Endothelin-1 decreases endothelial PPARγ signaling and impairs angiogenesis after chronic intrauterine pulmonary hypertension

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For infants who fail to respond to iNO, extracorporeal membrane oxygenation therapy is often necessary but is costly and often associated with increased morbidity and mortality. How function and structure of the fetal pulmonary circulation are altered in utero leading to the failure to adapt at birth is fundamental toward understanding and treating PPHN. Pulmonary artery endothelial cell (PAEC) dysfunction contributes to impaired angiogenesis in experimental PPHN; however, mechanisms responsible for PAEC dysfunction in PPHN are poorly understood.

ET-1 is strongly expressed in the developing lung and maintains high PVR in the normal fetus (28, 63). ET-1 has also been implicated in the pathogenesis of PPHN in experimental models as well as in the clinical setting (12, 13, 29–31, 42, 62). Human newborns with severe PPHN have high circulating ET-1 levels (53), and lung ET-1 content is markedly increased in infants who died with severe PPHN and congenital diaphragmatic hernia (CDH) (34, 38). Although impaired vascular growth contributes to the pathobiology of severe PPHN, the potential role of ET-1 in the developing lung is controversial. Studies of adult rats with chronic hypoxia-induced pulmonary hypertension suggest that lung angiogenesis is increased after chronic hypoxia (27). In addition, in vitro studies of isolated human umbilical vein endothelial cells, have demonstrated that ET-1 increases tube formation in vitro (55). In striking contrast, angiogenesis and lung vascular density is decreased in experimental PPHN (24), and we have previously shown in this model that increased ET-1 production impairs fetal PAEC tube formation in vitro and nonselective ET-1 blockade is protective (20). These differences are in part due to differences in species and developmental timing and demonstrate the importance of studying PAECs isolated from a developmentally relevant model. In addition, they confirm the importance of ET-1 in the pathogenesis of severe PPHN, especially in the setting of lung hypoplasia. Mechanisms through which increased ET-1 production decrease PAEC function and impair angiogenesis in the developing lung remain unknown.

Peroxisome proliferator-activated receptor (PPAR) is an early phylogenetic member of the nuclear receptor superfamily (40). There are three isotypes of PPAR: α, δ, and γ (14, 18, 28). PPARγ is abundantly expressed in normal lung, with the highest levels in the lungs compared with other organs (1). Through different stages of fetal development, PPARγ is present at constant levels in the lung (1) and decreases during adulthood. In adult patients with pulmonary hypertension, lung PPARγ expression is markedly reduced (3, 8). In experimental models of pulmonary hypertension and chronic lung disease, activation of PPARγ prevents pulmonary hypertension and reduces lung injury (3, 8, 35, 47, 48, 56, 58).

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an important role in lung and alveolar development as PPARγ knockout mice demonstrate decreased alveolarization (56) and PPARγ agonists improves lung structure after both hypoxia-(8) and hyperoxia-induced neonatal lung injury (58). However, the role PPARγ signaling plays in contributing to PAEC dysfunction and impaired angiogenesis in PPHN remains unknown.

Past studies have shown that partial ligation of the ductus arteriosus (DA) in late gestation fetal sheep provides a useful animal model for studying the pathogenesis and treatment of PPHN (2). In this model, partial DA ligation increases pulmonary artery pressure without causing sustained elevations of pulmonary blood flow or hypoxemia (2). PVR remains elevated at birth, with extrapulmonary shunting and hypoxemia (2), closely mimicking severe PPHN in human newborns. Along with changes in vascular tone, DA ligation impairs lung vascular and alveolar growth in vivo and causes sustained abnormalities of PAEC growth and tube formation in vitro (21, 24), providing a useful in vitro model to study how hemodynamic stress induced by hypertension disrupts normal endothelial cell signaling and function and impairs vascular growth in PPHN.

