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Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase

Karen A. Fagan, 1 Masahiko Oka, 1 Natalie R. Bauer, 1 Sarah A. Gebb, 4 D. Dunbar Ivy, 2 Kenneth G. Morris, 1 and Ivan F. McMurtry 1

1 Cardiovascular Pulmonary Research Laboratory and 2 Department of Pediatrics, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Fagan, Karen A., Masahiko Oka, Natalie R. Bauer, Sarah A. Gebb, D. Dunbar Ivy, Kenneth G. Morris, and Ivan F. McMurtry. Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. Am J Physiol Lung Cell Mol Physiol 287: L656–L664, 2004. First published February 20, 2004; 10.1152/ajplung.00990.2003.—RhoA GTPase mediates a variety of cellular responses, including activation of the contractile apparatus, growth, and gene expression. Acute hypoxia activates RhoA and, in turn, its downstream effector, Rho-kinase, and previous studies in rats have suggested a role for Rho/Rho-kinase signaling in both acute and chronically hypoxic pulmonary vasoconstriction. We therefore hypothesized that activation of Rho/Rho-kinase in the pulmonary circulation of mice contributes to acute hypoxic pulmonary vasoconstriction and chronic hypoxia-induced pulmonary hypertension and vascular remodeling. In isolated, salt solution-perfused mouse lungs, acute administration of the Rho-kinase inhibitor Y-27632 (10 M) attenuated hypoxic vasoconstriction as well as that due to angiotensin II and KCl. Chronic treatment with Y-27632 (30 mg·kg⁻¹·day⁻¹) via subcutaneous osmotic pump decreased right ventricular systolic pressure, right ventricular hypertrophy, and neomuscularization of the distal pulmonary vasculature in mice exposed to hypobaric hypoxia for 14 days. Analysis of a small number of proximal pulmonary arteries suggested that Y-27632 treatment reduced the level of phospho-CPI-17, a Rho-kinase target, in hypoxic lungs. We also found that endothelial nitric oxide synthase protein in hypoxic lungs was augmented by Rho-kinase target, in hypoxic lungs. We also found that endothelial nitric oxide synthase (eNOS). In endothelial cells, activation of Rho-kinase decreases eNOS by reducing eNOS mRNA stability (8, 13, 36). It is not known whether Rho-kinase signaling regulates expression of eNOS in the hypoxic lung.

RhoA: Y-27632

RHOA IS A MEMBER of the Rho family of small GTPases, including Rac1, Cdc42, and others, that are important in regulating a variety of cellular responses, including cell contraction, migration, growth, gene expression, and differentiation (7). RhoA is activated by exchange of GDP for GTP and translocated to the plasma membrane where it stimulates its downstream effectors such as Rho-kinase. RhoA is regulated by activators, guanine nucleotide exchange factors, inactivators, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors. Activation of RhoA can occur via stimulation of G protein-coupled receptors or by receptor and nonreceptor tyrosine kinases. There is evidence that hypoxia also activates RhoA in pulmonary artery (PA) smooth muscle and endothelial cells, but the mechanisms are unknown (23, 36, 39).

Rho-kinase activation increases Ca²⁺ sensitivity of contraction in vascular smooth muscle cells by inhibiting myosin light chain phosphatase, which increases the phosphorylation of myosin light chain and augments contraction at a given level of cytosolic Ca²⁺ and activity of myosin light chain kinase. Rho-kinase inhibits myosin light chain phosphatase by phosphorylating the myosin-binding subunit of myosin light chain phosphatase (MYP-T-1) and/or the myosin light chain phosphatase inhibitor protein CPI-17 (32). In some cases, phosphorylation of CPI-17 by Rho-kinase may be more significant in sustained vasoconstriction than is phosphorylation of MYP-T-1 (12, 18).

