Chronic Intrauterine Pulmonary Hypertension Increases Endothelial Cell Rho-Kinase Activity and Impairs Angiogenesis in vitro.

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Abstract

Persistent pulmonary hypertension of the newborn (PPHN) is characterized by endothelial dysfunction and decreased vascular growth. The role of rho-kinase activity in modulating endothelial function and regulating angiogenesis during normal lung development and in PPHN are unknown. We hypothesized that PPHN increases rho-kinase activity in fetal pulmonary artery endothelial cells (PAECs) and impairs angiogenesis in vitro. Proximal PAECs were harvested from fetal sheep with partial ligation of the ductus arteriosus in utero (PPHN) and age-matched controls. Rho-kinase activity, was measured by rhoA, RhoGTP and P-MYPT-1 protein content. The effects of rho-kinase activity on angiogenesis, eNOS protein expression and NO production were determined in normal and PPHN PAECs. Angiogenesis was assessed by tube formation in vitro with/without Y-27632, (rho-kinase inhibitor), and calpeptin, (rho-kinase activator), in presence/absence of L-NA (NOS inhibitor). RhoA, rho-GTP and P-MYPT-1 protein were increased in PPHN PAECs. Tube formation was reduced by 29% in PPHN PAECs (p<0.001) and increased with Y-27632 treatment in normal and PPHN PAECs with PPHN PAECs achieving similar values to normal PAECs. L-NA inhibited the Y-27632-induced increase in tube formation in normal but not PPHN PAECs. Calpeptin reduced tube formation in normal and PPHN PAECs. eNOS expression, was reduced by 42% in PPHN PAECs (p<0.01). Y-27632 increased eNOS protein and NO production in normal and PPHN PAECs. Calpeptin decreased eNOS protein only in normal PAECs, but reduced NO production in
normal and PPHN PAECs. We conclude that rho-kinase activity is increased in PPHN PAECs, which down-regulates eNOS protein and NO production and impairs angiogenesis \textit{in vitro}.

\textbf{Key terms:} Persistent pulmonary hypertension of the newborn, pulmonary hypertension, angiogenesis, vasculogenesis, rho-kinase, nitric oxide, endothelial nitric oxide synthase, endothelial cells, lung vascular development.
Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome characterized by elevated pulmonary vascular resistance (PVR) that persists after birth, leading to extrapulmonary right to left shunting and profound hypoxemia. Mechanisms responsible for elevated PVR in PPHN include increased vascular tone, hypertensive remodeling and in the most severe cases, impaired angiogenesis or vascular growth (12). Impaired angiogenesis is usually seen in the setting of PPHN with lung hypoplasia, such as with congenital diaphragmatic hernia (12,14). In the presence of lung hypoplasia, decreased arterial number plays an especially prominent role in maintaining high PVR, resulting in disease that is often refractory to vasodilator therapies, such as inhaled nitric oxide (14). In this setting, novel strategies that can stimulate vascular growth and increase arterial number may improve outcomes of neonates with severe PPHN and lung hypoplasia. However, mechanisms that impair angiogenesis and enhance lung vascular growth in severe PPHN are poorly understood.

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 (3,29,54). In this model, partial DA ligation increases pulmonary artery pressure without causing sustained elevations of pulmonary blood flow or hypoxemia (1). At delivery, PVR remains elevated and causes hypoxemia due to extrapulmonary shunting despite mechanical ventilation with supplemental oxygen (1). Physiologically, this model of PPHN is characterized
by marked endothelial dysfunction, as reflected by the early loss of endothelium-dependent vasodilation, with down-regulation of lung endothelial NO synthase (eNOS) expression, impaired NO production, increased superoxide generation and increased ET-1 expression (16,17,22,41,48,49). Overall, these and other findings suggest that disruption of normal endothelial function in the fetal lung increases pulmonary vasoconstriction and causes abnormal vasoreactivity in PPHN. In addition to its role in the regulation of vascular tone, the endothelial cell also modulates vascular structure and growth. Recent studies in this experimental model have shown that chronic intrauterine pulmonary hypertension impairs lung angiogenesis and cause lung hypoplasia (11). We recently demonstrated that endothelial cells from PPHN fetal sheep maintain an abnormal phenotype in vitro, which is characterized by decreased growth and impaired tube formation (9). However, how hemodynamic stress induced by hypertension alters endothelial cell function and impairs vascular growth in PPHN is unknown.

