Pulmonary hypertension impairs alveolarization and reduces lung growth in the ovine fetus

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Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome characterized by abnormal pulmonary vascular tone, reactivity, and structure, leading to sustained elevation of pulmonary vascular resistance (PVR) and severe hypoxemia at birth (19, 24, 31). Mechanisms that cause PPHN are uncertain, but clinical and pathologic studies suggest that chronic intrauterine stress alters the pulmonary circulation during late fetal life, causing failure of PVR to fall at birth (19, 24, 31). Experimental models of PPHN caused by chronic intrauterine constriction of the ductus arteriosus (DA) are characterized by physiological changes such as elevated pulmonary artery pressure and PVR and abnormal vasoreactivity to several physiological and pharmacological stimuli (1). In addition, structural changes characteristic of this experimental model include development of right ventricular hypertrophy (RVH), medial hypertrophy, and distal muscularization of small pulmonary arteries, and an overall reduction in pulmonary vascular density (8, 28, 29).

PPHN is closely associated with diverse clinical diseases, including meconium aspiration syndrome, pneumonia, and congenital heart disease (27, 32, 33, 35). In addition, severe pulmonary hypertension often accompanies several disorders characterized by lung hypoplasia, including congenital diaphragmatic hernia and primary lung hypoplasia (4). Pulmonary vascular disease associated with lung hypoplasia, including congenital diaphragmatic hernia, consists of decreased vessel density and severe structural remodeling of small pulmonary arteries, even in newborns who die in the first days of life (7, 25). However, mechanisms linking impaired lung growth in utero and pulmonary hypertension at birth are unknown.

Because the growth of small pulmonary arteries is closely linked with alveolarization in the developing lung, it has been speculated that impaired vascular growth can directly impair alveolarization (17). Indeed, experimental models have shown that inhibition of pulmonary vascular growth with angiogenic agents (fumagillin and thalidomide) leads to profound impairments of alveolarization and lung growth in the early postnatal period (17). Vascular endothelial growth factor (VEGF), which is critical for normal embryonic vascular development (9, 17), is also necessary for normal pulmonary vascular and alveolar growth, since treatment with a selective VEGF receptor (VEGF-R) 2 inhibitor (SU-5416) causes alveolar simplification in neonatal and adult rats (13, 17, 22). Furthermore, a genetic strain of rat (Fawn-Hooded rat) known for its predisposition to develop pulmonary hypertension has abnormalities in both vascular density and alveolar number and complexity throughout development (21). These studies suggest that normal vascular development is essential for alveolarization and that disruption of angiogenesis during critical periods of lung development may contribute to the development of pulmonary hypoplasia.

Because pulmonary hypertension impairs normal pulmonary vascular growth and is associated with clinical disorders of lung hypoplasia in the newborn, we hypothesized that chronic intrauterine pulmonary hypertension itself can directly impair vascular growth and inhibit lung growth and development. To test this hypothesis, we studied the effect of partial constriction of the DA in utero on lung vascular and alveolar growth in fetal sheep. We found that chronic intrauterine pulmonary hypertension impaired pulmonary vessel growth and reduced alveolar...
larization, as demonstrated by decreased radial alveolar counts (RAC), increased mean linear intercepts (MLI), and reduced lung weight-to-body weight ratios. We conclude that chronic intrapartum pulmonary hypertension directly impairs normal alveolarization and lung growth during late gestation. We speculate that pulmonary hypertension during critical periods of lung growth disrupts normal signaling pathways coordinating pulmonary vascular and alveolar growth and contributes to the development of pulmonary hypoplasia.

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 19 mixed-breed Columbia-Rambouillet pregnant ewes according to previously published methods (1). Animals were assigned to one of two groups: DA ligation [surgery performed at 112–125 days (canalicular/early saccular phase) n = 10] or sham surgery (n = 9). Under isoflurane inhalational anesthesia, the fetal forelimb was exposed through a hysterotomy, and a left thoracotomy was performed. A polyvinyl catheter was placed in the left axillary artery and advanced into the ascending aorta to obtain daily fetal arterial blood gas tensions. In ligated 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. Age-matched control animals underwent the same surgery, including isolation of the DA, but in contrast with PPHN animals, the DA was not ligated. Animals were killed 8–12 days after surgery.

