Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus

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Balasubramaniam, Vivek, Timothy D. Le Cras, D. Dunbar Ivy, Theresa R. Grover, John P. Kinsella, and Steven H. Abman. Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 284:L826–L833, 2003. First published January 17, 2003; 10.1152/ajplung.00199.2002.—Platelet-derived growth factor (PDGF) is a potent smooth muscle cell mitogen that may contribute to smooth muscle hyperplasia during the development of chronic pulmonary hypertension (PH). We studied changes in PDGFα- and β-receptor and ligand expression in lambs with chronic intrauterine PH induced by partial ligation of the ductus arteriosus (DA) at gestational age 124–128 days (term = 147 days). Western blot analysis performed on whole lung homogenates from PH animals after 8 days of DA ligation showed a twofold increase in PDGFα- and β-receptor proteins compared with age-matched controls (∗P < 0.05). Lung PDGF-A and -B mRNA expression did not differ between PH and control animals. We treated PH animals with NX1975, an aptamer that selectively inhibits PDGF-B, by infusion into the left pulmonary artery for 7 days after DA ligation. NX1975 reduced the development of muscular thickening of small pulmonary arteries by 47% (∗P < 0.05) and right ventricular hypertrophy (RVH) by 66% (∗P < 0.02). Lung PDGFα- and β-receptor expression is increased in perinatal PH, and NX1975 reduces the increase in wall thickness of small pulmonary arteries and RVH in this model. We speculate that PDGF signaling contributes to structural vascular remodeling in perinatal PH and that selective PDGF inhibition may provide a novel therapeutic strategy for the treatment of chronic PH.

smooth muscle; NX1975; aptamer

PERSISTENT PULMONARY HYPERTENSION of the newborn (PPHN) is a clinical syndrome characterized by sustained elevation in pulmonary vascular resistance after birth, causing right-to-left shunting of blood across the ductus arteriosus (DA) or foramen ovale and severe hypoxemia (14). Clinical and experimental studies suggest that intrauterine stimuli, such as chronic hypoxia or hypertension, contribute to the pathogenesis of PPHN (2, 14, 26). Abnormalities of the pulmonary circulation that contribute to PPHN include increased vascular tone and reactivity, hypertensive structural remodeling, including smooth muscle cell hyperplasia, adventitial thickening, and decreased vascular growth (1, 21, 22).

In pulmonary hypertension, hemodynamic stress due to increased shear and stretch stress contributes to abnormal pulmonary vascular function and structure (1, 26). Mechanisms by which changes in hemodynamic stress result in abnormal vascular structure are unclear. Platelet-derived growth factor (PDGF) is a potent mitogen to vascular smooth muscle cell growth (8, 9, 23, 24). Although PDGF contributes to smooth muscle cell hyperplasia in the systemic circulation, the role of PDGF and its receptors in pulmonary hypertension have not been studied.

PDGF consists of dimers that include two genetically distinct but structurally similar polypeptides (A chain and B chain) (9, 23). PDGF stimulates cell growth through the activation of cell surface receptors α and β (9, 23). The PDGF receptors belong to a family of transmembrane receptor tyrosine kinases that include the epidermal growth factor receptor and vascular endothelial growth factor receptors. In vitro studies suggest that PDGF-B has affinity for both the α- and β-receptors, whereas PDGF-A shows affinity for only the α-receptor (9, 23). PDGF and its receptors play a key role in embryonic development, as inactivation of the genes for PDGF and its receptors causes abnormal kidney, lung, cardiac, and vascular development, which are almost always embryonic lethal (9, 13, 16, 17).

Several animal models have been developed to explore the pathogenesis and pathophysiology of PPHN. Clinical studies have shown striking hypertensive remodeling of pulmonary arteries of newborns with PPHN who died on the first day of life, suggesting that intrauterine events cause PPHN (21). To better understand the pathogenesis of PPHN, we have studied an intrauterine model of PPHN caused by partial ligation of the DA. Chronic DA ligation in fetal lambs causes marked elevation of pulmonary vascular resistance, right ventricular hypertrophy (RVH), abnormal vaso-reactivity, and hypertensive remodeling of small pul-