Increased ET-1 production and decreased PPARγ signaling contribute to the pathogenesis of adult pulmonary hypertension, but whether these two pathways interact to decrease PAEC function and impair angiogenesis in PPHN remains unknown. We therefore hypothesized that increased ET-1 production by PPHN PAECs decreases PPARγ activity, which impairs PPHN PAEC tube formation in vitro. Our findings support the hypothesis that ET-1 plays a critical role in the pathogenesis of PPHN and that its effects are partially mediated by decreased PPARγ signaling, which contributes significantly to impaired angiogenesis in PPHN.

### METHODS

#### Isolation and culture of fetal ovine PAECs.

All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University, Colorado Health Sciences Center. Left and right pulmonary arteries were isolated from late-gestation normal fetal sheep [mixed-breed Columbia Rambouillet pregnant ewes at 135 days gestation (n = 4), full term 147 days] and from fetal sheep that had undergone partial ligation of the DA in utero 7–10 days before euthanasia (PPHN; n = 4), as previously described (7, 43, 61).

Proximal PAECs were isolated and characterized as previously described (21, 39). PAEC phenotype was confirmed by positive immunostaining for von Willebrand factor, endothelial NO synthase (eNOS), vascular endothelial cadherin, and vascular endothelial growth factor receptor type 2 (KDR), positive uptake of acetylated low density lipoprotein (LDL), and negative staining for desmin. For all experiments, PAECs from four normal and four PPHN animals were used at passages 4 and 5, and cells from each animal were kept separate throughout all passages and for all experiments. All experiments are repeated two or three times to confirm reproducibility. Since PAECs were harvested from fetal sheep, for all experiments PAECs were incubated in 3% oxygen conditions to simulate the low-oxygen environment in the normal fetus in utero (5, 21).

#### siRNA transfection.

PPARγ siRNA (ovine) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA; catalog no. SC-156097), and siRNA transfection was performed per manufacturer’s recommendations and protocol. PPARγ siRNA is an ovine-specific pool of three target-specific 19–25 nt siRNAs designed to knock down PPARγ gene expression. Briefly, in a six-well tissue culture plate, PAECs from four normal and four PPHN animals were incubated at 3 × 10^5 cells per well in 2 ml antibiotic-free normal growth medium supplemented with FBS at 37°C in 5% CO2 and allowed to adhere overnight. Growth medium was removed and transfection reagents (siRNA and Scramble) were added to duplicate wells for a final volume of 1 ml. Duplicate wells were utilized, allowing for molecular analysis of transfection efficacy and utilization of transfected cells for the experiments outlined below. PPARγ siRNA (4 μM) and control siRNA (Santa Cruz Biotechnology, catalog no. SC-37007) were mixed with equal parts of transfection reagent (Santa Cruz Biotechnology, catalog no. SC-29528) and added to the appropriate wells for 6 h. Multiple doses of siRNA (0–8 μM) were tested in preliminary experiments and 4 μM siRNA utilized as this dose produced the greatest knockdown of PPARγ expression and activity without causing apoptosis. Two control wells were also prepared receiving only antibiotic-free normal growth medium supplemented with FBS to ensure that the scramble RNA was not having an effect on cellular signaling and function. After the 6-h incubation, normal growth medium with 20% FBS was added to each well without removing the transfection mixture. Cells were incubated for an additional 18–24 h and subsequently passaged for study. After passage, there was no further exposure to siRNA, and transfected cells were handled utilizing the same conditions as controls. Western blot analysis was performed after passage on whole cell lysates and revealed a 56% decrease in total PPARγ protein expression and 53% decrease in the ratio of phosphorylated to total PPARγ protein (PPARγ activity) in normal PAECs after siRNA treatment.

