Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats

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SUSTAINED ABNORMAL VASOCONSTRICTION is one of the major causes of many cardiovascular diseases, including pulmonary hypertension (PH). The degree of phosphorylation of the 20-kDa regulatory myosin light chain (MLC) generally determines the degree of vascular muscle cell (VSMC) contraction. Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) essentially regulates the activity of Ca\(^{2+}\)/calmodulin-dependent MLC kinase (MLCK), which phosphorylates MLC. At the same time, phosphorylated MLC is dephosphorylated by Ca\(^{2+}\)-independent MLC phosphatase (MLCP). Thus the balance in activities of MLCK (contraction) and MLCP (relaxation) regulates VSMC tone (30, 42). Inhibition of MLCP promotes MLC phosphorylation and contraction at a constant or decreasing cytosolic [Ca\(^{2+}\)]. This is referred to as Ca\(^{2+}\) sensitization (9, 43). Recent evidence indicates that although Ca\(^{2+}\)/calmodulin-dependent MLCK-mediated MLC phosphorylation is the key factor for triggering VSMC contraction, Ca\(^{2+}\) sensitization is important for the sustained phase of contraction (9, 43).

The small GTPase RhoA, a member of the Rho family of small GTP-binding proteins, and its downstream effector Rho kinase (Rho/Rho kinase signaling) play a major role in the regulation of MLCP activity and, thus, Ca\(^{2+}\) sensitization (9, 30, 43). RhoA is activated by various vasoconstrictors, including thromboxane, endothelin-1 (ET-1), and serotonin, the receptors of which are coupled to G proteins. Thus Rho/Rho kinase-mediated Ca\(^{2+}\) sensitization is thought to be a major component in the sustained vasoconstriction induced by G protein-coupled receptor agonists. Selective Rho kinase inhibitors, such as Y-27632 (45) and fasudil (HA-1077) (2, 41, 45), effectively reverse the sustained vasoconstriction induced by many agonists (3, 25, 34).

The pathogenesis of hypoxic PH comprises sustained vasoconstriction and structural remodeling of pulmonary arteries (PA). In systemic vascular diseases, such as hypertension, there is considerable evidence for significant involvement of Rho/Rho kinase signaling (15, 18, 22, 45). Furthermore, recent studies indicate that activation of Rho kinase plays an important role in pulmonary vasoconstriction induced by G protein-coupled receptor agonists (6, 12) and acute hypoxia (32, 48). However, little is known about the contribution of this signaling pathway to the sustained pulmonary vasoconstriction of chronically hypoxic PH. Thus the purpose of this study was to test whether Rho/Rho kinase-mediated Ca\(^{2+}\) sensitization contributed to the increased pulmonary vascular tone of hypoxic PH. We tested the acute pulmonary hemodynamic effects of Y-27632 in conscious normoxic and chronically hypoxic rats. We also examined the effects of Y-27632 on basal perfusion pressure and N-nitro-L-arginine (L-NNA)- and KCl-induced vasoconstriction in lungs isolated from control and chronically hypoxic rats and on basal tone and vasoconstrictor responses to KCl in isolated normotensive and hypertensive PA.
METHODS

Animals

All experimental procedures were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center (Denver, CO). Experiments were performed with two groups of adult male Sprague-Dawley rats (240–400 g). The normoxic, pulmonary-normotensive group was kept at Denver’s altitude of 5,280 ft (~630 mmHg barometric pressure, ~120 mmHg inspired O2 tension). The chronically hypoxic, pulmonary-hypertensive group was exposed to hypobaric hypoxia (~410 mmHg barometric pressure, ~76 mmHg inspired O2 tension) for 3–4 wk in a chamber flushed continuously with room air to prevent accumulation of CO2, NH3, and H2O. Hypoxic exposure was 24 h/day, except when the chamber was opened for 10–15 min every 2 days to remove rats or clean cages and replenish food and water. All rats were exposed to a 12:12-h light-dark cycle and allowed free access to standard rat food and water.