Rho-kinase is also involved in growth of systemic vascular smooth muscle cells (4, 27, 29, 41) and plays a role in injury-induced vascular remodeling (28). Rho signaling alters expression of several genes known to be important in regulating pulmonary vascular tone and structure, including endothelial nitric oxide synthase (eNOS). In endothelial cells, activation of Rho-kinase decreases eNOS by reducing eNOS mRNA stability (8, 13, 36). It is not known whether Rho-kinase signaling regulates expression of eNOS in the hypoxic lung.

Hypoxia activates Rho/Rho-kinase signaling in PAs of rats, and Rho-kinase activity is important in mediating the sustained phase of acute hypoxic pulmonary vasoconstriction (23, 39). Inhibition of Rho-kinase abolishes the sustained phase of hypoxic vasoconstriction in rat PAs and perfused lungs (23, 39) presumably via activation of myosin light chain phosphatase and decreased phosphorylation of myosin light chains (39). A recent study shows that acute inhibition of Rho-kinase with the Rho-kinase inhibitors Y-27632 and fasudil elicits marked pulmonary vasodilation in chronically hypoxic catheterized rats and perfused rat lungs, suggesting a role for activation of Rho-kinase in the increased basal pulmonary vascular tone of hypoxic pulmonary hypertension (17). The Rho-kinase inhibitor fasudil was recently reported to prevent...
and reverse monocrotaline-induced pulmonary hypertension in rats (1). Neither acute nor chronic use of Rho-kinase inhibitors has been reported in mice.

Thus we hypothesize that Rho/Rho-kinase activation is important in mediating both acute hypoxic pulmonary vasoconstriction and chronic hypoxia-induced pulmonary hypertension and vascular remodeling in mice. To test this hypothesis, we examined whether acute inhibition of Rho-kinase with Y-27632 attenuated pulmonary vasoreactivity in isolated mouse lungs and whether chronic treatment with Y-27632 reduced the development of pulmonary hypertension and vascular remodeling in chronically hypoxic mice.

MATERIALS AND METHODS

Mice
C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME) ages 10–12 wk at the time of study and weighing ~25 g were acclimatized to Denver altitude for 2 wk before experimentation. Mini-osmotic pumps containing either vehicle (saline) or Y-27632 (see below) were implanted, and the mice were then housed for 14 days in either ambient conditions or hypobaric hypoxia simulating an altitude of 17,000-foot elevation (FiO₂ ~10%). They were fed and watered ad libitum, and cages were changed twice weekly. Interruption of hypobaric hypoxia was <20 min twice weekly. Upon removal from hypobaric hypoxia, animals were maintained in cages flushed with 10% O₂ until experimentation. Mice exposed to hypoxia lost ~1 g in body weight, whereas normoxic mice gained ~1.5 g. There was no difference between vehicle- or Y-27632-treated mice with respect to weight changes during hypoxia or normoxia. All animal manipulations were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee.

Insertion of Osmotic Pumps

Twenty-four hours before initiation of hypoxic exposure (see above), mice were anesthetized with ketamine/xylazine (100 mg and 15 mg/kg), the skin was shaved and cleaned with iodine soap, and a subcutaneous pocket through a small incision made in the skin near the scapulae. These pumps delivered a volume of 0.25 μl/h, which was equivalent to 30 mg·kg⁻¹·day⁻¹ of Y-27632 for a 25-g mouse. There were no complications of insertion of the pumps, including bleeding, infection, or deaths, in the 30·mg·kg⁻¹·Y-27632- or vehicle-treated animals.