#### Tube formation assay.

The ability of fetal PAECs to form vascular structures in vitro was assayed by plating of PAECs on type I bovine collagen (5 mg/ml; R&D Systems, Minneapolis, MN; catalog no. 3442-050-01) for 5–7 days. PAECs from four normal and four PPHN animals were utilized for all experiments, and for each condition four replicates were studied. A collagen-cell suspension consisting of collagen (1.25 mg/ml) and PAECs from normal and PPHN fetal sheep (2.5 × 10^5 cells/well) was pipetted into 24-well tissue culture dishes (500 μl/well) and allowed to polymerize at 37°C for 1 h. After polymerization, DMEM with 10% FBS, supplemented with rosiglitazone (Rosi; 100 μM; Cayman Chemical, Ann Arbor, MI catalog no. 71740), 15deoxy-Δ12,14 prostaglandin J2 (PG-J2; 5 μM; Cayman Chemical, catalog no. 18570), with and without N-nitro-l-arginine (L-NAME) 4 mM; Sigma, St. Louis, MO; catalog no. N5501) and ET-1 (100 nM; Sigma, catalog no. E7764) was added to each well depending on study conditions. Tube formation was also assessed after exposure to PPARγ siRNA and control. Doses for each drug were determined by preliminary experiments and published studies (20, 21, 49, 50, 53, 56). The lowest dose for which an effect was seen was used for all drugs. Because of the concern for off-target effects with Rosi, a second known PPARγ agonist, PG-J2, was used to determine whether similar effects would be seen with each agent. Tube formation was quantified via Fovea 3 software analysis (Microsoft, Redmond, WA) by counting the number of branch points per high-power field (HPF) and measuring tube length (inches).

#### Western blot analysis.

PAECs from normal and PPHN fetal sheep (n = 4) were grown on 150-mm cloning dishes in DMEM supplemented with 5% serum. At 70% confluence, PAECs were treated with Rosi (100 μM), PG-J2 (5 μM), and ET-1 (100 nM) with or without bosentan (1 μM; Actelion Pharmaceuticals, San Francisco, CA) for 24 h. Cell lysates were also collected after exposure to PPARγ siRNA and control siRNA, and 70% confluence was chosen because prior studies have demonstrated downregulation of important cell signaling pathways with confluence (63). At the time of harvest PAECs were 95% confluent. Cells were washed twice with ice-cold PBS, trypsinized, and centrifuged, and the supernatant was removed. The cell pellet was lysed via sonication in radioimmunoprecipitation buffer [PBS, 1% Nonidet P-40, 0.5%, sodium deoxycholate, 0.1% SDS, PMSF (10 mg/ml), aprotinin (16 μl/ml), and 1 mM sodium orthovanadate]. The supernatant was centrifuged at 10,000 g for 30 min at 4°C. Protein content in the supernatant was determined by the BCA assay.
samples were plated for each condition and each clone of cells. Cells were allowed to adhere overnight; then normal and PPHN PAECs were incubated with DAF-FM and with and without 100 μM Rosi and 5 μM PG-J2 in PBS for 1 h. VEGF (25 ng/ml) and LNA (4 mM) were used as positive and negative controls to ensure specificity of DAF-FM for the detection of intracellular NO. Green fluorescence intensity was measured with excitation at 495 nm and emission at 515 nm. The single-wavelength dye DAF-FM increases fluorescence emission intensity concomitant with an increase in NO production. Differences in fluorescence between normal and PPHN cells were compared.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed with the Prism 4 software package (GraphPad Software, San Diego, CA). Statistical comparisons were made by analysis of variance for tube formation, NO assays, and Western blot analysis with Bonferroni posttest analysis and unpaired t-test where appropriate.

RESULTS

Decreased PPARγ in PAECs from PPHN lambs. When compared with controls, PPARγ protein expression and activity were decreased in PPHN PAECs. By Western blot analysis on whole cell lysates, total PPARγ protein expression was decreased by 35% (P < 0.01; Fig. 1A) and phospho-PPARγ (p-PPARγ)-to-PPARγ ratio decreased by 33% (P < 0.05; Fig. 1B) in PAECs from PPHN lambs.

Effect of PPARγ inhibition on tube formation in normal PAECs. Knockdown with PPARγ siRNA decreased tube formation in normal PAECs. Tube length and the number of branch points were each decreased by 34% in normal PAECs after siRNA treatment (P < 0.01; Fig. 2).

