Chronic intrauterine pulmonary hypertension increases endothelial cell Rho kinase activity and impairs angiogenesis in vitro

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Gien J, Seedorf GJ, Balasubramaniam V, Tseng N, Markham N, Abman SH. Chronic intrauterine pulmonary hypertension increases endothelial cell Rho kinase activity and impairs angiogenesis in vitro. Am J Physiol Lung Cell Mol Physiol 295: L680–L687, 2008. First published July 11, 2008; doi:10.1152/ajplung.00516.2007.—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 is 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 fetuses with partial ligation of the ductus arteriosus in utero (PPHN) and age-matched controls. Rho kinase activity was measured by RhoA, Rho GTP, and phosphorylated MYPT-1 protein content. The effects of Rho kinase activity on angiogenesis, endothelial nitric oxide (NO) synthase (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 (a Rho kinase inhibitor) and calpeptin (a Rho kinase activator) in the presence/absence of N-nitro-L-arginine (L-NA, an NOS inhibitor). RhoA, Rho GTP, and phosphorylated MYPT-1 protein were increased in PPHN PAECs. Tube formation was reduced 29% in PPHN PAECs (P < 0.001) and increased with Y-27632 treatment in normal and PPHN PAECs, with PPHN PAECs achieving levels similar to those of 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 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 and impairs angiogenesis and downregulates eNOS protein and NO production in vitro.

Persistent pulmonary hypertension of the newborn; vasculogenesis; nitric oxide; endothelial nitric oxide synthase; lung vascular development

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, a decrease in the number of arteries plays an especially prominent role in maintaining high PVR, resulting in disease that is often refractory to vasodilator therapies, such as inhaled nitric oxide (NO) (14). In this setting, novel strategies that can stimulate vascular growth and increase the number of arteries 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 study of the pathogenesis and treatment of PPHN (3, 29, 54). In this model, partial DA ligation increases pulmonary arterial 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 downregulation of lung endothelial NO synthase (eNOS) expression, impaired NO production, increased superoxide generation, and increased endothelin 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 causes lung hypoplasia (11). We recently demonstrated that endothelial cells from PPHN fetal sheep maintain an abnormal phenotype in vitro that 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, and 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

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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. Rho GTPases 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). In contrast to 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 pulmonary artery endothelial cells (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 are due to increased Rho kinase activity. Specifically, we hypothesized that chronic intrauterine pulmonary hypertension would increase Rho kinase activity in PAECs, 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 as a result of reduced NO production. In this study, we report that Rho kinase activity is increased in PAECs from PPHN lambs and that Rho kinase inhibition increases eNOS expression and NO production and enhances tube formation 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 PAECs. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of 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 (3, 29, 54). Proximal PAECs were isolated as previously described (9, 22), and endothelial cell phenotype was confirmed by positive immunostaining for von Willebrand factor, eNOS, vascular endothelial cadherin, and vascular endothelial growth factor receptor type 2 (KDR), positive uptake of acetylated LDL, and negative staining for desmin. Cells from passages 4 and 5 were used for each of the 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 (catalog no. BK124, Cytoskeleton, Denver, CO), which was performed according to the manufacturer’s instructions. Briefly, PAECs from control and PPHN lambs were grown to 50–70% confluence in 150-mm dishes, the dishes were scraped, and cell lysates was collected. Lysates were snap frozen in liquid nitrogen and stored at −80°C. After the samples were thawed, protein concentrations were determined and samples were prepared with identitical protein concentrations. The RhoA activation assay was performed in triplicate, and the Rho GTP signal was determined by measurement of absorbance at 490 nm 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 (catalog no. 444810, Calbiochem, San Diego, CA). Briefly, PAECs from normal and PPHN fetal sheep were grown to 95% confluence in 150-mm dishes, and cells were detached from the dishes using 0.25% trypsin. Membrane protein was extracted from whole cell lysates according to the manufacturer’s instructions.

The cytosolic fraction was extracted using the Mem-PER eukaryotic membrane extraction kit (catalog no. 89826, Pierce Biotechnology, Rockford, IL). PAECs from normal and PPHN fetal sheep were grown to 95% confluence in 150-mm dishes, and cells were detached from the dishes using 0.25% trypsin. Cytosolic protein was extracted from whole cell lysates according to the manufacturer’s instructions.

Protein content in the membrane and cytosolic samples was determined by the bichinchoninic acid assay (catalog no. 23225, Pierce Biotechnology), with bovine serum albumin used as the standard. Twenty micrograms of protein sample per lane were resolved by SDS-PAGE. Proteins from the gel were transferred to nitrocellulose membrane, and RhoA protein was detected by Western blot analysis (see below).

