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Effects of Rho-Kinase Inhibition on Pulmonary Hypertension, Lung Growth, and Structure in Neonatal Rats Chronically Exposed to Hypoxia

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ABSTRACT: Rho-kinase (ROCK) inhibitors prevent pulmonary hypertension (PHT) in adult rodents, but little is known about their effects on the neonatal lung. Our objective was to examine the effects of ROCK inhibition on chronic hypoxia (CH)-induced PHT and abnormal lung structure in the neonatal rat. Pups were exposed to air or CH from postnatal d 1–14 while receiving Y-27632 (5 or 10 $mg \cdot kg^{-1} \cdot d^{-1}$), fasudil (20 $mg \cdot kg^{-1} \cdot d^{-1}$), or saline intraperitoneally. Relative to air, CH-exposed pups had increased pulmonary vascular resistance, right ventricular hypertrophy, arterial medial wall thickening, and abnormal distal airway morphology characterized by septal thinning and decreased secondary septation. Treatment with 10 mg/kg Y-27632 or fasudil attenuated the structural and hemodynamic changes of PHT while having no effect on septal thinning or inhibited secondary septation. In addition, Y-27632 (10 mg/kg) and fasudil augmented CH-induced somatic growth restriction. Pulmonary arteries of CH-exposed pups had increased ROCK activity, up-regulated expression of PDGF-BB and increased smooth muscle DNA synthesis, all of which were attenuated by treatment with 10 mg/kg Y-27632. Systemically administered ROCK inhibitors prevented PHT in the CH-exposed neonatal rat but at the cost of inhibited somatic growth. Limiting effects on vascular remodeling likely resulted, in major part, from attenuated vascular PDGF-BB/β-receptor signaling. (***Pediatr Res* 67: 177–182, 2010**)

Chronic pulmonary hypertension (PHT) that has its origins during fetal or neonatal life is characterized pathologically in both humans (1) and experimental animals (2) by sustained vasoconstriction and rapid remodeling of pulmonary resistance arteries in which hyperplasia of medial wall smooth muscle is a major feature (3,4). Recent studies have implicated the RhoA/Rho-kinase (ROCK) pathway as central to the initiation and perpetuation of chronic PHT (5), based largely on the effects of two ROCK-specific kinase inhibitors: Y-27632 and fasudil (6). ROCK inhibitors have been reported to inhibit pulmonary artery myogenic responses in hypoxia-exposed adult rats (7) and fetal sheep (8) and to reverse sustained pulmonary vasoconstriction in response to hypoxia (9,10), bleomycin (10), or the infusion of vasoconstrictors, such as endothelin-1 (11). Systemic administration of ROCK inhibitors, commenced at the onset of injury, has been reported to prevent PHT induced either by hypoxia (9) or monocrotaline (12) in adult rodents. Finally, pilot studies in humans have shown that systemically administered fasudil acutely decreases pulmonary arterial pressure in adults with idiopathic PHT (13) and in children with PHT secondary to congenital heart disease (14). Together, these findings indicate that ROCK inhibitors hold great promise as a uniquely effective treatment for chronic PHT, however, little is known about their effects on the neonatal lung and pulmonary vasculature.

We have recently reported evidence of RhoA/ROCK pathway activation in the pulmonary arteries of neonatal rats with NO-unresponsive chronic PHT, secondary to either chronic hypoxia (CH) or bleomycin exposure (10). We have further shown that a single bolus of either Y-27632 (15 and 30 mg/kg) or fasudil (30 mg/kg) completely normalized pulmonary vascular resistance (PVR) in both models of chronic neonatal PHT (10). These findings suggest that ROCK is central to sustained vasoconstriction in chronic neonatal PHT, but the question about its role in the structural changes of chronic PHT remains unexplored. We hypothesized that sustained ROCK inhibition from birth would prevent CH-induced vascular remodeling through inhibition of smooth muscle cell (SMC) proliferation. Because growth factors, particularly PDGFs (15,16), are known to mediate smooth muscle proliferation in immature pulmonary vessels (16) and have been implicated in the persistence and progression of chronic PHT in humans (17), we also hypothesized that attenuation of hypoxia-induced SMC proliferation by inhibition of ROCK would be associated with changes in expression of PDGFs and their receptors. Finally, given that ROCK is highly expressed throughout the hypoxia-exposed neonatal rat lung (10) and has been shown to play a major role in proliferation of epithelial cells and other cell types

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **MLC_{20},** myosin regulatory light chain; **%MWT,** percentage arterial medial wall thickness; **PAAT,** pulmonary arterial acceleration time; **PHT,** pulmonary hypertension; **PVR,** pulmonary vascular resistance; **ROCK,** Rho-kinase; **RVET,** right ventricular ejection time; **RVH,** right ventricular hypertrophy; **SMC,** smooth muscle cell

(18,19), we examined whether sustained ROCK inhibition would impact lung growth and alveologenesis.