In addition, to determine whether prolonged chronic intrapartum pulmonary hypertension increases the severity of pulmonary hypoplasia, we performed surgery on an additional eight mixed-breed Columbia-Rambouillet pregnant ewes according to the procedure outlined above. Fetal lambs (111–116 days gestation) underwent either surgical constriction of the DA (n = 4) or sham surgery (n = 4) and were examined near term (132–137 days), 21 days after surgery. Data collected from this group of animals were analyzed separately.

At the time of death, the left lung was rapidly frozen in liquid nitrogen and stored at −70°C until time of study. The right lung was inflated through the trachea with 10% buffered formalin at a constant pressure of 30 cmH2O, ligated while under pressure, stored in formalin overnight, and then transferred to 70% EtOH.

RVH was quantified as described below. Morphometric analysis included measurements of wall thickness of small pulmonary arteries, pulmonary vessel density, RAC, and MLI. Western blot analysis was performed for VEGF protein content. In addition, to determine whether pulmonary hypertension inhibited lung epithelial or vascular protein expression, we performed Western blot analysis for an epithelial cell protein, prosurfactant protein C (pro-SP-C), and an endothelial cell protein, platelet endothelial cell adhesion molecule (PECAM).

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 to left ventricle plus septum weight (RV/LV+S) was used as an index of RVH (1).

Morphometric analysis. Formalin-fixed lung tissue was paraffin embedded. Hematoxylin and eosin (H&E) staining was performed on paraffin sections from the lungs of three to seven animals in each study group, and six to ten sections were examined for each animal. The investigator was blinded to the identity of the sections at the time of analysis. Morphometry was performed on small pulmonary arteries (10–40 μm) on H&E-stained lung sections using a Zeiss Interactive Digital Analysis System. Wall thickness and external diameter were directly measured, and percent wall thickness was calculated as (2 × wall thickness/vessel diameter) × 100 to assess medial hypertrophy (1).

We determined vessel density after factor VIII staining by counting the number of positively stained vessels per high-powered field (hpf, ×200). Sections of peripheral lung adjacent to the pleural surface were examined, and fields with large airways or major vessels were avoided.

Alveolarization was measured by the RAC methods of Cooney and Thurlbeck (11) and Emery and Mithal (12) according to previously published methods. MLI were measured on images of each section captured at ×100 magnification as a high-resolution PICT image by a Magnafire digital camera (Optronics, 1,928 × 1,450 pixel resolution) and were analyzed with the use of Stereology Toolbox software (Davis, CA) as previously described (5). The intra-alveolar distance was measured as the MLI, which we determined by dividing the total length of 42 lines drawn across the lung section by the number of

Fig. 1. Effects of chronic intrapartum pulmonary hypertension on right ventricular hypertrophy (A), percent wall thickness of small pulmonary arteries (% WT, B), and pulmonary vessel density (C). RV/LV+S, right ventricle/left ventricle plus septum; PPHN, persistent pulmonary hypertension of the newborn; hpf, high-powered field.
intercepts encountered as determined by the investigator. Lines that crossed large airways or vessels were excluded from analysis. MLI is inversely proportional to the surface area of the lung (37, 38).