Effect of PPARγ stimulation on tube formation in normal and PPHN PAECs in vitro. Compared with controls, tube length was decreased by 27% (P < 0.01) and the number of branch points per HPF was decreased by 32% (P < 0.01) in PPHN PAECs at baseline (Fig. 3). Treatment with Rosi (100 μM) and PG-J2 (5 μM) increased tube formation in normal and PPHN PAECs. In normal PAECs, tube length was increased by 29 and 24% and number of branch points per HPF were increased by 45 and 37% with Rosi and PG-J2 treatments, respectively (P < 0.01; Fig. 3A). In PPHN PAECs, tube length was increased by 34 and 40% (P < 0.01; Fig. 3B) and number...
increased NO production in normal and PPHN PAECs. NO production was increased by 31 and 34% with Rosi and PG-J2 treatment, respectively, in normal PAECs. In PPHN PAECs, NO production increased by 128 and 86% with Rosi and PG-J2 treatment (P < 0.01; Fig. 4B).

Effect of PPARγ inhibition on eNOS protein expression in normal PAECs. PPARγ inhibition by siRNA decreased eNOS expression in normal PAECs. eNOS protein expression decreased by 38% (P < 0.01; Fig. 4C) with PPARγ siRNA.

Increase in tube formation with PPARγ activation is NO dependent in normal and PPHN PAECs. NO synthase inhibition with LNA (4 µM) prevented the increase in tube formation in normal and PPHN PAEC seen with PPAR agonists Rosi and PG-J2. In both normal and PPHN PAECs, Rosi and PG-J2 increased tube length and number of branch points per HPF. With the addition of LNA to Rosi and PG-J2 treatments both number of branch points per HPF and length were restored to untreated values (P < 0.01; Fig. 5). LNA alone decreased tube length and number of branch points per HPF in normal PAEC. With LNA treatment, number of branch points per HPF decreased by 31% (P < 0.05) and tube length by 25% (P < 0.05) in normal PAECs. In PPHN PAECs, no further decrease in tube length and number of branch points per HPF was seen with LNA treatment.

ET-1 decreases PPARγ protein expression and activity in normal and PPHN PAECs. Effect of ET-1 on PPARγ protein expression and activity was assessed in normal and PPHN PAECs by Western blot analysis on whole cell lysates. At baseline PPHN PAECs, total PPARγ protein expression was decreased by 27% and activity was decreased by 50% (P < 0.01; Fig. 4A). Treatment with ET-1 decreased total PPARγ protein expression and PPARγ activity by 39 and 42% (P < 0.01 for each comparison) in normal PAECs and 35 and 36% in PPHN PAECs, respectively (P < 0.01 for each comparison; Fig. 6A).

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Fig. 2. Decreased 3-dimensional tube formation by normal PAECs after PPARγ silencing with siRNA (4 µM). Silencing of PPARγ expression by use of siRNA decreased PPARγ activity by 53% (P < 0.01) and PPARγ expression by 56% in normal PAEC (A). PPARγ siRNA decreased tube length and number of branch points per high-power field (HPF) in normal PAEC (P < 0.01, respectively) (B). Values are means ± SE.

