we placed lungs on an X-ray film and imaged them using X-ray radiography. For microscopic analysis, one transverse section was taken from the midplane of the upper, middle, and lower lobes of the formalin-fixed right lung. Sections from each animal were processed and embedded in paraffin wax. Paraffin sections (5 μm thick) were cut from each block and stained with hematoxylin and eosin.

**Apoptosis assay.** We identified lung cells undergoing apoptosis by labeling their DNA 3’-OH nick ends by a variant of TUNEL staining. Staining was performed according to the manufacturer’s instructions with ApopTag Peroxidase in Situ Apoptosis Detection Kit (Intergen). Tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, and 70%). Tissue sections were then digested with proteinase K (20 μg/ml) for 15 min. Endogenous peroxidase activity was quenched with 3% H2O2 in PBS for 5 min. The slides were immersed in terminal deoxynucleotidyl transferase buffer containing digoxigenin-labeled nucleotides at 37°C for 1 h. After washing the slides, we added antidigoxigenin-peroxidase to cover the slides and incubated them in a humid chamber at room temperature for 30 min. After washing them with PBS, we stained the slides with diaminobenzidine and then counterstained them with 1% methyl green. We performed light microscopy to quantify the apoptotic cells by counting cells in 10 randomly selected fields (×100 magnification) of each slide with the following formula: number of apoptotic cells/total number of cells counted × 100.

**Morphometric analysis.** Analysis of each section was carried out in a blinded fashion. Wall thickness of small pulmonary arteries was measured on pulmonary arteries accompanying the terminal or respiratory bronchioles. At least 10 pulmonary arteries were measured for each animal. Arterial wall thickness and external diameter were measured with a Zeiss Interactive Digital Analysis System on hematoxylin- and eosin-stained lung sections as previously described (1). The percent medial thickness of an artery was calculated by the following formula: (2 × medial thickness × 100)/external diameter. We determined pulmonary artery density by counting barium-filled arteries per high-power field (×200 magnification). Alveolarization was assessed by the RAC of Emery and Mithal as described (11). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall (42). From the center of the respiratory bronchiole, a perpendicular was dropped to the edge of the acinus connective tissue or septum or pleura, and the number of septa intersected by this line was counted. At least 15 counts were performed for each animal.

**Statistical Analysis**

Statistical comparison was made by analysis of variance and Fisher’s protected least significant difference test and the Statview software package (Abacus Concepts, Berkeley, CA). Data are presented as means ± SE. Differences were considered significant at P < 0.05.

**RESULTS**

**Protocol 1: Does Neonatal Treatment With a VEGF Receptor Inhibitor Reduce Lung eNOS Expression and NO Production in the Developing Lung?**

In comparison with the vehicle-treated control rats, the ratio of RV/LV+S was greater after neonatal treatment with SU-5416 throughout the study period. The ratio of RV/LV+S was 23% greater after SU-5416 treatment than controls at day 5 of age (P < 0.05) and progressively increased through day 14 of age (at day 7, 143% of controls, P < 0.05; at day 14, 169% of controls, P < 0.01) (Fig. 1).

Two days after neonatal treatment with SU-5416, treated rats exhibited an 89% reduction in lung eNOS protein expression compared with controls (P < 0.05, Fig. 2). As shown, lung eNOS protein progressively returned toward control values toward the end of the study period (Fig. 2). Despite the lack of difference in eNOS protein content at 2 wk after neonatal treatment with SU-5416, the NOx concentration in perfusate from isolated lungs was decreased by 25% at day 14 of age compared with controls (P < 0.05, Fig. 3). IPL studies from day 14 rats also demonstrated increased baseline pulmonary artery pressure (30.2 ± 6.4 vs. 15.7 ± 1.0 mmHg, P < 0.05) after SU-5416 treatment. There were no differences in expression of lung VEGFR-2 and PECAM expression between SU-5416-treated and control rats at days 5 and 14 of age (Fig. 4). TUNEL assay showed similar proportions of immunoreactive cells between SU-5416-treated rats (1.7 ± 0.2%) and controls (1.3 ± 0.2%) at day 14 (P = 0.24).