Isolated, Salt Solution-Perfused Mouse Lung

With the use of techniques similar to those previously described (10), untreated, normoxic mice, and normoxic mice after 14 days of treatment with vehicle or Y-27632 via subcutaneous osmotic pump mice were anesthetized with phenobarbital (6–7 mg ip). After confirmation of deep anesthesia, a thoracotomy was performed, and 100 units of heparin was injected into the right ventricle (RV). The trachea was cannulated with an 18-G blunt-tipped catheter and ventilated (Harvard Apparatus, Holliston, MA) with 21% O₂, 5% CO₂, balance N₂ at 60 breaths per minute with peak inspiratory and expiratory pressures of 9 and of 2.5 cmH₂O, respectively. The RV was opened, and P10 double lumen tubing (Becton Dickinson, Sparks, MD) was placed in the main PA and sutured in place. After placement of P90 catheter tubing (Becton Dickinson) in the left ventricle (LV), the lung was perfused with recirculated physiological salt solution (PSS) by peristaltic pump (Minipulse; Gilson, Middleton, WI) via one lumen of the PA catheter at 0.04 ml/g body wt. The PSS (Earle’s balanced salt solution) contained (in mM/l) 116.3 NaCl, 5.4 KCl, 0.83 MgSO₄, 19.0 NaHCO₃, 1.04 NaH₂PO₄, 1.8 CaCl₂ H₂O, and 3.5 γ-glucose, pH 7.35–7.45. Ficoll (4%) and 3.1 μM sodium meclofenamate were added to act as an oncotic agent and to inhibit prostaglandin synthesis, respectively. Temperature was maintained at 37°C with a heated table and warming light, and the preparation was covered with a plastic hood to prevent desiccation. Pulmonary perfusion pressure was continuously monitored and recorded at constant flow through a second lumen of the PA catheter (p23 ID transducer; Gould Statham, Oxnard, CA and MP100 recorder/amplifier; Biopac Systems, Santa Barbara, CA). A baseline perfusion pressure while being ventilated with 21% O₂ was established, after which the lung was challenged twice with acute hypoxia (10 min of 0% O₂) followed by return to 21% O₂ and with a bolus injection of angiotensin II (0.2 μg) into the perfusate. The lung was then treated with vehicle or Rho-kinase inhibitor (Y-27632, 1 × 10⁻⁵ M) and exposed again to hypoxia and angiotensin II. Last, the lungs were stimulated with 40 mM KCl. The use of 21% O₂ results in an effluent perfusate PΟ₂ of ~100, and 0% O₂ results in a PΟ₂ of ~35 mmHg (26).

Right Ventricular Systolic Pressure

Right ventricular systolic pressure (RVSP) was measured as previously described (9, 42). Briefly, mice were anesthetized with ketamine/xylazine (100 mg and 15 mg/kg) and placed supine while spontaneously breathing room air. A 26-G needle attached to a pressure transducer (Sorenson Transpac; Abbott Critical Care Systems, Chicago, IL) was introduced percutaneously into the thorax via a subxyphoid approach. RVSP waveform was identified on a multichannel recorder and recorded. Blood was withdrawn from the heart, and animals were killed by exsanguination.

Right Ventricular Hypertrophy and Hematocrit

With the use of standard techniques, the RV was dissected from the LV and septum (LV+S) after removal of the atria. Tissue was weighed, and the ratio of RV to LV+S was calculated. Hematocrits were determined using standard capillary tube techniques.

Western Blotting

PA CPI-17 and β-actin. Freshly isolated mouse PAs from vehicle- and Y-27632-treated normoxic and chronically hypoxic mice were equilibrated for 1 h in either normoxic (21% O₂) or hypoxic (10% O₂) bath. Vessels were then transferred to a buffer of 10% trichloroacetic acid in acetone and stored at −70°C overnight. Vessels were then rinsed in 100% acetone, allowed to air dry, and homogenized in a glass Dounce homogenizer for 2 min on ice in ice-cold lysing buffer [PBS + 10 mM HEPES + 2 mM EDTA + 1 mM MgCl₂ + 10 mM Na₃P₃O₁₀ + 500 μM NaVO₄ + protease inhibitor cocktail (Sigma Chemical mix: 6.9 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μM aprotonin, 0.13 mM leupeptin, 0.26 mM bestatin, 0.1 mM pepstatin, 0.09 mM E-64] + 1 mM DTT + 1 mM PMSF + phosphatase inhibitor cocktail (proprietary mix, Sigma Chemical) + 1% Triton X-100]. Samples were then sonicated for 10 s on ice and centrifuged at 1,500 g for 10 min, and the pellet was discarded. Western blot analysis was performed on the supernatant for phospho-CPI-17 (anti-phospho-CPI-17 no. 36-006 antibody, 1:100 dilution; Upstate Bio-technologies, Lake Placid, NY). β-Actin (monoclonal anti-β-actin A5441, 1:15,000 dilution; Sigma Chemical) was probed to insure equal protein loading. Membranes were washed and incubated with secondary antibody (horseradish peroxidase-conjugated anti-mouse;
Vector Laboratories, Burlingame, CA) 1:2,000 in blocking solution. Horseradish peroxidase was detected using the ECL+ plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ). Relative density of CPI-17 to β-actin was determined using NIH Image software.

