Hypoxia-inducible factors HIF-1α and HIF-2α are decreased in an experimental model of severe respiratory distress syndrome in preterm lambs

Theresa R. Grover,* Tiina M. Asikainen,* John P. Kinsella, Steven H. Abman, and Carl W. White

University of Colorado School of Medicine, Pediatric Heart Lung Center, Department of Pediatrics, and National Jewish Medical and Research Center, Denver, Colorado

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Grover TR, Asikainen TM, Kinsella JP, Abman SH, White CW. Hypoxia-inducible factors HIF-1α and HIF-2α are decreased in an experimental model of severe respiratory distress syndrome in preterm lambs. Am J Physiol Lung Cell Mol Physiol 292: L1345–L1351, 2007. First published February 16, 2007; doi:10.1152/ajplung.00372.2006.— Respiratory distress syndrome (RDS) secondary to preterm birth and surfactant deficiency is characterized by severe hypoxemia, lung injury, and impaired production of nitric oxide (NO) and vascular endothelial growth factor (VEGF). Since hypoxia-inducible factors (HIFs) mediate the effects of both NO and VEGF in part through regulation by prolyl-hydroxylase-containing domains (PHDs) in the presence of oxygen, we hypothesized that HIF-1α and -2α in the lung are decreased following severe RDS in preterm neonatal lambs. To test this hypothesis, fetal lambs were delivered at preterm gestation (115-day gestation, term = 145 days; n = 4) and mechanically ventilated for 4 h. Lambs developed respiratory failure characterized by severe hypoxemia despite treatment with mechanical ventilation with high inspired oxygen concentrations. Lung samples were compared with nonventilated control animals at preterm (115-day gestation; n = 3) and term gestation (142-day gestation; n = 3). We found that HIF-1α protein expression decreased (P < 0.05) and PHD-2 expression increased (P < 0.005) at birth in normal term animals before air breathing. Compared with age-matched controls, HIF-1α protein and HIF-2α protein expression decreased by 80% and 55%, respectively (P < 0.005 for each) in preterm lambs with RDS. Furthermore, VEGF mRNA was decreased by 40%, and PHD-2 protein expression doubled in RDS lambs. We conclude that pulmonary expression of HIF-1α, HIF-2α, and the downstream target of their regulation, VEGF mRNA, is impaired following RDS in neonatal lambs. We speculate that early disruption of HIF and VEGF expression after preterm birth and RDS may contribute to long-term abnormalities in lung growth, leading to bronchopulmonary dysplasia.

lung development; neonatal lung injury; vascular endothelial growth factor

Despite recent advances in neonatal medicine, respiratory distress syndrome (RDS) after preterm birth remains a major cause of neonatal morbidity and mortality (39). Neonatal RDS in premature infants is characterized by surfactant deficiency, which may constitute a primary or secondary process, and often benefits from exogenous surfactant therapy (8, 14). Lung function in premature infants with RDS may be further impaired by ongoing ventilator-induced lung injury, caused by barotrauma, volutrauma, and exposure to supraphysiologic concentrations of oxygen (11). Furthermore, human and animal models of bronchopulmonary dysplasia (BPD) have shown impaired expression of critical growth factors such as vascular endothelial growth factor (VEGF) within the lung (7, 24) and that early disruption of VEGF signaling can contribute to late abnormalities of lung structure. VEGF is a growth factor found early in fetal life that has been shown in numerous studies to play a critical role in pulmonary vascular and alveolar development (9, 12, 24, 25). In sheep, lung VEGF expression increases dramatically during the late stages of lung development and falls shortly after birth, and inhibition of VEGF impairs pulmonary alveolar and vascular development (13, 18, 25). In addition, neonates with severe BPD have decreased VEGF expression in the lung (7, 24), and treatment with VEGF preserves or restores normal lung architecture in rat models of BPD (22, 23, 37). Together, these data suggest an important role for VEGF in modulating lung growth and function during lung development. The expression of VEGF in the lung is tightly regulated, in part by oxygen tension, through the actions of the hypoxia-inducible factors (HIFs) (27).