MATERIALS AND METHODS

Materials. Y-27632 and fasudil were from Alexis Biochemicals (San Diego, CA) and Enzo Life Sciences (Plymouth Meeting, PA), respectively. Plexiglas animal exposure chambers and automated O_2 controllers (Oxy Cycler model A84XOV) were from BioSpherix Ltd. (Redfield, NY). Acids, alcohols, organic solvents, paraformaldehyde, Permount, and Superfrost/Plus microscope slides were from Fisher Scientific (Whitby, ON, Canada). 5-Bromo-2′-deoxyuridine (BrdU) and an *in situ* BrdU immunostaining kit were from BD Biosciences (Mississauga, ON, Canada). Rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12; sc-25778), PDGF-receptor β (PDGF-Rβ; sc-339), phospho-tyrosine 1021 PDGF-Rβ (sc-12909-R) PDGF-Rα (sc-338), anti-myosin regulatory light chain (MLC_{20}; sc-15370), and goat anti-mouse and -rabbit IgG-biotin secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-human PDGF-BB (RB-9257) was from Neomarkers (Fremont, CA). Polyclonal goat anti-human PDGF-AA (AB-221-NA) was from R&D Systems (Minneapolis, MN). A goat anti-rabbit IgG-peroxidase antibody was from Cell Signaling Technology (Beverly, MA). Phos-tag acrylamide was from NARD Institute (Amagasaki City, Japan).

Animal exposures and interventions. All procedures involving animals were performed in accordance with the standards of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Sunnybrook Research Institute. Each litter, maintained at $n = 10$–12 pups to control for nutritional effects, was nursed in either air (21% O_2) or hypoxia (13% O_2) from postnatal d 1–14, as previously described in detail (2). Pups received either Y-27632 (1 or 2 mg/mL suspended in 0.9% saline vehicle; 5 μL/g body weight = 5 or 10 mg/kg) or an equivalent volume of vehicle by daily intraperitoneal injection. In initial experiments, a higher dose of Y-27632 (15 $mg \cdot kg^{-1} \cdot d^{-1}$) previously found to be acutely effective in reversing sustained pulmonary vasoconstriction (10) was found to be fatal in ~40% of pups by d 14. In additional studies, treatment with a structurally dissimilar and ~50% less potent ROCK inhibitor (6), fasudil (20 $mg \cdot kg^{-1} \cdot d^{-1}$), was also found to increase mortality (~20% by 14 d). At the end of each 14-d exposure period, pups were either killed by pentobarbital overdose or exsanguinated after anesthesia. Some pups received 20 mg/kg BrdU intraperitoneally 2 h before sacrifice.

Right ventricular hypertrophy (RVH). RVH was quantified by measuring the right ventricle to left ventricle and septum dry weight ratio, as previously described (20).

Two-dimensional echocardiographic analyses. Evaluation of PVR was performed in anesthetized animals spontaneously breathing room air, as previously described in detail (2,10). Briefly, a short axis view at the level of the aortic valve was obtained and the pulmonary artery was identified using color flow Doppler. The pulmonary arterial acceleration time (PAAT) was measured as the time from the onset of systolic flow to peak pulmonary outflow velocity and the right ventricular ejection time (RVET) as the time from onset to completion of systolic pulmonary flow. A surrogate of PVR was calculated according to the formula: [1/(PAAT/RVET)].

Immunohistochemistry. Lungs were inflation-fixed, embedded in paraffin, cut into 5-μm sections and immunostained using an avidin-biotin-peroxidase method, as previously described (2,20). Concentrations of the primary antisera were 1:1000 for PDGF-BB, 1:200 for PDGF-AA, 1:200 for PDGF-Rβ and -Rα, and 1:100 for phospho-PDGF-Rβ. For PDGF-BB, PDGF-Rβ, and phospho-PDGF-Rβ, negative controls were generated by preadsorption with a 10-fold excess of blocking peptide. For quantitation of BrdU-labeled SMC nuclei, eight BrdU-stained sections were examined from each animal (four from the right lung and four from the left), also as previously described (16).

Morphometric analyses. For all analyses, measurements were carried out on four sections per animal and four animals (representing two litters) per treatment group by an observer blinded to group identity. For assessment of percentage arterial medial wall thickness (%MWT), pulmonary arteries were identified by the presence of both inner and outer elastic lamina using Hart's stain, as previously described in detail (2). Analyses of distal airway structure, including tissue density, mean linear intercept, and secondary crest volume density were carried out on hematoxylin and eosin stained sections, as previously described (21).