Fig. 3. Effect of PPARγ agonist treatment on tube formation in normal and PPHN PAECs. At baseline, tube length decreased by 27% (P < 0.01) and the number of branch points per HPF was decreased by 32% (P < 0.01) in PPHN PAECs. Treatment of normal PAEC with PPARγ agonist rosiglitazone (Rosi; 100 µM) and 15d-prostaglandin-J2 (PG-J2; 5 µM) increased branch points per HPF and tube length in normal PAECs (P < 0.01 for each comparison) (A). In PPHN PAECs, Rosi and PG-J2 increased branch points per HPF and tube length (P < 0.01 for each comparison), restoring tube formation by PPHN PAECs to normal values. Values are means ± SE.
Fig. 4. Effect of PPARγ agonists [Rosi (100 μM) and PG-J2 (5 μM)] on endothelial NO synthase (eNOS) activity (p-eNOS-to-eNOS ratio) and NO production in normal and PPHN PAECs. Rosiglitazone and PG-J2 treatment increased eNOS activity (p-eNOS-to-eNOS protein ratio) (A) and NO production (B) in normal and PPHN PAECs (P < 0.01 for each comparison). VEGF (25 ng/ml) (positive control) increased NO production and N-nitro-L-arginine (LNA; 4 μM) decreased NO production, indicating specificity of 4-amino-5-methylamino-2,7-difluorofluorescein (DAF-FM) compound for detecting intracellular NO production. C: PPARγ silencing with siRNA (4 μM) decreased eNOS expression in normal PAEC (P < 0.01). Values are means ± SE.
ET receptor blockade (bosentan) prevents the decrease in PPARγ protein expression and activity after ET-1 treatment. Effect of bosentan on PPARγ protein expression and activity was assessed in normal and PPHN PAECs in the presence and absence of ET-1 (Fig. 6B). ET-1 decreased total PPARγ protein expression (data not shown) and PPARγ activity by 39 and 42% (P < 0.01 for each comparison) in normal PAECs and 35 and 36% in PPHN PAECs, respectively (P < 0.01 for each comparison). Bosentan alone had no effect on PPARγ protein expression (data not shown) and activity. Bosentan prevented the decrease in PPARγ protein expression (data not shown) and activity after ET-1 treatment in both normal and PPHN PAECs (Fig. 6B).

Effect of ET-1 treatment on tube formation is prevented by PPARγ agonists. ET-1 treatment decreased tube formation by normal and PPHN PAECs. After ET-1 treatment (100 nM), tube length was decreased by 30% in both normal and PPHN PAECs (P < 0.05) and branch points per HPF by 38% in normal and 44% in PPHN PAECs (Fig. 7; P < 0.05 for each comparison). Treatment with Rosi and PG-J2 restored tube formation to normal values in normal and PPHN PAECs after ET-1 treatment. In normal PAECs, Rosi and PG-J2 increased tube length by 54% (P < 0.01) and branch points by 63 and 67% (P < 0.01 for each comparison) after ET-1 treatment. In PPHN PAECs, Rosi and PG-J2 increased tube length by 48 and 50% (P < 0.01 for each comparison) and branch points by 66 and 60% (P < 0.01 for each comparison; Fig. 7) after ET-1 treatment restoring tube formation by PPHN PAEC to similar values seen in normal controls.

DISCUSSION

Decreased PPARγ signaling contributes to the pathogenesis of pulmonary hypertension in adult models (3), but whether PPARγ regulates angiogenesis in the developing lung and contributes to impaired angiogenesis in PPHN has not been previously studied. We report decreased PPARγ protein and activity and decreased tube formation by PPHN PAECs. PPARγ agonists, Rosi and PG-J2, increased tube formation in normal PPHN PAECs, restoring tube formation by PPHN PAECs to normal values. Rosi and PG-J2 increased eNOS protein and activity and NO production and, conversely, PPARγ silencing decreased eNOS protein expression. The effects of PPARγ agonists on tube formation were NO dependent, since these effects were absent during concurrent treatment with a NOS inhibitor (LNA). ET-1 decreased PPARγ protein and activity, which was prevented with nonselective ET receptor blockade. ET-1 decreased tube formation in normal and PPHN PAECs, and PPARγ agonists restored tube formation to normal after ET-1 treatment. Our findings suggest that ET-1 plays an important role in the regulation of PPARγ and that increased ET-1 production decreases PPARγ signaling, resulting in PAEC dysfunction and impaired angiogenesis in PPHN.