Western blot analysis. Western blot analysis for lung VEGF, pro-SP-C, and PECAM protein was performed according to previously published methods (23). Protein assay was performed using the Bradford method, and 20 μg of lung protein were loaded per lane. Samples were run on two separate Bis Tris 4–12% polyacrylamide gels, using Rainbow (Amersham Biosciences, Piscataway, NJ) marker to determine molecular weight. Gels were transferred to a single membrane with a semidry blot transfer apparatus (Bio-Rad Trans-Blot SA; Bio-Rad Laboratories, Hercules, CA). The blot was stained with Ponceau S to ensure consistent loading and transfer. Recombinant human VEGF (Genentech) was run as a positive control on the VEGF Western blot and demonstrated a single band at 17 kDa. Immunodetection was performed with a rabbit polyclonal antibody to VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody to pro-SP-C (Chemicon International, Temecula, CA), or a goat polyclonal antibody to PECAM (Santa Cruz Biotechnology). ECL Plus (Amersham, Arlington Heights, IL) detection was utilized, and luminescence was determined by exposure to X-ray film for 5–60 s. Densitometry was performed with a UMAX scanner and National Institutes of Health Image J software (NIH Research Services Branch, Bethesda, MD). Blots were probed for actin (Sigma, St. Louis, MO), and densitometry values for VEGF, pro-SP-C, and PECAM are presented as normalized to actin. A single band was detected for each protein, at 17 kDa for VEGF, 43 kDa for pro-SP-C, and at 130 kDa for PECAM.

Data analysis. Statistical analysis was performed by one-way analysis of variance or unpaired t-tests. Where significant differences were identified, post hoc analysis was performed by Student-Newman-Keuls test. All statistical measurements were performed with a commercially available statistics package (GraphPad Prism, Graph-
Pad Software). The level of statistical significance was set at $P < 0.05$; results are reported as means $\pm$ SE.

**RESULTS**

**Pulmonary hypertension.** Short-term DA constriction during late gestation (canalicular/saccular phase) caused severe RVH and increased wall thickness of small pulmonary arteries. RVH, as expressed by the RV/LV+S ratio, was increased by 29% after DA ligation (0.71 $\pm$ 0.02 PPHN vs. 0.55 $\pm$ 0.02 control, $P < 0.0001$) when compared with age-matched sham surgery controls (Fig. 1A). Smooth muscle cell hyperplasia of distal pulmonary arteries was evident on histological examination (Fig. 2). Percent wall thickness of small pulmonary arteries increased by 36% after DA ligation (65.4 $\pm$ 4 PPHN vs. 48.1 $\pm$ 3 control, $P < 0.002$; Fig. 1B), and pulmonary vessel density decreased by 40% after DA ligation (21 $\pm$ 2 vessels per hpf, PPHN vs. 35 $\pm$ 3 control; $P < 0.005$; Fig. 1C).

**Morphometric analysis.** On gross examination, lungs after chronic intrauterine pulmonary hypertension appeared smaller (Fig. 3). Although body weight, lung weight, and lung weight-body weight ratios were not different after 8 days of intrauterine pulmonary hypertension late in gestation (Table 1), prolonged pulmonary hypertension (21 days) during the canalicular stage caused a reduction in lung weight-body weight ratio (0.022 $\pm$ 0.002 PPHN vs. 0.029 $\pm$ 0.002 control, $P < 0.05$; Fig. 4 and Table 1).

Intrauterine pulmonary hypertension during late gestation decreased alveolar complexity, as seen on histological examination (Fig. 5), and reduced RAC by nearly 30% after DA ligation (4.9 $\pm$ 0.2 PPHN vs. 6.6 $\pm$ 0.3 control, $P < 0.001$; Fig. 6 and Table 1). In addition, MLI, an estimate of alveolar size, were increased by 20% after pulmonary hypertension during late gestation [16 $\pm$ 1 $\mu$m (PPHN) vs. 13 $\pm$ 1 $\mu$m (control), $P < 0.05$; Fig. 7 and Table 1].

Similar to brief pulmonary hypertension during late gestation (8 days), prolonged (21 days) intrauterine pulmonary hypertension caused alveolar simplification (Fig. 5), although the effect appeared more severe on histological examination. Prolonged pulmonary hypertension caused a 30% decrease in RAC (4.1 $\pm$ 0.1 PPHN vs. 5.8 $\pm$ 0.1 control, $P < 0.0001$; Fig. 6).
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**Fig. 7.** Effects of brief (8 days) late-gestation and prolonged (21 days) chronic intrauterine pulmonary hypertension on mean linear intercepts (MLI).