**Lung eNOS and β-actin.** Freshly isolated lung tissue was homogenized in ice-cold radioimmunoprecipitation assay buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS with 30 µl/ml aprotinin, 10 mg/ml PMSF, and 1 mM sodium orthovanadate). Samples were centrifuged for 10 min at 10,000 g at 4°C. Protein concentration was measured by the method of Lowry (Sigma Diagnostics, St. Louis, MO). Five micrograms of total protein were separated by SDS-PAGE under reducing conditions using 4–12% gradient gels and transferred to polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA). Membranes were blocked for 1 h in PBS, 1% nonfat dry milk, and 0.05% Tween 20 and then incubated overnight with primary antibody (anti-eNOS mouse monoclonal, diluted 1:1,500; BD Transduction Laboratories, San Diego, CA) and counterstained with hematoxylin as previously described (9). Airway anatomy was first identified at the level of the terminal bronchiole. Corresponding vessels were identified, and circumferential staining with myosin was determined as fully muscularized if >75% of the circumference was myosin positive. For each separate animal, 10 separate fields under high power (magnification ×200) were studied, and the number of fully muscularized vessels per field was counted.

**Statistical Analysis**

All data are expressed as means ± SE of the mean. Data were analyzed by ANOVA using Statview software (SAS Institute, Cary, NC) with P < 0.05 considered significant.

**RESULTS**

**Inhibition of Acute Pulmonary Vasoconstriction With Y-27632**

To test the effect of Rho-kinase inhibition on the response to hypoxia- and agonist-induced pulmonary vasoconstriction, we used PSS-perfused normoxic mouse lungs and measured vasoconstriction before and after treatment with Y-27632 (1 × 10⁻⁵ M). Figure 1A shows representative tracings of acute hypoxic vasoconstriction in lungs treated with vehicle (top) vs. Y-27632 (bottom) and demonstrates attenuation of the hypoxic pressor response by the Rho-kinase inhibitor. There was no difference in acute hypoxic or angiotensin II-induced pulmonary vasoconstriction before treatment with Y-27632 (1 × 10⁻⁵ M) or vehicle (Fig. 1, B and C). After treatment with Y-27632, there was a small, but significant, decrease in baseline pulmonary perfusion pressure compared with vehicle (−1.39 ± 0.5 vs. 0.3 ± 0.3 mmHg). Figure 1, B and C, demonstrates the significant attenuation of acute hypoxic and angiotensin II-induced pulmonary vasoconstriction after treatment with Y-27632 compared with vehicle. The Rho-kinase inhibitor also attenuated the vasoconstriction to 40 mM KCl (Fig. 1D).

To test whether chronic treatment with Y-27632 resulted in decreased responsiveness of the pulmonary circulation to exogenously administered Y-27632, normoxic mice were treated with 30 mg·kg⁻¹·day⁻¹ of Y-27632 or vehicle via osmotic pump. After 12–14 days of treatment, lungs were isolated as above. As shown in Fig. 1E, there was no difference in acute hypoxic vasoconstriction in lungs isolated from chronic Y-27632-treated mice compared with vehicle before or after administration of Y-27632 (1 × 10⁻⁵ M) in the perfusate, suggesting that tachyphylaxis to the Rho-kinase inhibitor had not occurred.