Past studies in adult rodents have suggested that lung angiogenesis is increased in chronic hypoxia-induced pulmonary hypertension (27). In striking contrast, extensive work with experimental models of PPHN has clearly shown that decreased arterial density contributes to high PVR in severe
PPHN, especially in the setting of lung hypoplasia (22, 24, 26). Past studies have shown that pulmonary hypertension during late gestation impairs fetal lung vascular growth in vivo (24) and causes abnormalities in PAECs that persist in vitro (21). The PAEC PPHN phenotype is characterized by decreased growth and tube formation, increased ET-1 production, and altered VEGF-NO signaling (20, 21). Although these findings provide some insights into mechanisms responsible for impaired angiogenesis in severe PPHN, how sustained elevations in pulmonary arterial pressure inhibit lung angiogenesis during development is poorly understood. In this study, we demonstrate endothelial dysfunction with decreased PPHN PAEC tube formation in vitro and decreased PPARγ protein and activity in PAECs from an experimental model of PPHN. PPARγ agonists rescue the in vitro PPHN PAEC phenotype restoring PPARγ activity to baseline values. Bosentan alone had no effect on PPARγ protein expression (data not shown) and activity. Values are means ± SE. For all figures representative blots are shown.

Fig. 6. Effect of endothelin-1 (ET-1) on PPARγ protein expression and activity was assessed in normal and PPHN PAECs by Western blot analysis on whole cell lysates. Compared with controls, total PPARγ protein expression and activity were decreased in PPHN PAECs. Treatment with ET-1 (100 nM) decreased total PPARγ protein expression and PPARγ activity in both normal and PPHN PAECs, respectively (P < 0.01 for each comparison) (A). Values are means ± SE. For all figures representative blots are shown. Bosentan (Bos; 1 μM) (nonselective ET receptor blocker) prevented the decrease in PPARγ protein expression (data not shown) and activity (B) after ET-1 treatment in both normal and PPHN PAECs, restoring PPARγ activity to baseline values. Bosentan alone had no effect on PPARγ protein expression (data not shown) and activity. Values are means ± SE. For all figures representative blots are shown.
ET-1-PPARγ INTERACTIONS IN PPHN

Fig. 7. Effect of ET-1 (100 nM) on tube formation with and without PPARγ agonists [Rosi (100 μM) and PG-J2 (5 μM)] was assessed in normal and PPHN PAECs. ET-1 treatment decreased tube length and number of branch points per HPF in normal and PPHN PAECs (P < 0.05 vs. control). After ET-1 treatment, Rosi and PG-J2 increased tube length and number of branch points per HPF in both normal and PPHN PAECs (P < 0.01 for each comparison), restoring tube formation to normal values in normal and PPHN PAECs after ET-1 treatment. Treatment with Rosi and PG-J2 restored tube formation by PPHN PAEC to similar values seen in normal controls. Values are means ± SE.

Eling and pulmonary hypertension (8, 48). PPARγ is present in the fetal lung (1) and is an important regulator of lung and alveolar development (56). In a recent study, knockout of PPARγ in mice resulted in decreased radial alveolar counts and lung hypoplasia (56). In this study the presence of pulmonary hypertension was not assessed; however, other studies have demonstrated the development of significant pulmonary hypertension and right ventricular hypertrophy after PPARγ knockdown (25). After neonatal hypoxia and hyperoxia, activation of PPARγ improves lung structure (8, 58) and prevents the development of pulmonary hypertension. Mechanisms by which PPARγ regulate lung alveolar growth are unknown. Past studies have suggested that inhibition of vascular growth impairs alveolarization in the developing lung (32), leading one to speculate that restoration of PAEC function has the potential to improve alveolar growth. The ability of PPARγ agonists to rescue the in vitro PPHN PAEC phenotype proposes therapeutic benefit in the setting of PAEC dysfunction and lung hypoplasia.

We have previously reported decreased eNOS expression in PAECs from PPHN fetal sheep and that exogenous NO treatment restores PPHN PAEC function to normal (21). PPARγ is an important regulator of eNOS protein expression and activity (9, 23, 37, 50). PPARγ knockout mice produce less NO than wild-type controls (37), and PPARγ activation in PAEC increases NO production in a PPARγ-dependent mechanism (50). PPARγ protein expression and activity are decreased in PPHN PAECs and silencing of PPARγ with siRNA decreases eNOS protein, suggesting that PPARγ plays an important role in regulating eNOS protein expression in fetal PAECs. PPARγ agonists increase endogenous NO production in PAECs in an NO-dependent manner and restores tube formation in PPHN PAECs, confirming that altered PPARγ regulation of eNOS and NO production contributes to PAEC dysfunction and impaired angiogenesis in PPHN.