Rho-kinase signaling is a complex pathway responsible for cellular proliferation, migration, differentiation and gene expression in diverse vascular beds (26). Rho-kinase activity has been shown to regulate smooth muscle cell contraction and vascular tone in systemic and pulmonary circulations (4,5,32,53). During lung development, rho-kinase activity maintains high PVR in the fetal lung (35) and may contribute to increased vascular tone in neonatal pulmonary hypertension. In adult animal models of pulmonary hypertension, rho-kinase activity is increased (21,26,32,52), however these studies have largely focused on the effects of rho-kinase activation on smooth muscle cell function, demonstrating
that increased rho-kinase activity elevates vascular tone, mediates calcium sensitization and contributes to hypertensive remodeling (5,32,42,51,53). In adult models of pulmonary hypertension, acute treatment with rho-kinase inhibitors causes potent pulmonary vasodilation and chronic therapy prevents vascular remodeling and improves survival (20,21,26,30,34).

In addition to its effects in smooth muscle cells, rho-kinase activity also modulates endothelial cell function. RhoGTPases are key regulators of endothelial permeability (2,50,55) and rhoA activation increases vascular permeability (10,39,47). Whether rho-kinase activity regulates angiogenesis, especially in the lung circulation, is controversial. In adult models of pulmonary hypertension due to chronic hypoxia, inhibition of rho-kinase activity prevents pulmonary hypertension and inhibits angiogenesis (13). Unlike these findings in the adult lung, experimental pulmonary hypertension in fetal sheep is associated with reduced vascular growth and impaired angiogenesis (11). Whether increased rho-kinase activity impairs endothelial cell function and reduces vascular growth in severe pulmonary hypertension, especially in the developing lung or in neonatal pulmonary hypertension, remains unknown.

Since eNOS protein expression is decreased in PPHN PAECs (9) and rho-kinase activity regulates eNOS protein expression and NO production (27,33,36), we proposed to test whether reduced eNOS protein expression and tube formation in PPHN PAECs is due to increased rho-kinase activity. Specifically, we hypothesized that chronic intrauterine pulmonary hypertension would increase rho-kinase activity in pulmonary artery endothelial cells, resulting in endothelial cell
dysfunction, decreased eNOS expression and impaired angiogenesis. We further hypothesized that increased rho-kinase activity would impair angiogenesis in fetal and hypertensive PAECs due to reduced NO production. In this study, we report increased rho-kinase activity in PAECs from PPHN lambs, and that rho-kinase inhibition increases eNOS expression and NO production and enhances tube formation \textit{in vitro}. Overall, these findings support the hypothesis that rho-kinase activation contributes to endothelial cell dysfunction and impaired angiogenesis in PPHN.
Methods

Isolation and culture of fetal ovine pulmonary arterial endothelial cells. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center, Denver, CO. The 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), term = 147 days) and from fetal sheep that had undergone partial ligation of the ductus arteriosus in utero 7-10 days prior to euthanasia (PPHN)(n=4)(as previously described, (3,29,54). Proximal PAECs were isolated as previously described (9,22) and endothelial cell phenotype confirmed by positive immunostaining for von Willebrands Factor (vWF), eNOS, vascular endothelial (VE)-cadherin. VEGF-R2 (KDR), positive uptake of ac-LDL and negative staining for desmin. Cells from passage 4 and 5 were used for each of the study experiments and cells from each animal were kept separate throughout all passages and for all experiments.

ELISA: ELISA was performed using the G-LISA RhoA Activation Assay (Cytoskeleton Inc, Denver, CO #BK124) and the assay was performed according to the manufacturers instructions. Briefly PAECs from control and PPHN lambs were grown to 50-70% confluence in 150mm dishes and cell lysate collected by
scraping the dishes. Lysates were snap frozen in liquid nitrogen and stored at -80°C. After thawing protein concentrations were determined and samples were prepared with identical protein concentrations. The rhoA activation assay was performed in triplicate and rho GTP signal was determined by measuring absorbance at 490nm using a microplate spectrophotometer. Differences in absorbance between normal and PPHN PAECs were measured and quantified.

**Membrane-cytosolic separation:** Membrane fraction separation was performed using the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, Cat#444810 San Diego, CA). Briefly, PAECs from normal and PPHN fetal sheep were grown to 95% confluence in 150mm dishes and cells were detached from the dishes using 0.25% trypsin. Membrane protein was extracted from whole cell lysates per manufacturer instructions.