HIFs are constitutively expressed in fetal tissues and are expressed by a variety of pulmonary cell types, including pulmonary epithelial, smooth muscle, and endothelial cells (41). Downstream targets of HIFs modulate such diverse actions as angiogenesis, vascular tone and remodeling, and cell proliferation and survival (32). In addition, gene deletion studies have shown that both HIF-1α and HIF-2α are critical for normal lung development, as deletion of the HIF-1α isoform causes lethal defects in embryonic vascular development, and deletion of HIF-2α leads to fatal RDS early in neonatal life (10, 17, 35).

HIF expression is influenced not only by oxygen tension but also by the actions of other growth factors, barotrauma, and inflammation (11, 32). Exposure to oxygen at the time of preterm birth, followed by mechanical ventilation, likely disrupts normal HIF expression, which may lead to downstream alterations in critical angiogenic factors such as VEGF and endothelial nitric oxide synthase (eNOS). Indeed, premature baboons with prolonged mechanical ventilation demonstrate decreased HIF-1α expression, and pharmacological stabilization and upregulation of HIF in vivo upregulates VEGF mRNA and improves pulmonary angiogenesis (1, 5). Whether short-term ventilation following preterm birth decreases HIF expression and leads to impaired lung development is unknown. We designed the current study to examine the normal pattern of HIF expression during late gestation, to determine whether preterm birth followed by short-term ventilation and RDS decreases pulmonary HIF and VEGF expression, and to determine the mechanisms underlying these changes.

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MATERIALS AND METHODS

All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Two study protocols were followed: 1) ontogeny of lung HIF expression, and 2) HIF expression in an experimental model of RDS in extremely preterm lambs.

Protocol 1: Ontogeny of Lung HIF Expression

Lung tissue was obtained from six fetal Columbia-Rambouillet sheep at 115-day gestation (n = 3; term = 145 days) and 140–142 days (“term”; n = 3). All animals were euthanized with a rapid injection of intravenous pentobarbital sodium. For biochemical assays, 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. For histological studies, the trachea was cannulated for fixation, and the right lung was inflated with 10% buffered formalin at 30 cmH2O pressure. Lung sections were embedded in paraffin for storage, and upon histological analysis, sections were cut and stained with hematoxylin and eosin.

Protocol 2: Lung HIF Expression in Preterm Lambs with RDS

Surgical preparation. Ewes were sedated with intravenous pentobarbital sodium (2–4 g total dose) and anesthetized with intrathecal tetracaine hydrochloride (1% solution, 3 mg; n = 4). A uterine incision was made under sterile conditions. The fetal head was exteriorized and placed in a rubber glove containing warm saline to prevent fetal breathing before controlled ventilation. A right paramedian skin incision was made in the neck after local infiltration with lidocaine (1% solution, 2–3 ml). Polyvinyl catheters (20-gauge; Mar-tech Medical Products, Lansdale, PA) were advanced into the ascending aorta through the carotid artery and into the superior vena cava through the jugular vein. The aortic catheter was connected to a MP 100A Biopac System (Santa Barbara, CA). Calibrations of pressure transducers were performed with a mercury column manometer. Pancuronium (0.1 mg/kg) was administered to the fetus, and a tracheotomy was performed with placement of a 3.0-mm internal diameter endotracheal tube (HiLo Jet tube; Mallinckrodt). All animals were treated with exogenous surfactant (Infasurf, provided by Dr. E. A. Egan, ONY, Amherst, NY) at an estimated dose of 3 ml/kg before the first breath. Mechanical ventilation was initiated with a continuous flow, time-cycled, pressure-limited neonatal ventilator (Infant Star; Infrasonics, San Diego, CA) at the following settings: peak inspiratory pressure (PIP), 35 cmH2O; positive end-expiratory pressure (PEEP), 6 cmH2O; rate, 30 breaths/minute; inspiratory time, 1.0 s; and FIO2 = 1.00. After 10 min of mechanical ventilation, the umbilical cord was ligated, and the animal was transferred to a radiant warmer. A solution of 5% dextrose in normal saline and pentobarbital (1 mg·kg⁻¹·h⁻¹) was continuously infused at 10 ml/h. Sodium bicarbonate (1 meq/kg) was infused to correct metabolic acidemia if the arterial pH was less than 7.0.