Western blot analyses. Third or fourth generation intrapulmonary arteries were dissected from four litters per group (the pooled vessels of 2–3 animals from each litter representing one sample), lysed in RIPA buffer containing protease and phosphatase inhibitors, fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, blotted and band densities measured as previously described (16). Compensation for differences in protein loading was achieved by reblotting for GAPDH. Dilutions of primary antisera were 1:1000 for PDGF-BB and PDGF-Rβ and 1:5000 for GAPDH. MLC_{20} phosphorylation, used as a marker of ROCK activity (22), was quantified by Phos-tag acrylamide SDS-PAGE (23), according to a method previously reported in detail (24). After electrophoresis and transfer, membranes were blotted with anti-MLC_{20} (dilution 1:1000), yielding two bands with Phos-tag (an upper band representing phosphorylated and a lower band representing unphosphorylated MLC_{20}) and only one band when Phos-tag was omitted from the resolving gel. Density of the upper band was expressed as a percentage of the combined densities of both upper and lower bands.

Data presentation and analysis. All values are expressed as means ± SEM. Statistical significance ($p < 0.05$) was determined by two-way ANOVA followed by pair-wise multiple comparisons using the Tukey test (SigmaStat, Systat software, San Jose, CA).

RESULTS

Body weight and lung weight. As shown in Table 1, and reported previously (2), neonatal rats chronically exposed to hypoxia had reduced body weight compared with air-exposed controls. Daily treatment with 10 mg/kg Y-27632 or 20 mg/kg fasudil augmented hypoxia-induced somatic growth restriction but had no effect on body weight in air-exposed animals (Table 1). In contrast, inhibitory effects on growth were not observed in hypoxia-exposed animals treated with 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632. Lung weight was significantly decreased in CH-exposed animals treated with vehicle or 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632, when compared with air, but not in animals treated with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 or 20 $mg \cdot kg^{-1} \cdot d^{-1}$ fasudil (Table 1).

Right ventricular hypertrophy. Chronic exposure to hypoxia led to significant RVH, which was almost completely attenuated by treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 (Fig. 1*A*). A similar degree of attenuation of hypoxia-induced RVH was observed after treatment with 20 $mg \cdot kg^{-1} \cdot d^{-1}$ fasudil. In contrast, treatment with 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 had no significant effect on this parameter.

Arterial wall remodeling. As shown in Figure 1*B*, chronic exposure to hypoxia led to arterial wall remodeling, as demonstrated by increased %MWT. Treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 or 20 $mg \cdot kg^{-1} \cdot d^{-1}$ fasudil almost completely attenuated the hypoxia-induced increase in %MWT, whereas 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 had no significant effect. Differences in medial wall thickness between vehicle- and Y-27632-treated groups are further illustrated by Hart's elastin staining in Figure 1*C*.

Table 1. *Body weight and lung weight at 14 d*

	Air	Hypoxia
Body weight (g)		
Vehicle	30.4 ± 1.02	27.0 ± 0.87*
Y-27632 (5 $mg \cdot kg^{-1} \cdot d^{-1}$)	28.4 ± 0.34	26.0 ± 0.58*
Y-27632 (10 $mg \cdot kg^{-1} \cdot d^{-1}$)	29.9 ± 0.56	22.0 ± 0.71*†
Fasudil (20 $mg \cdot kg^{-1} \cdot d^{-1}$)	28.3 ± 0.78	21.3 ± 0.65*†
Lung weight (mg)		
Vehicle	511 ± 23	448 ± 16*
Y-27632 (5 $mg \cdot kg^{-1} \cdot d^{-1}$)	529 ± 21	454 ± 20*
Y-27632 (10 $mg \cdot kg^{-1} \cdot d^{-1}$)	516 ± 12	470 ± 12
Fasudil (20 $mg \cdot kg^{-1} \cdot d^{-1}$)	503 ± 18	465 ± 22

Values represent means ± SEM for eight animals (representing two litters)/group.

* $p < 0.05$, by two-way ANOVA, compared with respective air-exposed group.

† $p < 0.01$, by two-way ANOVA, compared with hypoxia-exposed vehicle-treated group.