The cytosolic fraction was extracted using the Mem-PER Eukaryotic membrane extraction kit (Pierce Biotechnology Inc (catalog # 89826) Rockford, IL). PAECs from normal and PPHN fetal sheep were grown to 95% confluence in 150mm dishes and cells were detached from the dishes using 0.25% trypsin. Cytosolic protein was extracted from whole cell lysates per manufacturer instructions.

Protein content in the membrane and cytosolic samples was determined by the Bicinchoninic acid assay (BCA) (Pierce Biotechnology Inc (catalog # 23225) Rockford, IL), using bovine serum albumin as the standard. Twenty µg of protein sample per lane was resolved by SDS polyacrylamide gel
electrophoresis. Proteins from the gel were transferred to nitrocellulose membrane and Rho A protein was detected by western blot analysis (See protocol below).

**Tube Formation Assay:** The ability of fetal PAECs to form vascular structures in vitro was assayed by plating PAEC’s on type 1 collagen. Collagen was pipetted into 24 well tissue culture dishes (250 µl/well) and allowed to polymerize at 37°C for one hour. PAEC from normal and PPHN fetal sheep were seeded at a density of 5 x 10^4 cells/well in serum free DMEM supplemented with and without Y-27632 (1µM; rho-kinase inhibitor), calpeptin (100µg/ml; rho-kinase activator) and Y-27632 (1µM) with nitro-L-arginine (LNA; a NOS inhibitor; 4mM).

Doses for each drug were determined by preliminary experiments and published studies (40). The lowest dose for which an effect was seen was used for all drugs. PAECs were incubated in 3% oxygen conditions in order to simulate the low oxygen environment in the normal fetus (9). After 6 hours, branch point counting was performed in blinded fashion under 10X magnification from each of 4 wells, as previously described (38).

**Western Blot Analysis:** PAECs from normal and PPHN animals were grown on 150mm cloning dishes in DMEM supplemented with 5% serum. At 70% confluence, PAECs were treated with Y-27632 (1µM) for 24 hours and calpeptin (100µg/ml) were for 30 minutes per the manufacturer’s recommendations. Cells were washed with ice cold PBS x 2 and lysed in radioimmunoprecipitation (RIPA) buffer (PBS, 1% Nonidet P-40, 0.5%, sodium deoxycholate, 0.1% SDS, PMSF
[10mg/ml], aprotinin [16 µl/ml], and sodium orthovanadate [1mM]). Cell lysates were scraped off the dishes, sonicated, and centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was removed and protein content in the supernatant was determined by the BCA assay (Pierce Biotechnology Inc (catalog # 23225) Rockford, IL), using bovine serum albumin as the standard. 20 µg of protein sample per lane was resolved by SDS polyacrylamide gel electrophoresis, and proteins from the gel were transferred to nitrocellulose membrane.

*Rho A*: Blots were blocked for 30 minutes in 5% nonfat dry milk dissolved in buffer 1 (10mM tris-hcl, 150mM NaCl, 0.05% tween-20, PH 8.0) Blots were incubated for 2 hours at room temperature with anti ROCK-II/ROKα (BD610624 BD Biosciences, San Jose, CA) (1:500) diluted in 5% nonfat dry milk in buffer 1. After washing, blots were incubated for 1 hour at room temperature with goat anti-mouse HRP conjugated secondary (Chemicon, Billerica, MA)(1:10000). Bands of interest were visualized by enhanced chemiluminenscence (ECL+ kit; Amersham Pharmacia Biotech, Buckinghamshire, UK), identified by molecular weight as identified by the manufacturer for the protein of interest.

Phospho-MYPT-1: Blots were blocked for 30 minutes with 5% nonfat dry milk dissolved in buffer 1 (10mM tris-hcl, 150mM NaCl, 0.05% tween-20, PH 8.0). Blots were then incubated overnight with Phospho-MYPT1 (Thr853) Antibody (#4563 Cell Signaling, Danvers, MA) (1:500). After washing, blots were incubated for 1 hour at room temperature with goat anti-rabbit HRP conjugated secondary
(Santa Cruz Biotech, SC2054). Bands of interest were visualized by enhanced chemiluminencescence (ECL+ kit; Amersham Pharmacia Biotech, Buckinghamshire, UK), identified by molecular weight as identified by the manufacturer for the protein of interest.