![Fig. 1. Serial changes in right ventricular hypertrophy (RVH) after neonatal treatment with SU-5416, a VEGF receptor inhibitor. As shown, RVH, as determined by the right ventricle/left ventricle plus septum (RV/LV+S) ratio, progressively increases during infancy after SU-5416 treatment of newborn rats (CMC, carboxymethyl cellulose, vehicle controls; SU, SU-5416-treated rats).](http://ajplung.physiology.org/)
Protocol 2: Effects of Inhaled NO on Pulmonary Hypertension and Lung Growth in Infant Rats After Neonatal Treatment With a VEGF Receptor Inhibitor

In comparison with vehicle controls, the SU-5416-treated rats had a 56% increase in the ratio of RV/LV+S at 3 wk of age ($P < 0.05$, Fig. 5). Prolonged treatment with inhaled NO (10 ppm) markedly reduced the ratio of RV/LV+S to values measured in CMC control animals at 3 wk of age ($P < 0.05$, Fig. 5).

Barium arteriograms illustrate the reduction in the perfusion of small pulmonary arteries after SU-5416 treatment (Fig. 6). As shown, the background haze is reduced after SU-5416 treatment. Prolonged treatment with inhaled NO (10 ppm) increased perfusion of the distal pulmonary arteries.

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Fig. 2. Effects of SU-5416 treatment on lung endothelial nitric oxide synthase (eNOS) protein content. Compared with CMC controls, lung eNOS was markedly reduced after SU-5416 treatment at day 5 (A) but steadily increased toward control values during the study period (B).

Fig. 3. Effects of SU-5416 treatment on NOx levels in lung perfusate from isolated lungs obtained from 3-wk-old control and SU-5416-treated rats. As shown, lung NOx was reduced by 20% after SU-5416 treatment.

Fig. 4. Serial changes in lung VEGF receptor-2 (VEGFR-2, A) and PECAM (B) protein contents in control and SU-5416-treated rats. In contrast with the marked reduction in lung eNOS content, lung VEGFR-2 protein did not differ between SU-5416-treated rats and CMC controls. As shown, lung PECAM protein increases between days 5 (5d) and 14 (14d) in control rats and was not affected by SU-5416 treatment. NS, not significant.

Fig. 5. Effects of inhaled NO (INO) treatment on RVH after SU-5416 treatment. Compared with CMC controls, SU-5416 treatment increased the RV/LV+S ratio by 50% ($P < 0.05$). iNO treatment blocked the rise in RVH after neonatal SU-5416 injection.
In comparison with controls, wall thickness of small pulmonary arteries (external diameter between 20 and 50 μm) was increased by 116% after SU-5416 treatment at 3 wk of age (P < 0.05, Fig. 7). Treatment with inhaled NO reduced arterial wall thickness by 35% at 3 wk of age compared with those SU-5416-treated and raised in room air (P < 0.05, Fig. 7). SU-5416-treated rats had a 59% reduction in pulmonary artery density at 3 wk of age compared with controls (P < 0.05, Fig. 7). As shown, treatment with inhaled NO increased pulmonary artery density by 107% compared with SU-5416-treated animals (P < 0.05, Fig. 7).

In addition, SU-5416 treatment reduced RAC by 44% from control values at 3 wk of age (P < 0.05, Fig. 8). Compared with the SU-5416 treatment group, inhaled NO increased RAC by 54% at 3 wk of age (P < 0.05).

DISCUSSION

We found that neonatal treatment with SU-5416, a VEGF receptor inhibitor, caused progressive RVH, decreased vessel density, and reduced alveolarization in infant rats. We further report that VEGF receptor inhibition decreased lung eNOS protein expression and that despite the recovery of lung eNOS protein content over time, NOx production was decreased in IPL from infant rats at day 14. In addition, we found that prolonged inhaled NO therapy prevented RVH, reduced wall thickness of small pulmonary arteries, and increased artery density and RAC in the infant rats that were treated with SU-5416 in the newborn period. Overall, our study demonstrates that transient disruption of VEGF signaling in the neonates specifically downregulates lung eNOS expression and...
decreases lung NO bioavailability in infant rats, which likely contribute to the development of pulmonary hypertension and impaired lung growth. These findings collectively support our hypotheses that VEGF regulates angiogenesis and lung growth, at least partly through NO-dependent mechanisms in the newborn, and that early disruption of VEGF signaling decreases eNOS expression and NO production, which contributes to impaired lung growth and pulmonary hypertension throughout infancy.