**Attenuation of Chronic Hypoxia-Induced Pulmonary Hypertension With Y-27632**

To determine whether Rho-kinase activation contributes to development of chronic hypoxia-induced pulmonary hypertension, mice were treated with vehicle or Y-27632 via osmotic pump (30 mg·kg⁻¹·day⁻¹) during 14 days of hypobaric hypoxia. As shown in Fig. 2A, treatment with Y-27632, compared with vehicle, reduced but did not prevent the hypoxia-induced increase in RVSP. There was no effect of Y-27632 on right ventricular pressure in normoxic control mice. Consistent with decreased right ventricular pressure, Fig. 2B shows the attenuation in right ventricular hypertrophy (RVH) in Y-27632-treated vs. vehicle control in chronically hypoxic mice. There was no effect of Y-27632 on right ventricular mass in normoxic animals. LV/body weight ratios were no different between vehicle- and Y-27632-treated normoxic mice. Treatment with Y-27632 resulted in a slight increase in LV/body weight in hypoxic mice (3.360 ± 0.058 vs. 2.998 ± 0.054, P = 0.001). This might have overestimated the attenuation of RVH. However, RV/body weight ratios paralleled the RV/LV+S ratios and confirmed that Y-27632 decreased RVH in hypoxia but had no effect in normoxia (data not shown). Treatment with Y-27632 caused a small decrease in hematocrit compared with vehicle in hypoxic mice (54.2 ± 1.6 vs. 57.8 ± 1.2%, P = 0.04) but not in normoxic mice (44.2 ± 0.5 vs. 45.7 ± 0.5%, P = not significant).

**Decreased Phospho-CPI-17 With Y-27632 in Hypoxic Mouse Lung**

To test whether in vivo treatment with Y-27632 inhibited Rho-kinase activity in mouse lung, we measured level of phosphorylated CPI-17, a target of Rho-kinase, in a small number of normoxic and hypoxic mouse proximal PAs. As shown in Fig. 3, A and B, there was suggestive evidence for an increase in PA phospho-CPI-17 after chronic hypoxia, and treatment with Y-27632 appeared to decrease phospho-CPI-17 in the chronically hypoxic mouse PAs.

**Y-27632 Decreased Neomuscularization of Distal Pulmonary Circulation in Hypoxia**

To determine whether attenuation of chronic hypoxia-induced pulmonary hypertension by Y-27632 was associated with decreased pulmonary vascular remodeling, we measured whether the number of vessels in the distal pulmonary circu-
lation that were >75% circumferentially stained positive for myosin was decreased. As seen in Fig. 4, A–C, myosin staining is readily apparent in fixed lungs. Compared with normoxic mice, the number of positive pulmonary vessels per high-powered field is increased in chronically hypoxic mice (Fig. 4A vs. 4B). Treatment with Y-27632 decreased the number of myosin-positive vessels in chronically hypoxic lungs (Fig. 4, C and D).

Y-27632 Enhanced eNOS Induction in Hypoxia

To examine whether upregulation of eNOS might have been involved in the attenuation of hypoxic pulmonary hypertension by Rho-kinase inhibition, we measured eNOS protein in lungs from normoxic and chronically hypoxic mice with and without treatment with Y-27632. As seen in Fig. 5, A and B, treatment with Y-27632 decreased lung eNOS protein in normoxic mice.
but enhanced eNOS protein during exposure to hypoxia. Two separate gels were run (normoxia and hypoxia), and direct comparison between normoxic and hypoxic lungs is not possible.

**DISCUSSION**

Hypoxic pulmonary hypertension contributes to the morbidity and mortality of patients with advanced heart and lung disease. A combination of hypoxia-induced vasoconstriction and vascular remodeling increases pulmonary vascular resistance and pressure and can lead to cor pulmonale. The exact mechanisms by which hypoxia causes these changes in the pulmonary circulation are unclear but likely involve complex regulation of cell signaling.