Fig. 1. Ontogeny of pulmonary hypoxia-inducible factor (HIF) and prolyl-hydroxylase-containing domain (PHD)-2 protein expression. A: HIF-1α protein expression is decreased in lungs of term lambs (140-day gestation; n = 3) compared with nonventilated preterm lambs (115-day gestation; n = 3; *P < 0.05). B: HIF-2α protein expression is unchanged in full-term lambs compared with preterm control lambs. C: lung PHD-2 protein expression is increased in term lambs (n = 3) compared with preterm lambs (P < 0.05; n = 3). d, days.
than 7.25 with PaCO2 in the target range of 35–45 mmHg. Blood samples for pH, PO2, PCO2, oxygen saturation, and methemoglobin were withdrawn anaerobically into Natelson glass pipettes and analyzed at 39.5°C using a Radiometer OSM3 blood gas analyzer (Copenhagen, Denmark).

Experimental Design

After delivery to the radiant warmer, animals were treated with the following ventilator strategy. The approach to mechanical ventilation described below evolved from experience ventilating the very premature lamb and emphasizes optimizing gas exchange and PaO2 while minimizing the risk of air leak and adverse hemodynamic effects (19, 21). The following protocol was followed during the 4-h period of conventional mechanical ventilation, and mechanical ventilator settings were modified during the course of studies based on the results of serial arterial blood gas samples. Changes in PIP were determined by measurements of PaCO2. If PaCO2 was less than 35 mmHg (4.7 kPa), then PIP was reduced to 30 cmH2O (2.9 kPa). If subsequent measurements of PaCO2 were less than 35 mmHg (4.7 kPa), then PIP was reduced to 25 cmH2O (2.5 kPa). The maximum PIP delivered was 35 cmH2O (3.4 kPa). If PaCO2 was greater than 45 mmHg (6 kPa), then the ventilator rate was increased to a maximum of 60 breaths/min. The inspiratory time was then decreased to maintain an inspiratory-to-expiratory ratio of 1.0 or less. PEEP was changed according to PaO2. If PaO2 was less than 100 mmHg (13.3 kPa), PEEP was maintained at 6 cmH2O (0.6 kPa). With PaO2 greater than 100 mmHg (13.3 kPa), PEEP was decreased to 5 cmH2O (0.5 kPa). If PaO2 was greater than 200 mmHg (26.7 kPa), PEEP was decreased to 4 cmH2O (0.4 kPa).

After the study, animals were killed with T-61 euthanasia solution (American Hoechst, Summerville, NJ) and processed for analysis as described above. Expression of HIF-1α, HIF-2α, eNOS, and the prolyl-hydroxylase-containing domain (PHD) enzymes, which are responsible for initiating HIF degradation in the presence of molecular oxygen, was assessed by Western blot, and VEGF mRNA expression was determined by Northern blot analysis.

Western Blot Analysis

Lung tissues were homogenized and processed for Western blot according to previously published methods (1). Pilot studies indicated that HIFs were undetectable by Western blotting of whole cell homogenates. Therefore, homogenized tissue was pelleted by centrifugation, and the nuclear and cytosolic fractions were immediately extracted using a commercial kit (Pierce, Rockford, IL). Following electrophoresis, protein transfer, and blocking of nonspecific binding, the membranes were incubated with anti-HIF-1α (1:500 dilution; BD Biosciences, San Diego, CA), anti-HIF-2α (1:500 dilution; Novus Biologicals, Littleton, CO), and anti-PHD-1, -2, or -3 antibody (1:500 to 1:750; Novus Biologicals) overnight at 4°C or anti-eNOS (1:1,000, Sigma Aldrich) was used as a loading control. Hypoxia-exposed A549 cells were used as positive controls for both HIF-1α and HIF-2α blots as described (3). Films were scanned and then quantitated using the NIH Image 1.63 program.

On Western blot analysis, both HIF-1α and HIF-2α were detected as bands at 118 kDa, with a background band visible at 75 kDa. PHD-1, -2, and -3 were detected at 46, 46, and 25 kDa, respectively, with a background band visible at 75 kDa, and additional bands were visible at 50 and 150 kDa for PHD-3. Antibody specificity in ovine tissues has been previously reported (29, 33, 34).