Conscious Catheterized Rats

Normoxic and chronically hypoxic rats were anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg) for placement of catheters in the right jugular vein and pulmonary and right carotid arteries (27). The rats were allowed to recover for 48 h in room air at Denver’s altitude. After recovery, conscious rats were placed in a ventilated plastic box, and pulmonary and systemic arterial pressures were measured with pressure transducers. Cardiac output was determined by a standard dye-dilution method, and total pulmonary resistance (TPR) was calculated by dividing mean PA pressure (MPAP) by cardiac output.

Isolated Perfused Lungs

After pulmonary-hypertensive rats were removed from the hypobaric chamber, hypoxia was maintained by keeping the animals in a small plastic box flushed with 10% O2 and, after anesthesia with pentobarbital sodium (30 mg ip), by ventilating them with 10% O2 until lungs were isolated. Lungs were isolated from the anesthetized rats after intracardiac injection of 100 IU of heparin. The techniques of lung isolation, ventilation, and perfusion have been described in detail elsewhere (21). In the first experiment, the perfusate was 20 ml of heparinized blood collected by cardiac puncture of adult male normoxic rats anesthetized with isoflurane (Baxter). In subsequent experiments, the perfusate was a physiological salt solution (PSS) containing (in mM) 116.3 NaCl, 5.4 KCl, 0.83 MgSO4, 19.0 NaHCO3, 1.04 NaH2PO4, 1.8 CaCl2:2 H2O, and 5.5 D-glucose (Earle’s balanced salt solution; Sigma). Ficoll (4 g/100 ml; type 70 Sigma) was included as a colloid, and 3.1 μM moflofenam (Sigma) was added to inhibit synthesis of vasodilator prostaglandins. Perfusion rate was 0.04 ml/g body wt−1min−1. Blood- or PSS-perfused normotensive and hypoxic hypertensive lungs (NL and HL, respectively) were equilibrated at 37°C for 20 min during ventilation with 21% O2-5% CO2-74% N2 and 8% O2-5% CO2-87% N2, respectively. After equilibration, two hypoxic pressor responses were elicited by 10 min of ventilation with 0% O2-5% CO2-95% N2 and 3% O2-5% CO2-92% N2, separated by 10 min of normoxic ventilation, to induce hypoxic vasoconstriction. The drugs described in subsequent experimental protocols were added to the perfusate reservoir to achieve the calculated circulating concentrations.

Isolated PA Rings

Rings of the left first branch of the extralobar large PA (LPA) and fifth-branch small PA (SPA) were prepared as previously described (29). Briefly, after anesthesia with pentobarbital sodium (30 mg ip) and heparinization (100 IU), the heart and lungs were removed en bloc. The LPA and SPA (200–300 μm ID) were isolated using small scissors under observation with a dissecting microscope. Care was taken to avoid damage to the endothelium. LPA and SPA rings were placed on steel wires attached to a force transducer and suspended in baths containing 10 ml of PSS at 37°C. Resting passive force was adjusted to a previously determined optimal tension (determined by maximum response to 80 mM KCl; 400 mg for SPA and 750 mg for LPA from NL, and 750 mg for SPA and 1,500 mg for LPA from HL) (28, 29). Rings were gassed with 21% O2-5% CO2-74% N2 and allowed to equilibrate for 60 min.

Experimental Protocols

Conscious rats. All experiments, except those for acute hypoxic challenges, were done under normoxic (room air) conditions. After two 10-min hypoxic challenges (we exposed the rat to hypoxia by flushing the plastic box with 10% O2-90% N2), baseline pulmonary and systemic arterial pressures, cardiac output, and heart rate were measured. Normoxic and hypoxic rats were injected with Y-27632 (Biomol) at 1, 3, and 10 mg/kg iv at 30-min intervals. Pressures were measured 10 min after injection of each dose. Cardiac output measurements were repeated 10 min after injection of 1 and 10 mg/kg Y-27632. After the final dose, rats were again exposed to 10 min of hypoxia.