RhoA and one of its downstream effectors, Rho-kinase, have been recognized as important in cellular signaling in processes including cell growth, differentiation, gene expression, adhesion, actin cytoskeleton rearrangement, migration, and contraction (7). Recent reports have suggested a potential role for Rho/Rho-kinase in acute hypoxic vasoconstriction in isolated PAs and perfused lungs of rats (23, 39) and in the increased basal pulmonary vascular tone of chronically hypoxic rats (17). However, the role of Rho-kinase activation in the development of chronic hypoxia-induced pulmonary hypertension is not known. We report that the Rho-kinase inhibitor Y-27632 attenuated acute hypoxia-, agonist-, and KCl-induced vasoconstriction in perfused mouse lungs and that in vivo treatment of mice with Y-27632 attenuated the development of chronic hypoxia-induced pulmonary hypertension and vascular remodeling.

The balance between phosphorylated and dephosphorylated myosin light chains is important in contraction of vascular smooth muscle. Inhibition of myosin light chain phosphatase increases the phosphorylation of myosin light chains and thus promotes contraction. Because inhibition of myosin light chain phosphatase prevents dephosphorylation of myosin light chains, contraction can be sustained even if cytosolic Ca\(^{2+}\) levels fall, a process referred to as Ca\(^{2+}\) sensitization (32). In isolated PAs, hypoxia causes a transient peak in cytosolic Ca\(^{2+}\) that presumably activates myosin light chain kinase and initiates the transient initial contraction by increasing phosphorylation of myosin light chains. However, the progressive secondary phase of hypoxic contraction is associated with a constant elevated level of cytosolic Ca\(^{2+}\) and is likely due to Ca\(^{2+}\) sensitization of the contractile apparatus (23). A major pathway of Ca\(^{2+}\) sensitization is mediated via activation of Rho-kinase. Rho-kinase activation inhibits myosin light chain phosphatase via phosphorylation of MYPT-1 and/or CPI-17 (12, 18, 32). In the present study, we observed that chronic hypoxia may have led to increased phosphorylation of CPI-17 but enhanced eNOS protein during exposure to hypoxia. Two separate gels were run (normoxia and hypoxia), and direct comparison between normoxic and hypoxic lungs is not possible.

![A](https://example.com/image1.png) ![B](https://example.com/image2.png)

**Fig. 2.** A: right ventricular systolic pressure (RVP, mmHg) in mice treated with vehicle or Y-27632 (30 mg·kg\(^{-1}\)·day\(^{-1}\)) during 14 days of normoxia or hypoxia; n = 14 for vehicle-treated normoxic; n = 9 for Y-27632-treated normoxic; n = 11 for vehicle-treated hypoxic; n = 14 for Y-27632-treated hypoxic. *P < 0.05 compared with vehicle-treated hypoxic and vehicle- and Y-27632-treated normoxic mice. B: right ventricular (RV) weight compared with left ventricular plus septal weight (LV+S) in mice treated with either vehicle or Y-27632 (30 mg·kg\(^{-1}\)·day\(^{-1}\)) during 14 days of normoxia or hypoxia; n = 17 for vehicle-treated normoxic; n = 9 for Y-27632-treated normoxic; n = 16 for vehicle-treated hypoxic; n = 17 for Y-27632-treated hypoxic. *P < 0.05 compared with vehicle-treated hypoxic and normoxic mice.

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![A](https://example.com/image3.png) ![B](https://example.com/image4.png)

**Fig. 3.** A: phospho (P)-CPI-17 and β-actin immunoblotting of mouse proximal pulmonary arteries after 14 days of normoxia or hypoxia from vehicle (V) or Y-27632 (Y) chronically treated mice. B: CPI-17 density as % β-actin density. N = 2 different animals with normoxia vehicle; n = 1 normoxia Y-27632; n = 2 hypoxia vehicle; n = 2 hypoxia Y-27632.
in proximal PAs, and treatment of chronically hypoxic mice with the Rho-kinase inhibitor Y-27632 appeared to decrease phospho-CPI-17. This provides supportive evidence for activation of Rho-kinase during chronic hypoxia and for inhibition of Rho-kinase by Y-27632 in vivo. Thus inhibition of Rho-kinase-mediated Ca\(^{2+}\)/H\(_{11001}\) sensitization and decreased pulmonary vasoconstriction may have contributed to the attenuation of hypoxia-induced pulmonary hypertension.