ET-1 has been implicated in the pathogenesis of diverse neonatal diseases including bronchopulmonary dysplasia (BPD), a chronic lung disease of infancy characterized by decreased alveolar and vascular growth. In newborn infants with respiratory distress syndrome (RDS), the degree of elevation in plasma ET-1 concentrations predicted higher risk for the subsequent development of BPD (19, 49). In newborn lambs with RDS and PPHN, as well as human infants with PPHN and CDH, plasma ET-1 levels predict disease severity and degree of pulmonary hypertension (15, 34, 53). These studies suggest the importance of ET-1 in the pathogenesis of severe neonatal pulmonary hypertension, especially in the setting of lung hypoplasia. Kang et al. (33) recently reported a link between ET-1 and PPARγ in hypoxia-induced pulmonary hypertension, demonstrating in adult mice exposed to hypoxia and isolated human PAECs that PPARγ agonists were able to suppress hypoxia induced enhanced ET-1 signaling. We have previously shown that PPHN in fetal sheep increases PAEC ET-1 mRNA and protein expression and that ET-1 inhibition improves PAEC tube formation, suggesting that increased ET-1 production by PPHN PAECs is responsible for PAEC dysfunction and impaired angiogenesis in the developing lung (20). Our findings are consistent with those of Kang et al., demonstrating that PPARγ agonists were able to reverse the effect of ET-1 on PAEC function and tube formation in vitro. These findings confirm a link between ET-1 and PPARγ and suggest that ET-1-PPARγ interactions contribute to the pathogenesis of PAEC dysfunction in PPHN. Despite our prior work demonstrating that the effect of ET-1 on PAEC function was in part due to ET-1 activation of Rho-kinase (ROCK) (20), in this study we were unable to demonstrate a link between PPARγ and ROCK in PAECs (data not shown), proposing an alternate mechanism for ET-1-mediated impaired angiogenesis in PPHN.

Exogenous ET-1 treatment decreased tube formation and PPARγ expression and activity in both normal and PPHN PAECs, demonstrating that even in the setting of enhanced ET-1 production exogenous ET-1 administration was still able to suppress PPARγ expression and activity. This finding is interesting because it suggests that the cell signaling pathway responsible for ET-1 suppression of PPARγ protein expression and activity is not maximally downregulated at baseline in PPHN PAECs and that further increases in ET-1 production have the potential to further suppress PPARγ protein expression and activity. Nonselective ET receptor blockade with bosentan restored PPARγ expression and activity to normal values, confirming that ET-1 suppresses PPARγ expression
and activity through the ET<sub>B</sub> receptor on PAECs. ET-1 activation of ET<sub>B</sub> receptors increases NO production (10); however, other published reports propose ET<sub>B</sub> receptor-mediated effects other than NO release, demonstrating increases in reactive oxygen species (16) and thromboxane A2 release (17) with ET<sub>B</sub> receptor activation and supporting the idea that in some settings ET<sub>B</sub> receptor activation may not be beneficial. Consistent with these findings, our previous report demonstrated ROCK activation in PPHN PAECs, secondary to ET-1 activation of the ET<sub>B</sub> receptor (20). ET-1-mediated ET<sub>B</sub> receptor activation decreases PPAR<sub>γ</sub> expression and activity, suggesting that, in the setting of neonatal pulmonary hypertension with impaired vascular growth, combined ET<sub>A</sub>/ET<sub>B</sub> receptor blockade may be more beneficial than selective ET<sub>A</sub> receptor blockade alone.