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monary arteries that resembles those changes seen in the clinical syndrome of PPHN (2, 3, 15, 19). This model initially increases pulmonary blood flow during the first hour of DA ligation; however, pulmonary blood flow returns to basal levels, and pulmonary artery pressure remains elevated for the duration of DA ligation (2). This model has been used for extensive studies of the mechanisms underlying altered vascular reactivity, including downregulation of endothelial nitric oxide synthase (25, 28), but little is known about mechanisms by which hypertension results in smooth muscle cell hyperplasia. In particular, the expression of PDGF and its receptors in the late-gestation fetal lung and their contribution to the development of PPHN has not been studied. We hypothesize that altered PDGF signaling contributes to pulmonary arterial smooth muscle cell thickening in this experimental model of PPHN. To test this hypothesis, we measured PDGF ligand and receptor expression in the normotensive and hypertensive fetal lung. We also studied the effect of a selective PDGF inhibitor on vascular wall remodeling during chronic hypertension.

**METHODS**

**Study animals and protocols.** All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Three different protocols were employed in obtaining tissue samples for analysis.

1) **Maturation-related changes in PDGF ligand and receptor expression.** Studies were performed in fetal lambs from mixed-breed (Columbia-Rambouillet) pregnant ewes in the following groups: 70–101 days, 113–115 days, 123–128 days, 130–140 days of gestation (term = 147 days), and 4–7 days of postnatal age. Animals were anesthetized with pentobarbital sodium, and fetal lung tissues were harvested after cesarean section. Five neonatal lambs in each group (total 25 lambs) were anesthetized with pentobarbital sodium, and lung tissue was obtained after a rapidly performed thoracotomy. Distal lung tissue was rapidly frozen in liquid nitrogen and stored at −70°C until analysis. This tissue was utilized for isolation of mRNA and protein to determine PDGF ligand and receptor expression in these groups.

2) **Effects of intrauterine pulmonary hypertension on lung PDGF ligand and receptor expression.** Twenty-eight mixed-breed (Columbia-Rambouillet) pregnant ewes between 125 and 129 days of gestation (term = 147 days) were fasted 24 h before surgery. Ewes were sedated with intravenous pentobarbital sodium, and fetal lung tissues were harvested after cesarean section. Five neonatal lambs in each group (total 25 lambs) were anesthetized with pentobarbital sodium, and lung tissue was obtained after a rapidly performed thoracotomy. Distal lung tissue was rapidly frozen in liquid nitrogen and stored at −70°C until analysis. This tissue was utilized for isolation of mRNA and protein to determine PDGF ligand and receptor expression in these groups.

3) **Effects of PDGF inhibition on vascular remodeling after chronic intrauterine pulmonary hypertension.** A high-affinity DNA-based aptamer to the PDGF-B chain, NX1975 (7), was made under the left forelimb after local infiltration with lidocaine (2–3 ml, 1% solution). A left-sided thoracotomy was performed but did not have DA ligation and were used as controls.

**Western blot analysis.** Frozen lung samples were homogenized in ice-cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin A. The samples were centrifuged at 1,500 g for 20 min at 4°C to remove cellular debris. Protein content in the supernatant was determined by the Bradford method (4), using bovine serum albumin as the standard. Briefly, 25 μg of protein sample per lane for each were subjected to SDS-PAGE, and proteins from the gel were transferred to nitrocellulose membrane. Blots were blocked overnight in 5% nonfat dry milk in TBS (Tris buffered saline) with 0.1% Tween 20. These blots were incubated for 1 h at room temperature with either rabbit anti-human PDGF-receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human PDGF-receptor antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:1,000 in 5% nonfat dry milk in TBS with 0.1% Tween 20. Blots were incubated for 1 h at room temperature with a goat anti-rabbit IgG-horseradish peroxidase antibody (Santa Cruz Biotechnology). After being washed, bands were visualized by enhanced chemiluminescence (ECL+ kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). Densitometry was performed using NIH Image v1.61. Western blot analysis with increasing amounts of lung protein from a 123-day fetal lamb was used as a standard curve for comparison of the ontogeny samples (range: 5–35 μg). These studies showed that the signal fell within the linear range of Western blot analysis.