In previous studies in rat lungs and isolated PAs, Rho-kinase inhibitors prevented the sustained phase of acute hypoxic pulmonary vasoconstriction, possibly by decreasing Ca\(^{2+}\) sensitivity (23, 39). In the present study, we found that acute administration of Y-27632 also attenuated acute hypoxic pulmonary vasoconstriction in perfused mouse lungs from normoxic mice, suggesting that activation of Rho-kinase contributes to the acute hypoxic vasoconstriction. Additionally, inhibition of Rho-kinase attenuated angiotensin II-induced pulmonary vasoconstriction. Angiotensin II activates RhoA in rat smooth muscle cells (41), and reports suggest involvement of Rho-kinase in angiotensin II-induced smooth muscle contraction (16, 21). Given that both hypoxia- and angiotensin-induced pulmonary vasoconstriction likely involve Ca\(^{2+}\) sensitization in smooth muscle, inhibition of Ca\(^{2+}\) sensitivity may be one mechanism by which inhibition of Rho-kinase attenuated these responses in mouse lung. Recently, Rho-kinase activity has also been implicated in KCl-mediated vasoconstriction, at least in some arteries (24). In aorta, Y-27632 inhibited KCl-induced vasoconstriction despite having no direct effects on cytosolic Ca\(^{2+}\), calmodulin, myosin light chain kinase, or myosin light chain phosphatase. In the present study, Y-27632 also attenuated KCl vasoconstriction in perfused mouse lungs, suggesting that Rho-kinase activity is important in hypoxia-, agonist-, and KCl-induced vasoconstriction in this preparation. Additionally, inhibition of Rho-kinase decreased normoxic, baseline pulmonary perfusion pressure slightly, suggesting that Rho-kinase activity plays a role in modulating basal pulmonary vascular tone. Because Y-27632 appears to be selective in inhibiting Rho-kinase activity without direct effects on other components of the contraction pathway (24), this raises the possibility that Rho-kinase inhibition leads to a generalized decrease in mouse pulmonary vascular reactivity. In addition to inhibiting myosin light chain phosphatase via phosphorylation of MYPT-1 and/or CPI-17, Rho-kinase activates LIM kinase that, in turn, phosphorylates and inactivates coflin, an actin-depolymerization protein (15). Thus inhibition of Rho-kinase with Y-27632 may also decrease vasoreactivity by disruption of the actin cytoskeleton (24, 32). It should be noted that whereas Y-27632 inhibited an augmented KCl vasoconstriction in hypertensive rat lungs, it did not inhibit the KCl response in normotensive lungs (17).

The role of Rho-kinase activation in the development of chronic hypoxia-induced pulmonary hypertension is unclear. Our group has reported that inhibition of Rho-kinase markedly reverses increased pulmonary vascular resistance in chronically hypoxic rats and perfused lungs (17), suggesting increased Rho-kinase-mediated vasoconstriction in chronic hyp-
Pulmonary vascular remodeling during chronic hypoxia in mice results in increased distal muscularization of the pulmonary circulation (9, 11, 34, 42). Rho/Rho-kinase signaling is involved in growth and hypertrophy of smooth muscle cells (4, 27–29), and inhibition of Rho-kinase reduced the number of neomuscularized vessels in the distal pulmonary circulation of chronically hypoxic mice in the present study. Because one mechanism of hypertrophy of PAs may be due to sustained vasoconstriction, attenuated chronic vasoconstriction may partly explain the decrease in neomuscularization of the pulmonary arterioles in hypoxic mice treated with Y-27632. Additionally, Rho/Rho-kinase signaling might promote pulmonary vascular remodeling by mediating responses to mitogens such as endothelin-1 and serotonin (5, 6). Both endothelin-1 and serotonin activate RhoA in vascular smooth muscle (25), and the activities of both are increased in hypoxia (6, 14).