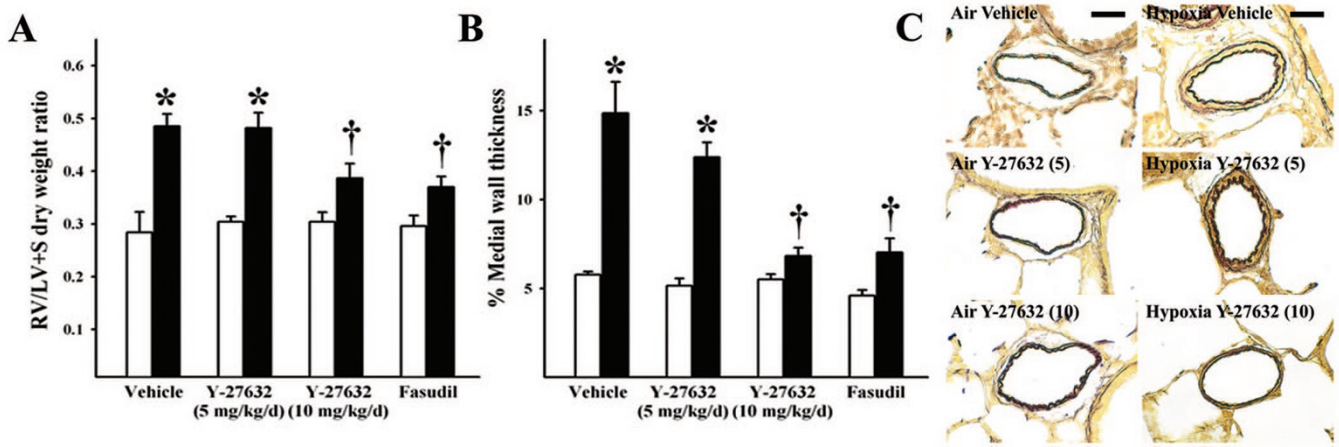

Figure 1. Structural changes of chronic PHT. (*A*) Right ventricle (RV)/left ventricle + septum (LV + S) weight ratio ($n = 8$ animals/group), as a marker of RVH, and (*B*) % arterial medial wall thickness in either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 4$ animals/group). $*p < 0.05$, by two-way ANOVA, compared with air-exposed groups. $\dagger p < 0.05$, by two-way ANOVA, compared with vehicle-treated hypoxia-exposed group. (*C*) Representative photomicrographs of Hart's elastin stain in the lungs of pups that were exposed to 21% O_2 (air) or 13% O_2 (hypoxia) and treated with either 0.9% saline (vehicle), 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 [Y-27632 (5)] or 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 [Y-27632 (10)] from postnatal d 1–14. Bar lengths = 25 μm.

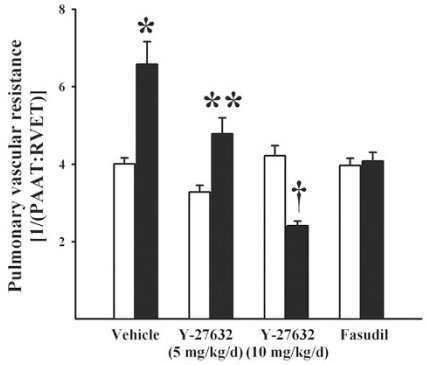

Figure 2. Pulmonary vascular resistance. Inverse PAAT:RVET [1/(PAAT:RVET)] ratio as a surrogate marker of PVR in either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 8$ animals/group). $*p < 0.01$, by two-way ANOVA, compared with respective air group. $**p < 0.05$, by two-way ANOVA, compared with respective air group. $\dagger p < 0.01$, by two-way ANOVA, compared with hypoxia groups.

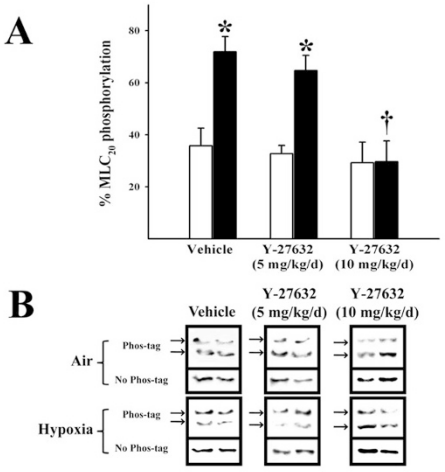

Figure 3. Rho-kinase activity. (*A*) Western blot analyses of % myosin regulatory light chain (MLC_{20}) phosphorylation, as a marker of Rho-kinase activity in lung tissue from either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 4$ samples/group). $*p < 0.01$, by two-way ANOVA, compared with respective air-exposed group. $\dagger p < 0.05$, by two-way ANOVA, compared with other hypoxia-exposed groups. (*B*) Representative immunoblots for MLC_{20} either with Phos-tag (upper phosphorylated band and lower unphosphorylated band, both highlighted by arrows) or without Phos-tag (single band).