This report directly demonstrates that neonatal inhibition of VEGF activity reduces lung eNOS expression, decreases lung NO bioavailability, and impairs distal lung growth during infancy. Grover et al. (16) have recently shown that intrapulmonary infusion of a VEGF antagonist (aptamer) downregulates eNOS expression and NO production in the late fetal sheep model. Previous studies have suggested that decreased lung VEGF expression is associated with BPD in two experimental models of BPD (34, 35) and in human infants with BPD (9, 29). In addition, we have previously shown that neonatal inhibition of VEGF receptors impairs vascular growth and alveolarization, mimicking key features of BPD (23, 30), further suggesting that impaired VEGF signaling can contribute to abnormal lung structure in infants with BPD. Importantly, lung eNOS expression is also reduced in the lamb (33) and primate (4) models of BPD, and our study demonstrates that early disruption of VEGF signaling downregulates eNOS expression and reduces NO production in the immature lung during the early postnatal period, further suggesting that the VEGF-NO signaling pathway is critical during normal lung development. Whether decreased eNOS expression and NO production due to altered VEGF activity play a major role in the pathogenesis of human BPD requires further study but is consistent with the findings among these diverse animal models of BPD.

Moreover, this is the first report demonstrating that inhaled NO preserves normal lung growth and prevents pulmonary hypertension after disruption of VEGF signaling. This study indicates that prolonged therapy with inhaled NO can enhance alveolarization and vascular growth in infant rats susceptible to disrupted lung growth. Our results suggest that inhaled NO therapy may remarkably prevent development of BPD by improving lung growth in high-risk premature infants.

Past studies have shown that eNOS expression and NO production increase during development in the fetal lung (38, 45, 46). In contrast, eNOS protein is decreased in experimental models of BPD, including chronically ventilated preterm lambs and baboons (4, 33). Similarly, previous studies have shown abundant VEGF mRNA and protein expressed in the normal fetal lung (3, 47), but lung VEGF expression is consistently decreased in studies of BPD (9, 29, 34, 35). Although past studies showed that VEGF can upregulate eNOS expression and augment NO production in vitro (40, 49) and systemic vessels in vivo (37), whether impaired VEGF signaling downregulates eNOS expression and decreases NO production in the developing lung has been unclear. The present study demonstrates that inhibition of VEGF receptors reduces eNOS expression and NO bioavailability in the immature lung, which later develops structural and functional features of BPD. Although lung eNOS protein is reduced shortly after neonatal SU-5416 injection in this model, eNOS content recovers during the study period in this model. The mechanism of persistent reduction in NO production at 14 days despite the increase in lung eNOS protein in this model is unclear but may be related to substrate limitation, decreased cofactor bioavailability, increased oxidant stress, or other causes.

Although eNOS activity has been primarily studied and considered with regard to its effects on vascular tone and structure, recent studies suggest that NO may further modulate vascular growth and alveolarization (6, 32) and branching morphogenesis in the early fetus (49). Overall, these results along with previously published studies suggest a critical role of VEGF-NO signaling during lung growth and development and that VEGF signaling may regulate alveolar and vascular growth through modulating eNOS expression and NO production.

Inhaled NO has been demonstrated to be an effective treatment for term infants with persistent pulmonary hypertension, but its role in managing preterm infants is still controversial. As a pulmonary vasodilator, inhaled NO was expected to improve oxygenation in premature infants with BPD, in whom there may be coexisting pulmonary hypertension (1, 8). Inhaled NO showed modest improvement in oxygenation in infants with established BPD by reducing ventilation-perfusion mismatch (7). In animal studies, inhaled NO reduces hypoxia-induced lung injury, suggesting that NO may protect the developing lung against predisposing factors of BPD (18, 20, 22, 36). Early treatment with inhaled NO decreased ventilator days in survivors of premature infants with acute respiratory failure (28), suggesting that inhaled NO may decrease the risk of BPD. In this study, inhaled NO is demonstrated to preserve normal growth of the developing lung in an experimental model with structural and functional features of BPD. In the present study, inhaled NO therapy was initiated immediately after VEGF receptor inhibition in the newborn rats, suggesting that early treatment might be required for effectively preserving lung growth to prevent BPD in the high-risk premature neonates.

The mechanisms by which inhaled NO improves lung growth after VEGF receptor inhibition are unknown. Possibilities include decreased oxidant stress (17, 18) or apoptosis (20, 26, 48), decreased inflammation (20, 27), or improved gas exchange (14, 41). Alternatively, NO may promote vascular growth and alveolarization by a direct effect on angiogenesis (5, 6, 14, 16, 19, 23, 37, 40, 50). Further studies are needed to determine the mechanisms of NO effects in this model.

We conclude that transient disruption of VEGF signaling in the newborn rats downregulates eNOS expression and decreases NO bioavailability in the infant rat lungs, which may contribute to impaired lung growth and pulmonary hypertension in this experimental model of BPD. We speculate that the VEGF-NO pathway regulates alveolarization and angiogenesis and that NO is required for achieving normal lung development. We suggest that early intervention with prolonged therapy of low-dose inhaled NO may prevent BPD in high-risk premature infants.

GRANTS

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