**Northern blot analysis.** Total RNA was purified from hypertensive and control fetal lungs using TRI-Reagent (Molecular Research Center, Cincinnati, OH) and the method of Chomczynski and Sacchi (5). The RNA was quantified by measuring the absorbance at 260 nm. Twenty micrograms of total RNA per lung were analyzed using standard Northern
and hybridization techniques and cDNA probes. Rat PDGF-A and PDGF-B cDNA probes were labeled with [\( ^{32} \text{P} \)]dCTP using random-primed labeling (RTS Random Primer DNA Labeling System, GIBCO BRL, Gaithersburg, MD). The 583-bp rat PDGF-A and 534-bp rat PDGF-B were kindly provided by Drs. E. Hoyle and G. Sakuntala Warshamana (Tulane Univ., New Orleans, LA). 18S rRNA oligonucleotide was labeled using terminal deoxytransferase and [\( ^{32} \text{P} \)]dCTP. Blots were hybridized overnight at 65°C. After hybridization, the blots were washed in 1× SSC and 0.1% SDS (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and then at 50°C in 0.4× SSC and 0.1% SDS. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in methanol. The slides were rinsed with 1× PBS. The sections were covered in Dako serum-free block for 15 min. The sections were incubated with 10% goat/2% sheep serum and then with anti-PDGF\( \alpha \)-receptor, anti-PDGF\( \beta \)-receptor, and anti-\( \beta \)-catenin, and probed with the appropriate secondary antibody and visualized with DAB reagent (Dako, Carpinteria, CA) and counterstained with hematoxylin.

**Immunohistochemical staining.** Lung was fixed in 10% buffered formalin for 24 h and then stored in 70% ethanol. Small pieces, 2–6 mm, were paraffin embedded, sectioned 5-\( \mu \)m thick, and mounted on Plus slides. Slides were deparaffinized in HemoDe and rehydrated by immersion in 100% ethanol, 95% ethanol, 70% ethanol, and then 100% water. A 0.1% trypsin solution was placed on the sections for 5 min. The sections were washed with 1× PBS (2.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 138 mM NaCl, 8.1 mM Na\(_2\)HPO\(_4\)). Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in methanol. The slides were rinsed with 1× PBS. The sections were incubated in Dako serum-free block for 15 min. The sections were incubated with 10% goat/2% sheep serum and then with anti-PDGF\( \alpha \)-receptor, anti-PDGF\( \beta \)-receptor, and anti-\( \beta \)-catenin, and probed with the appropriate secondary antibody and visualized with DAB reagent (Dako, Carpinteria, CA) and counterstained with hematoxylin.
ceptor (R&D Systems), or mouse IgG diluted 1:100 in 1/100 PBS with 1% BSA and 0.1% sodium azide overnight at 4°C. After being incubated, the sections were rinsed with 1/100 PBS. The sections were incubated in 10% goat/2% sheep serum for 5 min, followed by incubation with biotin-labeled goat anti-mouse secondary antibody diluted 1:200 in 10% goat/2% sheep serum for 45 min at room temperature. After being incubated with the secondary antibody, sections were rinsed with 1/100 PBS. The sections were incubated with ABC complex (Vector) for 30 min at room temperature, rinsed in 1/100 PBS, and developed with diaminobenzidine (DAB) and hydrogen peroxide. Washing with water stopped the DAB reaction. A light hematoxylin counterstain was applied. Sections were dehydrated by sequential immersion in 70% ethanol, 95% ethanol, 100% ethanol, and then HemoDe before cover-slipping.

RVH. RVH was measured by weighing the right ventricular free wall (RV) and the left ventricle plus septum (LV+S). RVH was assessed as the ratio of the RV/LV+S weights.

Tissue fixation. Fetal sheep lungs were fixed for histology by tracheal instillation of 10% buffered zinc formalin under constant pressure (30 cmH₂O). The trachea was ligated after sustained inflation, and the lungs were excised and immersed in zinc formalin overnight. The lungs were transferred to 70% ethanol, and, after 24 h, lungs were cut into 4- to 5-mm-thick sections and embedded in paraffin. Paraffin sections (5-μm thick) were mounted and stained.

Pulmonary arteriolar wall thickness. Hematoxylin- and eosin-stained sections were coded and evaluated in a blinded manner. Measurements of wall thickness were made for pulmonary arteries with external diameter of <70 μm that were located at the level of the terminal bronchiole or respiratory bronchiole. Four animals per group were examined, and 10 measurements were obtained for each study animal. Measurements of external diameter and medial wall thickness were made with the Zeiss Interactive Digital Analyzer System (Carl Zeiss, Thornwood, NY) as previously described (11). The wall thickness of each artery is expressed as a percentage of the external diameter using the formula \[(\text{2 × medial wall thickness/external diameter}) \times 100\]. Measurements were performed only on vessels that were cut transversely.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed with the Statview software package (SAS Institute, Cary, NC). Statistical compar-

Fig. 3. Effects of intrauterine pulmonary hypertension on lung PDGFα-receptor (A) and β-receptor (B) protein expression. Pulmonary hypertension increased lung PDGFα-receptor and β-receptor protein levels nearly twofold (n = 5 in each group; **P < 0.01, hypertensive vs. control).