Pulmonary vascular resistance. Chronic exposure to hypoxia increased PVR (Fig. 2), even while breathing room air, as previously reported (2,10). Treatment with either dose of Y-27632 or 20 $mg \cdot kg^{-1} \cdot d^{-1}$ fasudil significantly attenuated PVR when compared with hypoxia-exposed vehicle-treated pups but with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 having a greater effect than 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 or fasudil (Fig. 2).

ROCK activity. As shown in Figure 3, increased % MLC_{20} phosphorylation (as a marker of increased ROCK activity) was observed in the lungs of animals chronically exposed to hypoxia. Treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 normalized ROCK activity in hypoxia-exposed pups, whereas 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 had no significant effect. A complete attenuation of ROCK activity was also observed in the lungs of animals treated with 20 $mg \cdot kg^{-1} \cdot d^{-1}$ fasudil (data not shown).

Arterial smooth muscle proliferation. As shown in Figure 4, we observed increased SMC proliferation in the medial wall of pulmonary arteries from animals chronically exposed to hypoxia, as demonstrated by increased BrdU labeling. Increased proliferation was completely attenuated by treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632.

PDGF ligand and receptor expression. Immunohistochemistry was used to screen for changes in pulmonary arterial expression of PDGF-AA and its -Rα, which were found not to differ between air- and hypoxia-exposed groups (data not shown). In contrast, a marked hypoxia-mediated increase in PDGF-BB immunoreactivity on pulmonary arteries was observed (Fig. 5*A*). Increased PDGF-BB immunoreactivity was attenuated by treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$, but not by 5 $mg \cdot kg^{-1} \cdot d^{-1}$, Y-27632 (Fig. 5*A*). These findings were confirmed by Western blot analyses on pulmonary arterial tissue-derived protein (Fig. 5*B*). Chronic exposure to hypoxia also

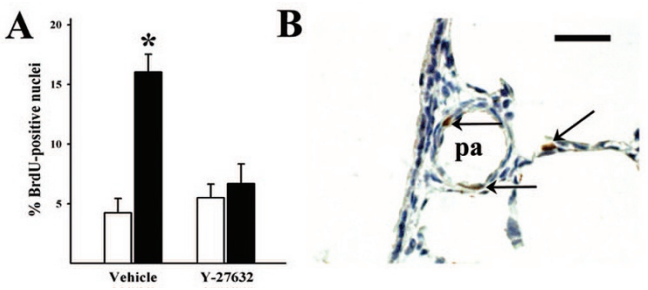

Figure 4. Arterial wall smooth muscle proliferation. (*A*) Bromodeoxyuridine-labeled medial wall nuclear counts in pulmonary arteries, expressed as a percentage of total medial wall nuclei in either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 4$ animals/group). *$p < 0.001$, by two-way ANOVA, compared with all other groups. (*B*) Representative high-power photomicrograph of a lung section from a hypoxia-exposed vehicle-treated pup, demonstrating BrdU-positive nuclei (brown stain), highlighted by arrows, two of which are located in the medial wall of a pulmonary artery (pa). Bar length = 25 μm.