Tube formation assay. The ability of fetal PAECs to form vascular structures in vitro was assayed by plating of PAECs on type I collagen. Collagen was pipetted into 24-well tissue culture dishes (250 μl/well) and allowed to polymerize at 37°C for 1 h. PAECs from normal and PPHN fetal sheep were seeded at a density of 5 × 10⁴ cells/well in unsupplemented serum-free DMEM and serum-free DMEM supplemented with 1 μM Y-27632 (a Rho kinase inhibitor), 100 μg/ml calpeptin (a Rho kinase activator), and 1 μM Y-27632 + 4 mM N-nitro-L-arginine (L-NA, an NOS inhibitor). 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 to simulate the low-oxygen environment in the normal fetus (9). After 6 h, branch point counting was performed in blinded fashion under ×10 magnification for each of four wells, as previously described (38).

Western blot analysis. PAECs from normal and PPHN animals were grown on 150-mm cloning dishes in DMEM supplemented with 5% serum. At 70% confluence, PAECs were treated with 1 μM Y-27632 for 24 h and calpeptin (100 μg/ml) for 30 min according to the manufacturer’s recommendations. Cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation buffer [PBS, 1% Nonidet P-40, 0.5%, sodium deoxycholate, 0.1% SDS, PMSF (10 mM), and protease inhibitor cocktail]. The supernatant was removed, and protein content in the supernatant was determined by the bichinchoninic acid assay, with bovine serum albumin used as the standard. Twenty micrograms of protein sample per lane were resolved by SDS-PAGE, and proteins from the gel were transferred to nitrocellulose membrane. RhoA. Blots were blocked for 30 min in 5% nonfat dry milk dissolved in buffer 1 [10 mM Tris·HCl, 150 mM NaCl, and 0.05% Tween 20 (pH 8.0)]. Blots were incubated for 2 h at room temperature with anti-RHO II/ROK (catalog no. BD610624, BD Biosciences, San Jose, CA; 1:500 dilution) diluted in 5% nonfat dry milk in buffer 1.
After they were washed, the blots were incubated for 1 h at room temperature with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Chemicon, Billerica, MA; 1:10,000 dilution). Bands of interest were visualized by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Buckinghamshire, UK) and identified by molecular weight as designated by the manufacturer for the protein of interest.

Phosphorylated MYPT-1. Blots were blocked for 30 min with 5% nonfat dry milk dissolved in buffer 1 [10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20 (pH 8.0)]. Blots were then incubated overnight with phosphorylated (Thr853) MYPT-1 antibody (catalog no. 4563 Cell Signaling, Danvers, MA; 1:500 dilution). The blots were washed and then incubated for 1 h at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (catalog no. SC2054, Santa Cruz Biotechnology). Bands of interest were visualized using the ECL Advance kit and identified by molecular weight as designated by the manufacturer for the protein of interest.

eNOS. Blots were blocked for 30 min in 2% ECL Advance (Amersham Pharmacia Biotech) dissolved in PBS with 0.05% Tween 20 and then incubated for 1 h with BD-610297 (eNOS/NOS III, 1:1,000 dilution in 2% ECL Advance). The blots were washed and then incubated for 1 h at room temperature with goat anti-mouse HRP-conjugated secondary antibody (Chemicon; 1:20,000 dilution). Bands of interest were visualized using the ECL Advance kit and identified by molecular weight as designated by the manufacturer for the protein of interest.

All blots were stripped and reprobed with an antibody to β-actin (catalog no. A5316, Sigma, St. Louis, MO). Densitometry was performed using NIH Image (version 1.61). Changes in protein expression were analyzed after normalization for β-actin expression.

NO assay. NO production was determined with the NO indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM; catalog no. D-23844, Molecular Probes, Eugene, OR). Normal and PPHN PAECs (5 × 10^5) were plated in 96-well plates in DMEM with 5% FBS under 3% oxygen conditions. Cells were allowed to adhere overnight; then normal and PPHN PAECs were incubated with DAF-FM with and without 1 μM Y-27632 and calpeptin (100 μg/ml) in PBS for 1 h. PBS containing DAF-FM was transferred to a black 96-well plate, and NO production in response to Rho kinase activation and inhibition was measured using a microplate reader with fluorescence excitation and emission maxima of 495 and 515 nm, respectively. NO production in normal and PPHN cells was compared.

Statistical analysis. Values are means ± SE. 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’s post test analysis. Unpaired t-test was used for Western blot analysis, ELISA, and NO production studies. P < 0.05 was considered significant.