Fig. 4. Lung PDGF-A (A) and PDGF-B (B) mRNA expression in control (sham operated) and hypertensive [ductus arteriosus (DA) ligation] animals. There is no change in PDGF-A or PDGF-B mRNA expression in hypertensive animals compared with control (n = 5 in each group).
isons were made using analysis of variance, and, in the case of multiple comparisons, a Fisher’s protected least significant differences post hoc test was used. $P < 0.05$ was considered significant.

RESULTS

Protocol 1: maturation-related changes in PDGF ligand and receptor expression. To evaluate the developmental expression of the PDGF ligand and receptors in late gestation, distal lung samples were obtained from fetal lambs in five groups: 70–101 days, 113–115 days, 123–128 days, 130–140 days of gestation, and 4–7 days of postnatal age. Due to a lack of a commercially available antibody for Western analysis on sheep tissue, we were unable to quantify PDGF-A and PDGF-B protein levels. PDGF-A mRNA expression increased in mid-late gestation and returned to baseline values in the near-term and postnatal animals ($P < 0.01$, Fig. 1). PDGF-B mRNA expression increased from early to middle gestation and then remained stable into the postnatal period ($P < 0.04$, Fig. 1). PDGFα-receptor protein level progressively increased from early to late gestation ($P < 0.02$, Fig. 2). PDGFβ-receptor protein content was highest in the mid-late gestation animal (123–128 days), representing a 7.5-fold increase in protein content compared with the late gestation animal ($P < 0.001$, Fig. 2).

Protocol 2: effect of intrauterine pulmonary hypertension on lung PDGF ligand and receptor expression. Intrauterine hypertension increased PDGFα-receptor protein levels by 93% ($P < 0.01$) when compared with lungs from sham age-matched controls (Fig. 3). Similarly, pulmonary hypertension increased lung PDGFβ-receptor protein levels by 97% ($P < 0.01$; Fig. 3). There was no difference in lung PDGF-A or PDGF-B mRNA expression between hypertensive and control fetal lambs (Fig. 4).

Positive immunoreactivity for the PDGFα-receptor was found in the airway epithelium, fibroblasts, and smooth muscle cells in the late gestation fetus (Fig. 5). PDGFβ-receptor staining is observed in the airway epithelium, fibroblasts, smooth muscle cells, and endothelial cells (Fig. 6). In the fetal lambs with pulmonary hypertension, immunoreactivity for PDGFα- and β-receptors appeared most intense in smooth muscle cells surrounding small vessels from hypertensive animals (Figs. 5 and 6).

Protocol 3: effects of PDGF inhibition on vascular remodeling after chronic intrauterine pulmonary hypertension. In saline-treated lambs, chronic hypertension increased pulmonary artery wall thickness in vessels associated with respiratory bronchiole and terminal bronchiole by 37% above nonhypertensive controls (Fig. 7). NX1975 (2 mg/day) reduced the increase in wall thickness to only 19 and 21% above nonhypertensive controls ($P < 0.05$) in small pulmonary arteries associated with respiratory and terminal bronchioles, respectively. A higher dose of NX1975 (4 mg/day) reduced wall thickness to 20 and 26% above nonhypertensive controls ($P < 0.05$) in small pulmonary arteries associated with respiratory and terminal bronchioles, respectively (Figs. 7 and 8). RVH, as measured by the ratio $RV/LV$$S$, was increased by 40% in the DA ligation animals. NX1975 treatment at a dose of 4 mg/day, but not at 2 mg/day, attenuated the severity of RVH by

![Fig. 5. PDGFα-receptor immunostaining in sham-operated late-gestation lambs (A and C) and after chronic intrauterine hypertension (DA ligation; B and D). There is increased PDGFα-receptor staining (brown color, arrows) around the hypertensive vessels. Magnification for A and B, ×200; magnification for C and D, ×400.](http://ajplung.physiology.org/)