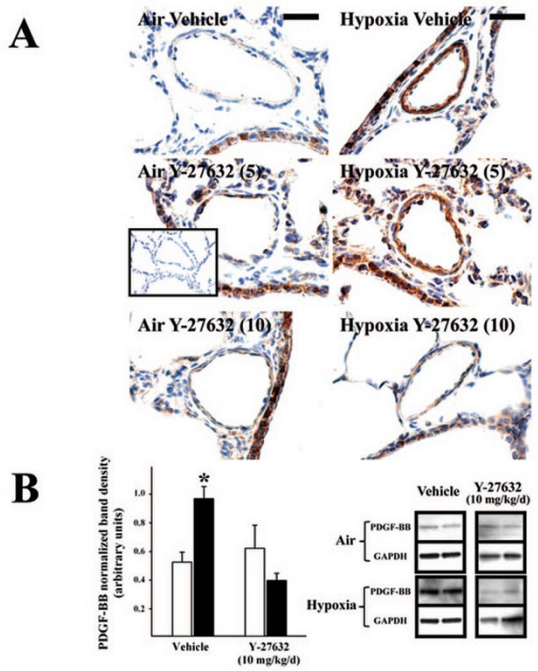

Figure 5. PDGF-BB expression. (*A*) Representative photomicrographs of PDGF-BB immunohistochemistry (brown stain) in pups that were exposed to 21% O_2 (air) or 13% O_2 (hypoxia) and treated with either 0.9% saline (vehicle) or Y-27632 (5 or 10 $mg \cdot kg^{-1} \cdot d^{-1}$) from postnatal d 1–14. Markedly increased arterial wall expression was evident in a hypoxia-exposed vehicle-treated pup, which was completely attenuated by treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 [Y-27632 (10)] but not 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 [Y-27632 (5)]. Inset: lack of staining in a section from a hypoxia-exposed vehicle-treated pup in which the primary antibody was preadsorbed with blocking peptide. Bar lengths = 25 μm. (*B*) Western blot analyses of arterial PDGF-BB expression, normalized to GAPDH, in either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 4$ samples/group). *$p < 0.05$, by two-way ANOVA, compared with air vehicle and hypoxia-exposed Y-27632-treated group.

led to increased arterial wall expression of PDGF-Rβ (Fig. 6*A* and *B*). In hypoxia-exposed animals, overall expression of PDGF-Rβ (as shown by Western blot analyses; Fig. 6*B*) was decreased to levels similar to air controls by treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632. Treatment with 5 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632 had no effect on PDGF-Rβ expression (data not shown). As shown in Figure 6*C*, PDGF-Rβ activation, using immunohistochemistry for phospho-tyrosine PDGF-Rβ as a marker (17), was increased by chronic exposure to hypoxia and completely attenuated by treatment with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ Y-27632.

Distal airway morphology. We observed septal thinning and "emphysematous" distal airspaces (Fig. 7*A*) in the lungs of pups chronically exposed to hypoxia, as evidenced by significantly decreased tissue density (Fig. 7*B*) and increased mean linear intercept (Fig. 7*C*), consistent with impaired alveolar formation, as previously reported by others (25). Neither Y-27632 nor fasudil had any effect on decreased tissue density induced by hypoxia (Fig. 7*B*). In contrast, there was a trend toward decreased mean linear intercept (suggestive of augmented secondary septation) in hypoxia-exposed animals treated with Y-27632 ($p = 0.065$ *versus* hypoxia-exposed vehicle-treated group). In comparison, treatment with fasudil led to a greater, and statistically significant, decrease in mean linear intercept. These findings led us to examine secondary crest number/unit area (mm^2) as a more sensitive marker of secondary septation. As shown in Figure 7*D*, neither Y-27632 nor fasudil had any impact on decreased secondary crest counts in hypoxia-exposed animals.

DISCUSSION

We have previously shown that a single bolus of ROCK inhibitor completely normalized PVR in neonatal rats with established chronic PHT (10). In this study, we extended these findings by examining the effects of sustained ROCK inhibition from birth in preventing the structural changes of chronic hypoxic PHT. We found that systemic treatment with Y-27632 or fasudil at doses, which completely attenuated hypoxia-induced up-regulation of ROCK activity in the lung, attenuated RVH and increased arterial medial wall thickening, but had no impact on changes in distal airways characterized by septal thinning and inhibited secondary septation.

A novel insight from this study was that attenuating effects of Y-27632 on SMC hyperplasia and arterial wall remodeling were paralleled by attenuated hypoxia-induced up-regulation of arterial PDGF-BB and PDGF-Rβ expression and consequently decreased PDGF-Rβ activation. PDGF is made up of homo or heterodimers consisting of A and B chains. PDGF-AA binds exclusively to the PDGF-Rα, whereas PDGF-Rβ binds all isoforms. We have previously reported that PDGF-BB (and not -AA) stimulates proliferation of neonatal rat primary cultured pulmonary arterial smooth muscle (16). Furthermore, we have shown, taking a soluble receptor approach, that increased expression of PDGF-Rβ ligands are critical to pathologic proliferation of neonatal rat pulmonary arterial smooth muscle *in vivo* (16). Coupled with these earlier observations, our current findings implicate inhibitory effects on the PDGF-BB/Rβ pathway as a major mechanism by which ROCK inhibitors limit pulmonary vascular remodelling. A caveat is that reversal of sustained vasoconstriction, which may be expected to reduce mechanical strain on vascular wall smooth muscle, can itself contribute to changes in PDGF receptor expression/activation