Northern Blot Analysis

Northern blot analysis was performed according to previously published methods (26, 38). Total RNA was extracted from frozen sheep lung using Tri reagent. Twenty micrograms of RNA was loaded per lane and electrophoresed in 1% SeaKem LE agarose/phosphate buffered saline. The membranes were then probed with probes specific for HIF-1α, HIF-2α, eNOS, phospho-eNOS, and VEGF. Films were scanned and then quantitated using the NIH Image 1.63 program.
Table 1. Arterial blood gas parameters and hemodynamic measurements during mechanical ventilation (RDS lambs)

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PaO2</th>
<th>PaCO2</th>
<th>OI</th>
<th>Mean Arterial Pressure, mmHg</th>
</tr>
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<tbody>
<tr>
<td>1 h</td>
<td>7.36±0.01*</td>
<td>152±50*</td>
<td>31±3</td>
<td>13±1†</td>
<td>36±3</td>
</tr>
<tr>
<td>2 h</td>
<td>7.26±0.02</td>
<td>54±15</td>
<td>45±4**</td>
<td>36±2</td>
<td>38±2</td>
</tr>
<tr>
<td>3 h</td>
<td>7.20±0.04</td>
<td>41±7</td>
<td>48±4**</td>
<td>48±5</td>
<td>35±3</td>
</tr>
<tr>
<td>4 h</td>
<td>7.28±0.02</td>
<td>38±6</td>
<td>40±6</td>
<td>53±3</td>
<td>34±2</td>
</tr>
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Values are means ± SD. pH, arterial pH; PaO2, arterial PaO2 (mmHg); PaCO2, arterial PaCO2 (mmHg); OI, oxygenation index (FeO2 × mean airway pressure-100)/PaO2; RDS, respiratory distress syndrome. *P < 0.05 vs. all time points, **P < 0.05 vs. 1 h, †P < 0.005 vs. all time points.

buffer and transferred to Hybond N+ membrane (Amersham, Piscataway, NJ). Twenty-five nanograms of sheep-specific VEGF probe (kindly provided by Dr. Elizabeth Perkett, University of New Mexico Health Sciences Center, Albuquerque, NM) was random prime labeled with [α-32P]dCTP to 8 × 106 cpm/μg (Amersham) and hybridized to the blot overnight, followed by increasingly stringent washes and detection on STORM phosphorimagery (Amersham).

Data Analysis

Statistical analysis of protein content was performed by one-way analysis of variance or Mann-Whitney nonparametric analysis. Where significant differences were identified, post hoc analysis was performed using Student-Newman-Keuls test. All statistical measurements were performed using commercially available statistics package (GraphPad Prism, GraphPad Software). The level of statistical significance was set at P < 0.05; results are reported as means ± SD.

RESULTS

Protocol 1: Ontogeny of Lung HIF Expression

Nuclear HIF-1α and HIF-2α protein expression. HIF-1α protein expression decreased by 40% in lungs of term lambs compared with preterm lambs (P < 0.05, Fig. 1A), whereas there was no change in HIF-2α protein expression between the groups (Fig. 1B).

PHD-1, -2, and -3 protein expression. Pulmonary expression of PHD-2 in RDS lambs was nearly double that of control fetal lambs (0.78 ± 0.10 control vs. 1.37 ± 0.22 RDS; P < 0.01; Fig. 5B). In contrast, PHD-1 expression was slightly decreased in RDS lambs (1.18 ± 0.05 control vs. 0.95 ± 0.08; P < 0.05; Fig. 5A), whereas PHD-3 expression was unchanged (Fig. 5C).

VEGF mRNA expression in RDS lambs. To determine whether impaired HIF expression was associated with altered VEGF gene transcription, VEGF mRNA was measured by Northern blot analysis. We found that, in addition to the decrease in HIF expression, VEGF mRNA was decreased by 40% in the lungs of preterm RDS lambs (0.14 ± 0.02 control vs. 0.08 ± 0.01 RDS; P < 0.001).

Arterial blood gas and hemodynamic measurements. We have previously published arterial blood gas and hemodynamic data in this model (20) and report a significant decrease in arterial PaO2 values after 2 h of mechanical ventilation with high inspired oxygen concentrations that persisted until the end of the study (P < 0.05, 1 h vs. all other time points; Fig. 3, Table 1). This hypoxemia was associated with a marked increase in oxygenation index (P < 0.0005, 1 h vs. all time points; Fig. 3B), a fall in arterial pH (P < 0.05 1 h vs. all time points), and an increase in PaCO2 (P < 0.05 1 h vs. 2 and 3 h; Table 1). In contrast, there was no significant change in mean systemic arterial blood pressure during mechanical ventilation.