$L830$ PDGF IN FETAL PULMONARY HYPERTENSION

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DISCUSSION

We report that lung PDGF receptor protein content increases with advancing gestational age in the fetal lamb and that the highest levels of protein content occur during the late canalicular stage and the early saccular stage of lung development in the fetal sheep. Chronic intrauterine pulmonary hypertension during late gestation increased PDGFα- and β-receptor protein by 93 and 97%, respectively. However, mRNA expression of the ligands PDGF-A and -B did not change after chronic pulmonary hypertension. Inhibition of PDGF-B with the selective PDGF-B aptamer NX1975 reduced smooth muscle cell thickening of small pulmonary arteries and reduced RVH. Overall, these findings demonstrate that chronic fetal pulmonary hypertension upregulates PDGF receptor protein content and that PDGF inhibition attenuates the severity of vascular remodeling in this experimental model of PPHN.

Little is known about the actual role of PDGF and its receptors in the pathogenesis of pulmonary hypertension. This study is the first to describe the ontogeny of PDGF receptor protein content in the fetal lamb, the increase in PDGF receptor protein content with pulmonary hypertension, and the effect of PDGF-B blockade on the development of pulmonary hypertension. PDGF expression has previously been investigated in experimental models of shear and stretch stress in the systemic circulation but not in the lung vasculature. PDGF mRNA levels are elevated by shear stress in human umbilical vein endothelial cells in vitro (10). In vivo studies using carotid arteries and mesenteric arteries have shown that hemodynamic stress increases PDGF mRNA expression (18, 27). The effects of hemodynamic force on PDGF receptor expression have not
been studied. Models of chronic hypoxic pulmonary hypertension in adult rats report an increase in PDGF mRNA (12). No published studies have examined the role of PDGF receptors in chronic pulmonary hypertension, and the effects of PDGF-B blockade on vascular structure have not been studied.

This model of perinatal pulmonary hypertension, created by partial ligation of the DA in the late fetal lamb, has been used to investigate mechanisms that lead to failure of postnatal adaptation of the pulmonary circulation in the early postnatal period, including impaired nitric oxide-cGMP and endothelin signaling (2, 3, 11, 19, 25, 28). Previous studies have not examined the role of PDGF in the development of neonatal pulmonary hypertension or the effects of hypertension on the expression of PDGF and its receptors. We report that chronic intrauterine pulmonary hypertension increases PDGF receptor expression, suggesting a role for PDGF in pulmonary arterial smooth muscle cell thickening in pulmonary hypertension. This concept is further supported by our findings that treatment with NX1975, a selective PDGF inhibitor, reduced pulmonary arterial wall thickness and...
RVH in this experimental model. Previous studies have shown that the NX1975 aptamer is a highly selective inhibitor of PDGF-B by binding to PDGF-B and preventing its interaction with PDGF receptors (6, 7). The NX1975 aptamer has been shown to have a high affinity for PDGF-B with a $K_d = 10^{-10}$ M (7). Intravenous injections in rats show a half-life of 12 h with the serum concentration not lower than $4 \times 10^{-8}$ M with this dosing plan (6).

Potential limitations of this study include the fact that we did not demonstrate direct effects of PDGF-B blockade on pulmonary vascular artery pressure in PPHN. Also, we were unable to measure PDGF ligand peptide levels in whole lung homogenates. These studies are primarily designed to examine the role of PDGF on vascular remodeling due to smooth muscle cell hyperplasia. In addition, we have only examined the contribution of PDGF-B in this model, and future work shall be done on the effect of combined PDGF-A and PDGF-B blockade, when a specific PDGF-A aptamer becomes available. Future work shall investigate the physiological correlates of the structural effects of PDGF inhibition in this model and the effects of PDGF inhibition on alveolar and pulmonary vascular development.

In summary, chronic intrauterine pulmonary hypertension caused by DA ligation increases PDGF-α-receptor and PDGFβ-receptor protein levels in the late-gestation fetal lung. We speculate that an imbalance in PDGF receptor expression contributes to the smooth muscle proliferation that is seen in perinatal pulmonary hypertension. Blockade of PDGF signaling in this model of intrauterine pulmonary hypertension reduces pulmonary vascular smooth muscle cell thickening and pathological RVH after DA ligation. Further studies are needed to define the exact consequence of PDGF blockade on vascular tone and reactivity and on lung vascular and alveolar development, but targeting the PDGF signaling system may be a novel therapy in the treatment of PPHN.

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