**eNOS:** Blots were blocked for 30 minutes in 2% ECL advance (Amersham Pharmacia Biotech, Buckinghamshire, UK) dissolved in PBS with 0.05% Tween 20 after which time the blots were incubated for 1 hour with BD610297 (eNOS/NOS III)(1:1000) diluted in 2% ECL advance. After washing, blots were incubated for 1 hour at room temperature with goat anti-mouse HRP conjugated secondary (Chemicon)(1:20000). Bands of interest were visualized using the ECL advance kit, identified by molecular weight as identified by the manufacturer for the protein of interest.

All blots were then stripped and reprobed with an antibody to β-actin (Sigma, St. Louis, A5316). Densitometry was performed using NIH Image (v1.61). Changes in protein expression were analyzed after normalizing for β-actin expression.

**Nitric Oxide Assay:** NO production was determined with the DAF-FM Nitric Oxide indicator (*Molecular Probes, Eugene, OR #D-23844*). 5 x 10³ normal and PPHN PAECs were plated in 96 well plates in DMEM with 5% FBS under 3% oxygen conditions. Cells were allowed to adhere overnight after which normal and PPHN PAECs were incubated with DAF-FM with and without Y-27632 (1µM) and calpeptin (100µg/ml) in PBS for 1 hour. PBS containing DAF-FM fluorescent
probe was transferred to a black 96 well plate and NO production measured in response rho-kinase activation and inhibition using a microplate reader with fluorescence excitation and emission maxima of 495 and 515 nm, respectively. Comparisons were made between normal and PPHN cells with respect to NO production.

**Statistical analysis.** Data are presented as means ± SEM. Statistical analysis was performed with the Prism 4 software package (GraphPad Software, San Diego, CA). Statistical comparisons were made using analysis of variance for tube formation assays with Bonferroni post test analysis. Unpaired t test was used for western blot and ELISA analysis and NO production studies. $P < 0.05$ was considered significant.
Results

*Increased Rho-kinase activity in fetal PAECs from PPHN Lambs.* In comparison with controls, PAECs from PPHN lambs exhibited increased rho-kinase activity. Western blot analysis on whole cell lysates from normal and PPHN PAECs demonstrated a 93% increase in total Rho A protein in PAECs from PPHN lambs (p<0.05)(fig 1a). As determined by ELISA, rho-GTP, the active form of rho, was increased by 53% in PPHN PAECs (p<0.001) (fig 1b.) In addition to increased rhoA and rho-GTP protein, phosphorylation of MYPT-1, another measure of rho-kinase activity, was increased 65% in PPHN PAECs (p<0.01) (fig 1c). When separated into membrane and cytosolic protein fractions, cell lysates from PPHN PAECs demonstrate increased rho A protein in both membrane and cystosolic fractions when compared with normal controls. In comparison with control PAECs, rho A membrane and cytosolic protein contents were increased by 34% and 52%, respectively (p<0.01) (fig 2).

*Activation of rho-kinase with calpeptin.* Western blot analysis on whole cell lysates from normal and PPHN PAECs demonstrated that rho-kinase activation with calpeptin increased phosphorylation of MYPT-1 by 85% in normal PAECs (p<0.01) (fig 3a). With calpeptin treatment there was no further increase in phosphorylation of MYPT-1 in PPHN PAECs (p=NS)(fig3b.).

*Effect of Rho-kinase inhibition and stimulation on tube formation in vitro.* Treatment with Y-27632, a rho-kinase inhibitor, increased tube formation in both
normal and PPHN PAECs. Tube formation was increased by 13% (p<0.01) and 31% (p<0.001) in normal and PPHN PAECs, respectively (fig 4a). Rho-kinase inhibition increased tube formation by PPHN PAECs to values achieved in normal PAECs. Treatment with calpeptin, decreased tube formation in both normal and PPHN PAECs by 29% (p<0.001) and 21% (p<0.01), respectively. (fig 4b). The addition of S-nitroso-N-acetylpenicillamine (SNAP) as an NO donor did not prevent the decrease in tube formation due to rho-kinase activation. Tube formation remained decreased by 25% (p<0.001) in normal and by 17% (p<0.001) in PPHN PAECs (fig not shown).