HIF-1α and HIF-2α protein expression in RDS lambs. We found that preterm lambs with RDS had dramatic reductions in HIF-1α and HIF-2α protein expression in the lung compared with age-matched nonventilated controls. HIF-1α protein expression decreased by nearly 80% (0.98 ± 0.22 control vs. 0.17 ± 0.02 RDS; P < 0.001; Fig. 4A). Similarly, HIF-2α protein expression was decreased by 55% in the lungs of RDS lambs (1.10 ± 0.2 control vs. 0.49 ± 0.1 RDS; P < 0.005; Fig. 4B) compared with control lambs.

PHD-1, -2, and -3 protein expression in RDS lambs. Pulmonary expression of PHD-2 in RDS lambs was nearly double that of control fetal lambs (0.78 ± 0.10 control vs. 1.37 ± 0.22 RDS; P < 0.01; Fig. 5B). In contrast, PHD-1 expression was slightly decreased in RDS lambs (1.18 ± 0.05 control vs. 0.95 ± 0.08; P < 0.05; Fig. 5A), whereas PHD-3 expression was unchanged (Fig. 5C).

Fig. 4. Pulmonary HIF protein expression in RDS lambs. HIF-1α (A; *P < 0.001) and HIF-2α (B; †P < 0.005) protein expression is decreased in preterm RDS lambs (115-day gestation; n = 4) compared with control preterm lambs (115-day gestation; n = 3).

Histology. We found that 4 h of mechanical ventilation induced histological changes consistent with severe lung injury in preterm lambs. Lungs from lambs with RDS were characterized by patchy atelectasis, overall poor lung inflation, marked interstitial thickening, areas of focal hemorrhage, and evidence of neutrophil influx (Fig. 2).
RDS in neonatal lambs. This decrease is likely related to a sudden increase in the activities of all PHDs due to the rapid rise in available molecular oxygen (3) and the ensuing surge in PHD protein PHD-2 expression. The decline in HIF expression in preterm lambs is also accompanied by a decrease in VEGF mRNA, which may contribute to the pathophysiological changes seen in neonatal RDS. In contrast, in nonventilated full-term lambs, lung HIF-1α expression decreased and PHD-2 expression increased at birth, whereas HIF-2α and PHD-1 and -3 expression in the lung remained unchanged.

The HIFs are highly regulated by oxygen tension (17, 32). At birth, exposure to oxygen with initiation of breathing rapidly and dramatically decreases steady-state levels of HIF expression within the lung (1). Although this decline in HIF expression at term serves an important function to control the production of several growth factors and vascular mediators, including VEGF and its receptors and nitric oxide (5, 15, 30), initiation of this cascade in the preterm lung may have critical and devastating long-term consequences. Suppression of VEGF and nitric oxide production during critical stages of lung vascular and alveolar development may lead to long-term impairment of lung growth and function (6, 18, 25), setting the stage for development of BPD. In addition, pharmacological augmentation of HIF expression after preterm birth in primates has been shown to upregulate angiogenic factors such as VEGF and platelet/endothelial cell adhesion molecule (5), and to improve alveolar surface area and lung function, thereby ameliorating some of the pathophysiological changes contributing to BPD (4).

HIF is a heterodimer composed of α- and β-subunits, with the α-subunit exquisitely sensitive to oxygen tension (5, 28, 36, 40). Hypoxia stabilizes the α-subunit, allowing dimerization and binding to a hypoxia-responsive element on various genes, including VEGF, thereby initiating gene transcription and promoting the downstream effects of HIF. In the presence of oxygen, the PHDs are activated, causing hydroxylation of the α-subunit, leading to proteolytic destruction and inhibition of transcriptional activity (32). Studies have suggested that PHDs

**DISCUSSION**

We report that pulmonary expression of HIF-1α and HIF-2α dramatically decreases following preterm birth complicated by

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**Fig. 5.** Lung PHD protein expression in RDS lambs. PHD-2 protein expression is nearly double in RDS lambs (115-day gestation; n = 4) compared with preterm controls (B; 115-day gestation; n = 3; *P < 0.005). PHD-1 protein expression is increased in preterm RDS lambs (A; *P < 0.05), and PHD-3 expression is unchanged (C) compared with control preterm lambs.