6 and Table 1) and a 30% increase in MLI (14.4 ± 1.2 PPHN vs. 11.2 ± 0.4 control, *P < 0.05; Fig. 7 and Table 1). Prolonged (21 days) intrauterine pulmonary hypertension caused more severe pulmonary hypoplasia, demonstrated by decreased RAC (4.1 ± 0.1 prolonged PPHN vs. 5.3 ± 0.2 brief late-gestation PPHN, *P < 0.005; Fig. 6 and Table 1) and lung weight-body weight ratio (0.022 ± 0.002 prolonged PPHN vs. 0.034 ± 0.004 brief late-gestation PPHN, *P < 0.05) compared with age-matched lambs exposed to only 8 days of chronic pulmonary hypertension.

Western blot analyses. Chronic intrauterine pulmonary hypertension impaired expression of both pulmonary epithelium- and endothelium-derived proteins. VEGF protein content was decreased by 45% after intrauterine pulmonary hypertension [0.55 ± 0.08 densitometry units (normalized to β-actin) PPHN vs. 0.80 ± 0.07 control, *P < 0.05; Fig. 8]. In addition, expression of both an epithelial cell (type II pneumocyte)-derived protein, pro-SP-C protein [0.90 ± 0.03 densitometry units (normalized to β-actin) PPHN vs. 1.02 ± 0.02 control, *P < 0.05] and an endothelial cell protein, PECAM [0.60 ± 0.07 densitometry units (normalized to β-actin) PPHN vs. 0.83 ± 0.07 control, *P < 0.05] was decreased after intrauterine pulmonary hypertension (Fig. 9).

**DISCUSSION**

Although angiogenesis and alveolarization are closely linked during pulmonary development, mechanisms that coordinate these processes are unknown. The aim of this study was to determine whether chronic pulmonary hypertension can directly impair not only pulmonary vascular growth but also alveolarization and lung growth during late fetal life. We report the novel finding that chronic intrauterine pulmonary hypertension during the late canalicular and saccular phases of lung development directly inhibits alveolar development, as demonstrated by reduced RAC, increased MLI, and decreased lung weight-to-body weight ratios. Prolonged intrauterine pulmonary hypertension increased the severity of both vascular and alveolar changes and impaired lung growth. Because, in this model of PPHN, pulmonary artery pressure is elevated while pulmonary blood flow and Paco2 remain unchanged (1), these findings suggest that chronic intrauterine hemodynamic stress, independently of changes in blood flow or oxygenation, not only impairs normal vascular growth and function but also inhibits normal alveolarization.

We further report that chronic intrauterine pulmonary hypertension not only disrupts normal endothelial production of PECAM protein but also has epithelial cell effects, demonstrated by a decrease in lung pro-SP-C protein content. In addition, chronic pulmonary hypertension decreases lung VEGF expression, thereby inhibiting pulmonary vascular growth, and likely disrupts signals coordinating angiogenesis and alveolarization, resulting in abnormal distal lung differentiation and growth.

Pulmonary hypertension is commonly associated with pulmonary hypoplasia in newborns, although the relationship between the two conditions is poorly understood. Pulmonary hypertension contributes to the high morbidity and mortality of newborns with disorders of lung growth such as congenital diaphragmatic hernia (4), primary pulmonary hypoplasia, and prolonged oligohydramnios (27, 36). In addition, children with bronchopulmonary dysplasia (BPD) (2) or Down syndrome (10) not only have impaired alveolar development and abnormal lung growth but also are at high risk to develop pulmonary hypertension. Pulmonary hypertension during a key window of lung development appears to have important effects on alveolar development and ultimate lung growth.