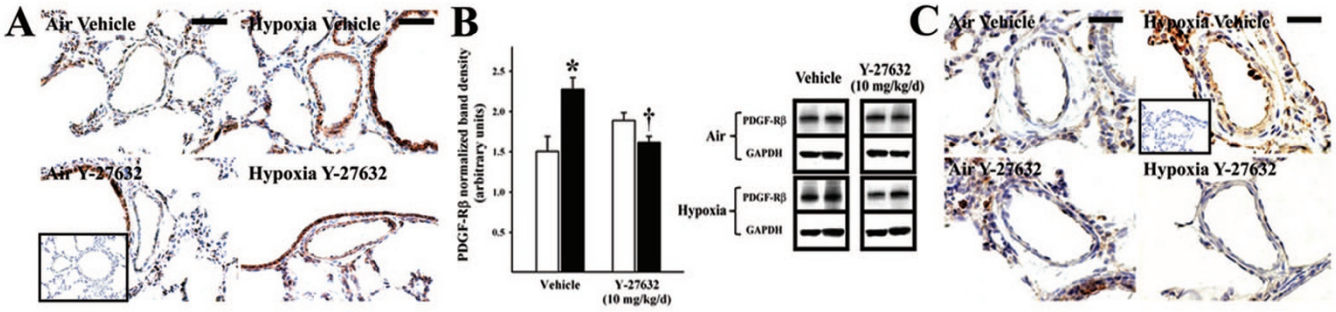

Figure 6. PDGF-Rβ expression and activation. (*A*) Representative photomicrographs of PDGF-Rβ immunohistochemistry (brown stain) in pups that were exposed to 21% O_2 (air) or 13% O_2 (hypoxia) and treated with either 0.9% saline (vehicle) or Y-27632 (10 $mg \cdot kg^{-1} \cdot d^{-1}$) from postnatal d 1–14. Markedly increased arterial wall expression is evident in a hypoxia-exposed vehicle-treated pup. Intensity of staining in the arterial wall was reduced by treatment with Y-27632. Inset: lack of staining in a section from a hypoxia-exposed vehicle-treated pup in which the primary antibody was preadsorbed with blocking peptide. Bar lengths = 50 μm. (*B*) Western blot analyses of arterial PDGF-Rβ expression, normalized to GAPDH, in either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 4$ samples/group). *$p < 0.05$, by two-way ANOVA, compared with respective air-exposed group. †$p < 0.05$, by two-way ANOVA, compared with hypoxia-exposed vehicle-treated group. (*C*) Representative photomicrographs of phospho-tyrosine PDGF-Rβ immunohistochemistry (brown stain), as a marker of PDGF-Rβ activation. Markedly increased immunoreactivity is evident in a hypoxia-exposed vehicle-treated pup, which was completely attenuated by treatment with Y-27632. Inset: lack of staining in a section from a hypoxia-exposed vehicle-treated pup in which the primary antibody was preadsorbed with blocking peptide. Bar lengths = 25 μm.