**Fig. 6.** Pulmonary VEGF mRNA expression in RDS lambs. VEGF mRNA is decreased in preterm RDS lambs (115-day gestation; n = 4) compared with nonventilated preterm controls (115-day gestation; n = 3; *P < 0.05). VEGF mRNA normalized to 18S rRNA.
are the cellular oxygen sensors, and whereas PHDs 1–3 may have variable effects, PHD-2 is the predominant HIF hydroxylase in most cells (16, 28). Through this nonheme, iron-dependent pathway, molecular oxygen directly regulates HIF-dependent transcription and angiogenic growth factor expression within the lung.

HIF-1α and HIF-2α are critical for normal development. Gene deletion studies have demonstrated that inactivation of HIF-1α is embryonic-lethal, with severe defects in vascular development (17), whereas deletion of HIF-2α leads to severe RDS that is generally fatal within hours of birth and causes severe systemic vascular remodeling (10, 31, 35). Previous studies from our group have shown that in the baboon, both isoforms of HIF (HIF-1α and -2α) are present during late gestation and HIF-1α declines postnatally (1). The present study confirms those findings in the fetal sheep model, as we demonstrate strong expression of both HIF-1α and HIF-2α in preterm lambs, and a decline in HIF-1α, but not HIF-2α, expression in the ovine lung after term birth. In addition to the striking effects of oxygen on HIF expression, pulmonary expression of HIF is also directly affected by inflammation and barotrauma. Long-term mechanical ventilation in baboon models of BPD has previously been shown to impair HIF-1α and HIF-2α expression in the lung and decrease angiogenic proteins and receptors (1, 5). Our study in preterm lambs demonstrates that short-term ventilation (4 h) in high oxygen concentrations has a similar striking effect, causing an 80% reduction in HIF-1α and 55% reduction in HIF-2α expression in preterm neonatal lambs. Furthermore, disruption of HIF signaling in preterm sheep with RDS also significantly impaired VEGF gene transcription, which has important implications for the effect of neonatal RDS and supplemental oxygen on long-term lung alveolar and vascular growth. Impairment of pulmonary VEGF expression during critical windows of lung development has been shown in numerous studies to cause abnormal vascular and alveolar development, contributing to lung hypoplasia, pulmonary hypertension, and BPD (10, 13, 18, 25).

Although this study clearly shows that short-term ventilation with high inspired oxygen disrupts pulmonary HIF expression and its downstream target VEGF, there are limitations. First, due to the severity of illness in these extremely preterm lambs, animals were exposed to high inspired oxygen concentration (FiO2 = 1.00). It is not clear whether exposure to lower oxygen concentrations would have the same effect on HIF and PHD expression. In addition, this model does not allow for distinction between the effects of oxygen exposure vs. ventilator-induced lung injury on HIF expression, although it is likely that both contribute to varying degrees, as studies in fetal primate lung explants have shown that oxygen alone degrades pulmonary HIFs (2). Finally, whereas the effects of RDS on HIF expression and VEGF gene transcription were marked, we found no change in lung eNOS protein expression. This may be related to the relatively short-term nature of this study, since changes in VEGF gene transcription would be expected to occur more rapidly than changes in eNOS protein expression. It is not clear whether longer exposure to mechanical ventilation or measurements at a later time point following ventilation would have altered eNOS protein expression within the lung.

Preterm birth followed by short-term mechanical ventilation with high inspired oxygen induces RDS and dramatically decreases both HIF-1α and HIF-2α nuclear protein expression in the lung. We believe that this rapid decline in HIF expression may be mediated in part by a surge in oxygen availability and PHD-2 expression, leading to enhanced HIF instability and degradation within the lung. Importantly, impaired HIF expression in preterm RDS lambs was also associated with decreased VEGF gene transcription, which has important long-term implications for lung alveolar and vascular growth and development. We speculate that disrupted HIF and VEGF expression in the lungs of preterm lambs causes long-term abnormalities in lung development and may contribute to the pathophysiological changes seen in BPD. We further speculate that preservation of HIF and VEGF expression after preterm birth may reduce lung injury and promote improved lung growth, therefore decreasing the incidence and severity of BPD in premature infants.

REFERENCES