**Effect of Rho-kinase inhibition and stimulation on eNOS expression.** Rho-kinase inhibition with Y-27632 increased eNOS protein content in both normal and PPHN PAECs. eNOS protein expression was increased by 30% (p<0.01) and 58% (p<0.05) in normal and PPHN PAECs, respectively (fig 5a). Rho-kinase activation with calpeptin decreased eNOS protein expression by 28% (p<0.01) in normal PAECs, however, calpeptin did not cause a further decrease in eNOS protein expression in PPHN PAECs ((fig 5b), which was decreased by 42% (p<0.01) at baseline.

**Effect of Rho-kinase inhibition and activation on nitric oxide (NO) production in normal and PPHN PAECs.** Rho-kinase inhibition with Y-27632 increased NO production in both normal and PPHN PAECs (fig 6a.). With rho-kinase inhibition NO production increased 63% (p<0.001) in normal and 64% (p<0.001) in PPHN
PAECs. Rho-kinase activation decreased NO production by 31% (p<0.05) and 25% (p>0.05) in normal and PPHN PAECs respectively (fig 6b).

Increase in tube formation with rho-kinase inhibition is nitric oxide dependent in normal but not PPHN PAECs. The increase in tube formation seen with rho-kinase inhibition was reversed with nitric oxide synthase inhibition using L-NA in normal PAECs. Tube formation decreased by 30% (p<0.001) with the addition of L-NA to Y-27632 (fig 7). Values achieved were 22% lower than that achieved by normal controls. In PPHN PAECs, L-NA had no effect on the increase in tube formation with rho-kinase inhibition (fig 7).
Discussion

In addition to increased pulmonary vascular tone and hypertensive remodeling, impaired angiogenesis also contributes to high PVR in severe PPHN, especially in the setting of lung hypoplasia (12). Previous studies have shown that pulmonary hypertension during late gestation impairs fetal lung vascular growth \textit{in vivo} (11), and causes abnormalities in endothelial cell phenotype that persist \textit{in vitro} (9). However, mechanisms through which sustained elevations of pulmonary arterial pressure inhibit lung angiogenesis during development are unknown. Since rho-kinase activity modulates eNOS protein expression and activity, we hypothesized that increased rho-kinase activity may account for the change in endothelial cell phenotype seen in PPHN. We found that rho kinase activity, as assessed by rhoA, and rhoGTP protein expression and phosphorylation of MYPT-1, was increased in PAECs harvested from PPHN lambs. We also found that treatment with Y27632, a rho kinase inhibitor, increased eNOS protein expression and NO production and rescued the abnormal \textit{in vitro} phenotype, restoring tube formation by PPHN PAECs to normal levels. In addition, treatment with calpeptin, a rho kinase activator, increased phosphorylation of MYPT-1, decreased eNOS protein expression and NO production, and decreased tube formation \textit{in vitro} in normal PAECs. These findings demonstrate that chronic intrauterine pulmonary hypertension increases rho kinase activity in lung vascular endothelium, which
contributes to impaired angiogenesis, reduced eNOS protein content and decreased NO production in PPHN.

This is the first study of rho-kinase activity in fetal PAECs and these findings demonstrate increased rho-kinase activity in PPHN PAECs, suggesting a role for the rho-kinase pathway in regulating angiogenesis in the developing lung. Prior studies in experimental pulmonary hypertension have demonstrated increased rho-kinase activity in the adult, but these reports have primarily focused on rho-kinase activity in the smooth muscle cell and its effect on vascular tone and hypertensive remodeling in pulmonary hypertension. With exposure to acute hypoxia inhibition of rho-kinase activity attenuates the constrictor response in adult rats (37,52), while after chronic hypoxia, rho-kinase inhibition decreases mean pulmonary artery pressure (30,32). Pulmonary hypertension induced by chronic hypoxia has previously been attributed to structural changes in the pulmonary vasculature including hypertensive remodeling which produces a fixed increase in resistance (13). Sustained inhibition of rho-kinase throughout the period of hypoxic exposure attenuates pulmonary hypertension and prevents vascular remodeling (20). Adult rats when treated with a single dose of monocrotaline develop severe pulmonary hypertension and vascular remodeling (8,46). Chronic rho-kinase inhibition in this setting prevents pulmonary vascular remodeling, by suppressing vascular smooth muscle cell proliferation and macrophage infiltration (19). These studies indicate that rho-kinase–mediated pathways are substantially involved in the pathogenesis of pulmonary hypertension contributing significantly to vascular tone and hypertensive remodeling in PPHN.
We recently reported that prolonged intrauterine pulmonary hypertension impairs angiogenesis and decreases alveolarization and lung weight in fetal lambs (11). Intrauterine pulmonary hypertension also directly alters endothelial cell function and impairs growth and tube formation by isolated PAECs in vitro. (9). Thus, pulmonary hypertension itself can impair endothelial cell function, reduce vascular growth and cause lung hypoplasia. How pulmonary hypertension alters endothelial cell function and contributes to impaired angiogenesis in PPHN remains unknown, but our results implicate the rho-kinase signal transduction pathway as contributing to endothelial cell dysfunction and impaired vascular growth in PPHN.