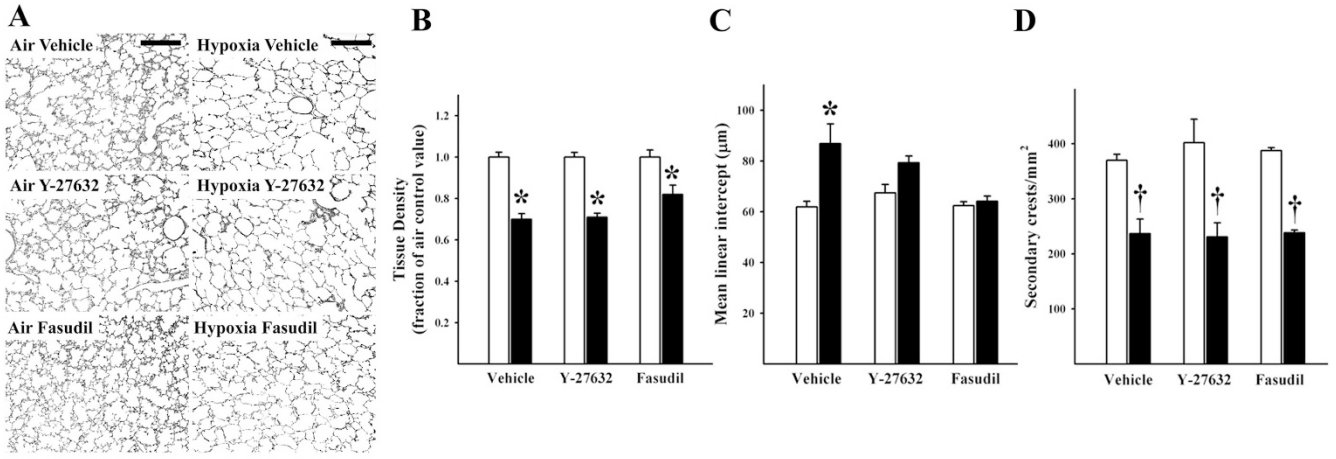

Figure 7. Distal airway morphology. (*A*) Representative low-power photomicrographs of hematoxylin and eosin-stained sections from pups that were exposed to 21% O_2 (air) or 13% O_2 (hypoxia) and treated with either 0.9% saline (vehicle) or Y-27632 (10 $mg \cdot kg^{-1} \cdot d^{-1}$) or fasudil (20 $mg \cdot kg^{-1} \cdot d^{-1}$) from postnatal d 1–14. Bar lengths = 250 μm. Morphometric analyses of (*B*) tissue density, (*C*) mean linear intercept and (*D*) secondary crests/mm^2 in either air- (*open bars*) or 13% O_2-exposed (*closed bars*) animals ($n = 4$ animals/group). *$p < 0.05$, by two-way ANOVA, compared with respective air-exposed groups. †$p < 0.001$, by two-way ANOVA, compared with air-exposed groups ($n = 4$ animals/group).

and consequent SMC proliferation (26). Therefore, it is not possible, taking the present pharmacological approach, to distinguish indirect consequences of ROCK inhibitor-induced vasodilatation from limiting effects on other ROCK-mediated pathways. Other mechanisms, not explored in this study, by which ROCK inhibitors may have limited vascular remodeling include attenuated vascular production of endothelin-1 (27), inhibited downstream signaling of G-protein-coupled receptor ligands (11,28), and augmented function of the NO/cyclic GMP pathway (29).

Importantly, we observed worsened somatic growth restriction in hypoxia-exposed pups receiving ROCK inhibitors, which has not been reported in mature rodents receiving either Y-27632 or fasudil. In addition, mortality was increased in neonatal rat pups at doses much lower than that previously reported to be nonlethal in adult rodents (9,30,31). Fagan *et al.* (9) reported increased mortality in adult mice given Y-27632 60 $mg \cdot kg^{-1} \cdot d^{-1}$ by s.c. infusion, with no apparent adverse effects reported at 30 $mg \cdot kg^{-1} \cdot d^{-1}$. Similarly, Hyvelin *et al.* treated adult rats with daily enteral boluses of Y-27632 30 mg/kg for up to 3 wk, without apparent toxic effects. Finally, Abe *et al.* (31) treated hypoxia-exposed mice with daily boluses of fasudil 100 mg/kg, also for 3 wk, without apparent toxicity. Our observation that a dose of Y-27632 (5 $mg \cdot kg^{-1} \cdot d^{-1}$) that did not attenuate ROCK activity also had no effect on somatic growth, coupled with the similarity in effects on growth between two structurally dissimilar ROCK inhibitors, strongly suggests that growth restriction was related to systemic inhibition of ROCK, rather than through an off-target effect. Whether growth restriction and mortality arose from the same downstream effect of ROCK inhibition is uncertain, as mortality affected both air- and hypoxia-exposed pups, whereas growth restriction was only seen in those chronically exposed to hypoxia. Systemic hypoperfusion, be-

cause of parallel dilating effects of ROCK inhibitors on the systemic vasculature (32) could contribute to both phenomena. Alternatively, ROCK is known to be expressed in both vascular and nonvascular cell types in many tissues (18,19); therefore, it is likely that ROCK modulates proliferation in many cell types and that its role is amplified under hypoxic conditions. Regardless of mechanism, given the apparent increased sensitivity of newborn animals to the systemic effects of ROCK inhibitors and the narrow therapeutic-toxic dose range, our results suggest that systemic treatment with this class of agents should only be undertaken in neonates with caution. A potentially safer and equally efficacious approach may be through delivery of smaller doses directly to the lung, either by intratracheal instillation (similar to exogenous surfactant) or by nebulization, which has been shown in adult animals to limit associated effects on the systemic vasculature (32).

ROCK signaling has been demonstrated to be critical for cardiac morphogenesis (33); however, its role in postnatal lung development has received limited attention. McMurtry *et al.* (34) reported that chronic PHT was attenuated by enteral (*via* mother's milk) fasudil over the first 8 wk of life in fawn-hooded rats at Denver altitude. It was further suggested that fasudil enhanced alveolar development, although morphometric analyses were not performed (34). Our observations differ in that neither Y-27632 nor fasudil had any impact on hypoxia-induced septal thinning or alveolarization, as assessed by secondary crest counts. Just as importantly, lung morphology in air-exposed animals was unaffected by either treatment, suggesting that ROCK is not critical to normal postnatal alveolarization. These observations provide a measure of reassurance regarding potential safety of ROCK inhibitors for the treatment of refractory PHT in neonates, should direct delivery to the lung prove efficacious in future studies.

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