Bosentan restored PPAR<sub>γ</sub> protein expression and activity to baseline values after ET-1 treatment; however, in PPHN PAECs, PPAR<sub>γ</sub> protein expression and activity remained decreased, compared with normal controls. ET-1 contributes to decreased PPAR<sub>γ</sub> protein expression and activity to the same values measured in normal controls suggests that cell signaling pathways other than ET-1 further contribute to decreased PPAR<sub>γ</sub> protein expression and activity in PPHN PAECs. Bosentan treatment of PPHN PAECs restores ROCK activity and PAEC function to normal (20). Despite studies of the systemic circulation demonstrating a link between ROCK and PPAR<sub>γ</sub> (4–6, 59), experiments to date in PAECs (data not shown) have failed to show a link between these cell signaling pathways. ET-1–ROCK interactions contribute to PAEC dysfunction in PPHN (20); however, the failure of bosentan to restore PPAR<sub>γ</sub> activity to normal control values confirms that the effect of ET-1 on PPAR<sub>γ</sub> activity is independent of ROCK and there are likely cell signaling pathways other than ROCK and ET-1 responsible for decreased PPAR<sub>γ</sub> expression in PPHN. ET-1 impairment of PAEC function and tube formation in vitro, through decreased PPAR<sub>γ</sub> signaling, suggests an alternate mechanism for decreased tube formation in vitro.

Potential limitations of this study include the use of fetal PAECs harvested from relatively large vessels and the possibility that the behavior of these cells might be different from that of microvascular PAECs. Although microvascular PAECs may better represent sites where lung angiogenesis occurs during development, studying organ-specific cells from a developmentally relevant model of PPHN and the use of multiple clones of cells from different animals is a strength of our study. Future studies are needed to compare and contrast ET-1-PPAR<sub>γ</sub> interactions in microvascular PAECs to determine whether similar cellular mechanisms are responsible for impaired microvascular endothelial cell function. Another potential limitation is the fact that angiogenesis was only measured in vitro, utilizing PAECs from fetal sheep with intrauterine pulmonary hypertension. Sustained phenotypic abnormalities in the early postnatal period, including increased ET-1 production and altered signaling pathways, are likely to continue in the postnatal period and are highly relevant for consideration for postnatal therapies. Although these findings provide insights into mechanisms responsible for altered lung development in utero, the possibility does exist that similar mechanisms may not exist postnatally. Whether ET-1-PPAR<sub>γ</sub> interactions impair angiogenesis and lung development after postnatal injury and whether prolonged treatment with PPAR<sub>γ</sub> agonists enhances angiogenesis in vivo remains unknown. The efficacy of PPAR<sub>γ</sub> agonist therapies has been established in postnatal rodent models of BPD (8, 56). These studies provide novel insights into mechanisms contributing to decreased lung vascular development and PPHN in a developmentally relevant model providing the basis for future studies, addressing the efficacy of chronic ET-1 inhibition and PPAR<sub>γ</sub> agonist therapy in this model.

In conclusion, we found that PAEC from experimental PPHN are characterized by impaired tube formation due in part to decreased PPAR<sub>γ</sub> protein expression and activity. We also report that exogenous ET-1 administration decreased tube formation and PPAR<sub>γ</sub> protein expression and activity, which is prevented by PPAR<sub>γ</sub> agonist treatment. Furthermore, these findings suggest that ET-1-PPAR<sub>γ</sub> interactions regulate eNOS activity, NO production, and tube formation. Previous work has suggested that the effect of ET-1 on PAEC function is mediated by ROCK (20). This study proposes an alternate mechanism by which ET-1 disrupts PAEC function and provides insights into the pathophysiology of PPHN. We speculate that therapies that inhibit ET-1 production or activity or restore PPAR<sub>γ</sub> signaling may enhance angiogenesis and lung growth in vivo and may be especially important in treating pulmonary hypertension in the setting of endothelial dysfunction and lung hypoplasia. Since ROCK is an alternate pathway through which ET-1 impairs PAEC function, we further speculate that combined therapy with ROCK inhibitors may be more beneficial than PPAR<sub>γ</sub> agonist therapy alone.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
ET-1-PPARγ INTERACTIONS IN PPHN