Our studies demonstrate a role for the rho-kinase pathway in regulating these important endothelial cell functions and contributing to normal blood vessel formation in the developing lung as well as impaired angiogenesis in PPHN. Whether decreased alveolarization in PPHN is mediated by rho-kinase is unknown. Prior studies have implicated rho-kinase signaling in regulating alveolarization during development (26,28), but mechanisms underlying these findings were not explored. Past studies have suggested that inhibition of vascular growth impairs alveolarization (18,44,45). We speculate that high rho-kinase activity in PPHN PAECs decreases vascular growth and subsequent alveolarization. Fetal lung explants incubated for 48 hours in 3% oxygen show increased branching as well as membrane associated rhoA when compared with room air controls (7,26).
Recent studies have demonstrated that under hypoxic condition rho-kinase is activated (26,37,43,52), suggesting that the increase in lung branching seen under hypoxic conditions may be mediated by rho-kinase. The fawn hooded rat (FHR) is a genetic model of pulmonary hypertension and is characterized by increased rho-kinase activity (31). FHRs when exposed to mild hypoxia seen at Denver’s altitude develop alveolar simplification and pulmonary hypertension (23,24). In this model chronic rho-kinase inhibition improves alveolarization and vascular growth. These studies support our findings that while rho-kinase activity during fetal life may regulate lung growth, inappropriate rho-kinase activation during fetal life or persistence of rho-kinase activation after birth, may impair angiogenesis and lung growth.

Prior studies have demonstrated that inhibition of rho-kinase upregulates and activates eNOS, increasing the production of NO (27,33,36). Long-term inhibition of rho-kinase activity is protective against pulmonary hypertension and right ventricular hypertrophy in hypoxia exposed adult mice (20), but was less effective in eNOS (-/-) mice (20), which suggests that eNOS activation after rho-kinase inhibition is responsible for these protective effects. We report that rho-kinase inhibition increases eNOS protein expression and activity in both normal and PPHN PAECs, which supports the concept that eNOS activation and increased NO production may be responsible for enhanced angiogenesis in vitro by normal and PPHN PAECs. However, the effect of rho-kinase inhibition on angiogenesis was lost in normal but not PPHN PAECs with NOS inhibition using
L-NA. This finding suggests an NO independent effect of rho-kinase inhibition and, raises the possibility that in conditions associated with dysfunctional eNOS, and impaired NO production, rho-kinase inhibitors may have even greater therapeutic benefit. Interestingly, Lohn et al demonstrated that in the models of genetically reduced endothelial NO production (eNOS-/- mice and spontaneous hypertensive rats) and in models of pharmacologically reduced endogenous NO production (LNAME treatment), rho-kinase inhibition produced a strong vasodilator response, which also suggests that inhibition of rho kinase has NO-independent effects as well (25).

Potential limitations of this study include the use of fetal PAECs harvested from relatively large vessels, and that differences may exist in the behavior of these cells as compared to microvascular PAECs. Since microvascular PAECs may primarily be involved in lung angiogenesis during development in vivo, future studies are needed to compare and contrast rho-kinase activity in microvascular PAECs. While calpeptin markedly increased phosphorylation of MYPT-1 in normal PAECs, there was no further increase in phosphorylation of MYPT-1 in PPHN PAECs. NO production, however, was significantly reduced with calpeptin treatment in both normal and PPHN PAECs. While the effects of calpeptin treatment may not be mediated through rho-kinase, the decrease in NO production with calpeptin treatment may support the concept that the detrimental effects of rho-kinase activation are the result of decreased eNOS activity. Another potential limitation is the fact that angiogenesis was only measured in vitro, and whether
rho-kinase inhibition enhances angiogenesis in vivo remains unknown.