RESULTS

Increased Rho kinase activity in fetal PAECs from PPHN lambs. Compared 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 RhoA protein in PAECs from PPHN lambs (P < 0.05; Fig. 1A). As determined by ELISA, Rho GTP, the active form of Rho, was increased 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 RhoA protein in membrane and cytosolic fractions compared with normal controls. Compared with
control PAECs, RhoA membrane and cytosolic protein contents were increased 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). Calpeptin treatment did not further increase phosphorylation of MYPT-1 in PPHN PAECs (not significant; Fig. 3B).

**Effect of Rho kinase inhibition and stimulation on tube formation in vitro.** Treatment with Y-27632, a Rho kinase inhibitor, increased tube formation in normal and PPHN PAECs. Tube formation was increased 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 normal and PPHN PAECs by 29% \((P < 0.001)\) and 21% \((P < 0.01)\), respectively (Fig. 4B). Addition of S-nitroso-N-acetylpenicillamine, 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 PAECs and by 17% \((P < 0.001)\) in PPHN PAECs (not shown).

Effect of Rho kinase inhibition and stimulation on eNOS expression. Rho kinase inhibition with Y-27632 increased eNOS protein content in normal and PPHN PAECs. eNOS protein expression was increased 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 42% \((P < 0.01)\) at baseline.

Effect of Rho kinase inhibition and activation on NO production in normal and PPHN PAECs. Rho kinase inhibition with Y-27632 increased NO production in normal and PPHN PAECs (Fig. 6A). With Rho kinase inhibition, NO production increased 63% \((P < 0.001)\) and 64% \((P < 0.001)\) in normal and PPHN PAECs, respectively. 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 NO dependent in normal, but not PPHN, PAECs. The increase in tube formation seen with Rho kinase inhibition was reversed by NO synthase inhibition with l-NA in normal PAECs. Tube formation decreased 30% \((P < 0.001)\) with the addition of l-NA to Y-27632 (Fig. 7). 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 showed that pulmonary hypertension during late gestation impairs fetal lung vascular growth in vivo (11) and causes abnormalities in endothelial cell phenotype that persist 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 Rho GTP protein expression and phosphorylation of MYPT-1, was increased in PAECs harvested from PPHN lambs. We also found that treatment with Y-27632, a Rho kinase inhibitor, increased eNOS protein expression and NO production and rescued the abnormal in vitro phenotype, restoring tube formation by PPHN PAECs to normal levels. In addition, treatment with calpeptin, a Rho kinase inhibitor, decreased eNOS protein expression in normal and PPHN PAECs (Fig. 5B). Values are means ± SE.

![Fig. 5. Effect of Rho kinase inhibition and activation on endothelial nitric oxide synthase (eNOS) protein expression. Cell lysates were collected from normal and PPHN PAECs with and without 1 μM Y-27632 and calpeptin (100 μg/ml). Rho kinase inhibition increased eNOS protein expression in normal and PPHN PAECs (A). Rho kinase activation decreased eNOS protein expression in normal, but not PPHN, PAECs (B). Values are means ± SE.](http://aijplung.physiology.org/)
Rho kinase activator, increased phosphorylation of MYPT-1, decreased eNOS protein expression and NO production, and decreased tube formation in vitro in normal PAECs. These findings demonstrate that chronic intrauterine pulmonary hypertension causes an increase in 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 regulation of angiogenesis in the developing lung. Previous studies of experimental pulmonary hypertension demonstrated increased Rho kinase activity in the adult, but these reports 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); after chronic hypoxia, however, Rho kinase inhibition decreases mean pulmonary arterial 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 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 imply that the Rho kinase signal transduction pathway contributes 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 implicated Rho kinase signaling in regulation of alveolarization during development (26, 28), but mechanisms underlying these findings were not explored. Earlier studies 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 h in 3% oxygen show increased branching, as well as membrane-associated RhoA, compared with room air controls (7, 26). Recent studies demonstrated that, during hypoxia, Rho kinase is activated (26, 37, 43, 52), suggesting that the increase in lung branching during hypoxia may be mediated by Rho kinase. The fawn-hooded rat is a genetic model of pulmonary hypertension and is characterized by increased Rho kinase activity (31). Fawn-hooded rats exposed to mild hypoxia 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 although 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.

Previous studies 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 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. (25) demonstrated that, in the models of genetically reduced endothelial NO production (eNOS\(^{-/-}\) mice and spontaneously hypertensive rats) and in models of pharmacologically reduced endogenous NO production (nitro-L-arginine methyl ester 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 the possibility that the behavior of these cells might be different from that of 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. Although 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 normal and PPHN PAECs. Although 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 measured in vitro; 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 downregulate 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.

GRANTS

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