In lungs isolated from mice treated with vehicle or Y-27632 for 12–14 days, acute hypoxic pulmonary vasoconstriction was not different, suggesting no residual effect of chronic Y-27632 in the PSS-perfused lung. However, the lung retained its responsiveness to exogenously administered Y-27632. This suggests that tachyphylaxis to Y-27632 did not occur in vivo and does not explain why treatment with Rho-kinase inhibitor failed to completely prevent the hypoxic pulmonary hypertension. Y-27632 appeared to decrease the Rho-kinase target phosphorylation of Y-27632 in hypoxic PAs, suggesting that at least some inhibition of Rho-kinase had occurred. It is possible that higher doses of Y-27632 might be needed to fully block Rho-kinase activity, but in additional preliminary experiments, a dose of 60 mg·kg⁻¹·day⁻¹ of Y-27632 resulted in early death of both normoxic and hypoxic mice (data not shown). Alternatively, fasudil, a Rho-kinase inhibitor with potential clinical usefulness, might be more effective in preventing hypoxic pulmonary hypertension (30, 31). A recent report indicates that fasudil prevents monocrotaline-induced pulmonary hypertension in rats (1), suggesting that either Rho-kinase activity is more important in the pathogenesis of monocrotaline-induced hypoxia-induced pulmonary hypertension, or fasudil is more effective in vivo at blocking Rho-kinase than is either treatment alone.

Compensatory increases in other GTPases, such as Rac1, might also be involved in why Y-27632 did not completely prevent hypoxic pulmonary hypertension (37, 40). Additionally, it has been previously found that inhibition of Ca²⁺ signaling with Ca²⁺ channel blockers does not completely attenuate hypoxic pulmonary hypertension in rats (33), and inhibition of both Ca²⁺ influx and Rho-kinase-mediated Ca²⁺ sensitization might be more effective in preventing hypoxic pulmonary hypertension than is either treatment alone.

Although a small effect, treatment with Y-27632 also decreased the polycythemia in chronically hypoxic mice. However, it is unlikely that this contributed to attenuation of right ventricular pressure and hypertrophy (22, 38). The role of Rho/Rho-kinase signaling in erythropoiesis is not known.

Hypoxic downregulation of eNOS expression and activity in cultured pulmonary endothelial cells is associated with Rho/Rho-kinase activation and is reversible by inhibition of Rho-kinase (36). In our study, treatment with Y-27632 augmented the eNOS protein in hypoxic lungs but decreased its expression in normoxic lungs. The upregulation in hypoxic lungs is compatible with the effects of Rho-kinase inhibition in endothelial cells, but the downregulation in normoxic lungs is not (36). We have no explanation for this apparent opposite effect of Y-27632 in hypoxic vs. normoxic mice. Whereas absence of eNOS expression is associated with increased vasoreactivity and hypoxia-induced neomuscularization of the pulmonary circulation (9, 35), increased eNOS expression inhibits hypoxic pulmonary hypertension, vascular neomuscularization, and RVH in mice (19, 20). Thus increased lung eNOS expression and activity is potentially one mechanism by which inhibition of Rho/Rho-kinase signaling reduced the chronic hypoxia-induced pulmonary hypertension in mice. However, a recent preliminary report indicates that fasudil attenuates hypoxic pulmonary hypertension in eNOS-deficient mice, suggesting that enhanced eNOS expression is not essential for a protective effect by inhibition of Rho-kinase (2).

In summary, Rho/Rho-kinase signaling appears to play a role in mediating the effects of both acute and chronic hypoxia on the pulmonary circulation. In mice, inhibition of Rho-kinase attenuated acute hypoxia-, agonist-, and KCl-induced pulmonary vasoconstriction and the development of
chronic hypoxia-induced pulmonary hypertension. Although the exact mechanisms by which Rho-kinase activation contributes to hypoxic pulmonary hypertension are unclear, we propose that it promotes pulmonary vasoconstriction and vascular remodeling via direct effects on smooth muscle cell contraction and via alterations in expression and activity of endogenous vasodilators, vasoconstrictors, and genes that regulate cellular growth.

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