In conclusion, we found that chronic intrauterine pulmonary hypertension increases rho-kinase activity in PAECs harvested from PPHN lambs and that this increase in rho-kinase activity directly contributes to downregulated eNOS, decreased NO production and impaired angiogenesis in vitro. Rho-kinase inhibition reversed the abnormal in vitro phenotype previously described in PPHN PAECs. This effect however was found to be NO independent. These findings suggest that treatment strategies that down regulate or inhibit rho-kinase activation in PPHN may enhance angiogenesis in vivo, and may be especially important in treating pulmonary hypertension in the presence of endothelial dysfunction and lung hypoplasia.
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Figure Legends

Figure 1. *Increased Rho-kinase activity in PAECs from PPHN Sheep.*

*RhoA, rhoGTP and phosphorylated MYPT-1 protein expression* was assessed in fetal ovine PAECs from normal and PPHN lambs by western blot analysis and ELISA. In comparison with normal PAECs total rhoA (fig 1a), rhoGTP (fig1b) and phosphorylated MYPT-1 (fig 1c.) protein was increased in PPHN PAECs.

Error bars represent SD from mean.

Figure 2. *Increased Membrane and Cystosolic Rho A Protein Expression in PAECs from PPHN Sheep.* Whole cell lysates were separated into membrane and cytosolic fractions and both membrane and cytosolic RhoA protein was increased in PPHN PAECs when compared with normal controls.

Error bars represent SD from mean.

Figure 3. *Effects of Calpeptin on Rho-Kinase Activation in Normal and PPHN PAECs.* *Phosphorylated MYPT-1 protein expression was assessed in normal and PPHN PAECs in response to calpeptin treatment (rho-kinase activator).* In response to calppeptin treatmet phosphorylation of MYPT-1 was increased in normal (fig 3a.) but not PPHN PAECs (fig 3b).
Figure 4. Effect of Rho-kinase Inhibition and Stimulation on Tube Formation in vitro.
Fetal ovine PAECs from normal and PPHN sheep were plated on collagen in serum free media under 3% oxygen conditions with and without Y-27632 (1μM) (rho-kinase inhibitor) and calpeptin (100μg/ml)(rho-kinase activator). Y27632 treatment increased the number of branch points in PPHN and normal PAECs (fig 4a), increasing the number of branch points by PPHN PAECs to similar values seen in normal PAECs. Calpeptin decreased tube formation in both normal and PPHN PAECs (fig 4b). Error bars represent SD from mean.

Figure 5. Effect of Rho-kinase Inhibition and Activation on eNOS Protein Expression.
Cell lysates were collected from normal and PPHN PAECs with and without Y-27632 (1μM) (rho-kinase inhibitor) and calpeptin (100μg/ml)(rho-kinase activator). Rho-kinase inhibition increased eNOS protein expression in both normal and PPHN PAECs (fig 5a.). Rho-kinase activation decreased eNOS protein expression in normal but not PPHN PAECs (fig 5b.). Error bars represent SD from mean.

Figure 6. Effect of Rho-kinase Inhibition and Activation on Nitric Oxide (NO) production in normal and PPHN PAECs. Rho-kinase inhibition with Y-27632 increased NO production in both normal and PPHN PAECs (fig 6a). Rho-kinase activation decreased NO production in both normal and PPHN PAECs (fig 6b).
Figure 7. *Increase in Tube Formation with Rho-kinase inhibition is Nitric Oxide Dependent in Normal but not PPHN PAECs.* Fetal ovine PAECs from normal and PPHN sheep were plated on collagen in serum free media under 3% oxygen conditions with and without Y-27632 (1μM) (rho-kinase inhibitor) in the presence and absence of L-NA(4mM). The addition of L-NA to Y-27632 decreased tube formation in normal but not PPHN PAECs (fig 7.). Error bars represent SD from mean.
Figure 1a.

Figure 1b.
Figure 1c.

The bar graph shows the phosphorylated MYPT-1 protein expression (densitometry units) with the following key points:

- **P-MYPT-1**
- **B Actin**

For the comparison between Normal and PPHN:
- P-MYPT-1 protein expression is significantly lower in the Normal group compared to the PPHN group with a p-value of less than 0.01.
Figure 2.

Membrane

Cytosolic

RhoA Protein in Densitometry Units

Normal  PPHN

p<0.01

RhoA Protein in Densitometry Units

Normal  PPHN

p<0.01
Figure 4a

![Figure 4a](image)

Figure 4b

![Figure 4b](image)
Figure 5a.

Figure 5b.
Figure 6a

![Figure 6a](image1)

Figure 6b

![Figure 6b](image2)
Figure 7.