Numerous growth factors and matrix proteins are involved in the process of lung development, including FGF, PDGF, matrix metalloproteinases, and VEGF (6, 34). The role of VEGF in pulmonary vascular development has been well established (9, 13), and its importance in pulmonary epithelial development is also emerging. VEGF is produced by the epithelium and mesenchyme early in development, but its expression later is localized primarily in the distal pulmonary epithelium (15). Although the receptors for VEGF (VEGF-R1 and -R2) are predominantly expressed in endothelium, VEGF-R2-positive cells have been found in the developing mesenchyme, maintaining close apposition with branching epithelium (15). The close spatial relationship between epithelial tissue and the developing vasculature suggests that cross talk between these, especially regarding proteins such as VEGF, is important for growth and differentiation of the lung.

Because the processes of pulmonary vascular growth and alveolarization are so closely coordinated, it has been speculated that disruption of signals that regulate vascular growth may directly impair alveolar growth as well. Previous studies in our laboratory have shown that treatment of newborn rats with inhibitors of angiogenesis (fumagillin and thalidomide) (17) or with specific inhibitors of VEGF-R2 (SU-5416) (17),
not only impaired vascular growth and caused pulmonary hypertension but also decreased alveolarization and adversely affected lung growth. VEGF appears to be essential for normal distal air space growth, since impaired expression of VEGF 164- and 188-isoforms in mice leads to impaired peripheral vascular growth but also delayed air space formation (14). In addition, VEGF expression is decreased in normoxic animal models of PPHN (3, 16), and clinical studies have shown a decrease in circulating VEGF levels in infants with severe PPHN or fatal BPD (20). We report that chronic intrauterine pulmonary hypertension decreases lung VEGF protein expression and impairs lung vascular growth, thereby disrupting normal alveolarization and lung growth.

Although chronic intrauterine pulmonary hypertension disrupts both pulmonary vascular and alveolar development in the fetus, the mechanisms underlying these findings are uncertain. We found that chronic intrauterine pulmonary hypertension disrupted vascular development and caused lung hypoplasia and that the effect of pulmonary hypertension on lung growth was most severe in animals exposed to prolonged periods of hypertension, suggesting that hemodynamic stress provides an ongoing stimulus that disrupts normal signaling pathways involved in lung development. Mechanical stress and “pulmonary stretch” have been shown to alter regulation of growth factor expression (including VEGF, PDGF) or activity in vitro by initiating cell signaling cascades involved in angiogenesis and alveolar epithelial development (26, 30). Chronic pulmonary hypertension may also impair alveolarization due to a direct effect on either the epithelium, seen as decreased pro-SP-C and VEGF expression, or endothelium, resulting in decreased PECAM expression. Further studies are needed to better define the cellular mechanisms through which chronic intrauterine pulmonary hypertension reduces alveolarization.

Although an association between pulmonary hypertension and impaired lung growth has been noted in both animal models (17, 18, 21, 22) and clinical settings (34), a direct effect of pulmonary hypertension on alveolar structure has not been previously described. Because vascular growth contributes to alveolarization and pulmonary hypertension is associated with clinical disorders of lung hypoplasia in the newborn, we hypothesized that chronic intrauterine pulmonary hypertension itself can directly impair vascular growth and inhibit lung growth and development. We found that chronic pulmonary hypertension induced by constriction of the fetal DA impaired vascular growth, resulted in larger, less complex alveoli, and decreased lung weight-to-body weight ratios. This effect was seen during the canalicular and saccular phases of lung development and was more severe after prolonged exposure to pulmonary hypertension. We conclude that chronic intrauterine pulmonary hypertension during critical periods of lung development impairs vascular and alveolar development and causes pulmonary hypoplasia. We speculate that pulmonary hypertension disrupts critical signaling pathways coordinating vascular and alveolar development and may contribute to the pathogenesis of pulmonary hypoplasia. Better understanding of the mechanisms by which lung vascular and alveolar growth are regulated may lead to new therapies for the treatment of pulmonary hypoplasia.

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