Isolated blood-perfused lungs. To test whether activation of Rho kinase contributed to increased basal vascular tone in HL, we compared effects of Y-27632 and nifedipine (Sigma), an L-type voltage-dependent Ca2+ channel (VDCC) blocker, on baseline perfusion pressure in blood-perfused NL and HL. Because our previous in vivo study (27) and a preliminary study of blood-perfused lungs indicated that nifedipine had little or no effect on basal pulmonary vascular tone of chronically hypoxic rats, we tested the effects of Y-27632 in the same lung preparation after the addition of nifedipine. After equilibration and two challenges with hypoxic ventilation, nifedipine was added to the perfusate at cumulative concentrations of 0.1, 1, and 10 μM at 10-min intervals. At 15 min after the final dose of nifedipine, lungs were exposed again to hypoxia (3% O2) for 10 min, and then Y-27632 was added at cumulative concentrations of 0.1, 1, and 10 μM at 10-min intervals.

Isolated PSS-perfused lungs. We previously found in PSS-perfused HL that acute inhibition of NO synthesis by L-NNa induced a marked sustained vasoconstriction that is dependent on extracellular Ca2+ and mediated partly by endogenetic ET-1 (20, 25). We examined the role of activation of Rho kinase and MLCK in the role of Ca2+ influx through VDCC in this L-NNa-induced vasoconstriction by comparing acute vasodilator effects of Y-27632, ML-9 (a selective MLCK inhibitor; Sigma) (33), and nifedipine. After development of the L-NNa-induced vasoconstriction (35 min after addition of 200 μM L-NNa; Aldrich), Y-27632 (0.1–10 μM), ML-9 (10–100 μM), or nifedipine (0.01–1 μM) was added cumulatively to the perfusate of separate lungs at 10-min intervals. Because of their possible involvement in vasoconstrictr signalizing, we also assessed the role of activation of protein kinase C (PKC), tyrosine kinase (TK), and phosphatidylinositol 3-kinase (PI 3-kinase) by examining effects of 0.1–3 μM GF-109203X (Calbiochem) (44), 100 μM tyrphostin A23 (Calbiochem) (11), and 10–100 μM MY-294002 (Sigma) (46) on the L-NNa-induced vasoconstriction. To confirm that the effect of Y-27632 was through inhibition of Rho kinase, we also examined effects of another Rho kinase inhibitor, HA-1077 (0.1–10 μM; Sigma) (2, 41, 45), on the L-NNa-induced vasoconstriction.

To test whether Rho kinase activity increases vasoconstrictor responsiveness to KCl in HL, we measured pressor responses to KCl in NL and HL with and without Y-27632 pretreatment. At 20 min after pretreatment with 10 μM Y-27632 or saline, 5–40 mM KCl was added to the perfusate in a concentration-response fashion. KCl at >40 mM was not used, because it caused severe edema.

We also investigated effects of the MLCK inhibitor ML-9 on sustained KCl vasoconstriction in PSS-perfused NL and HL. When
the KCl pressor response reached ~10 mmHg, ML-9 was added cumulatively to the perfusate at 10, 50, and 100 μM.

Isolated PA rings. To determine whether isolated hypertensive PA also show evidence of Rho kinase-mediated increased basal tone and Ca2+ sensitization, we compared effects of Y-27632 on resting tone and KCl contractile reactivity in LPA and SPA from NL and HL. After 60 min of equilibration, 10 μM Y-27632 or saline was added to the organ bath. After 30 min, concentration-response curves to 5–80 mM KCl were determined.

**Right Ventricular Hypertrophy**

To demonstrate the presence of PH in chronically hypoxic rats, hearts were dissected, and an index of right ventricular (RV) hypertension was calculated as the ratio of wet weight of the RV wall to wet weight of the left ventricular wall plus septum (LV + S).

**Statistical Analysis**

Values are means ± SE. Comparisons between groups were made with Student’s t-test, analysis of variance (ANOVA) with Fisher’s post hoc test for multiple comparisons, or repeated-measure ANOVA. Differences were considered significant at P < 0.05.

**RESULTS**

**RV Hypertrophy**

The presence of PH in the chronically hypoxic rats was reflected in RV/LV + S, which averaged 0.58 ± 0.01 (n = 69) vs. 0.30 ± 0.01 (n = 31) in normoxic rats (P < 0.05).

**Acute Hemodynamic Effects of Y-27632 in Normoxic and Chronically Hypoxic Rats**

Y-27632 dose dependently and markedly reduced MPAP in chronically hypoxic rats, whereas it caused only a small reduction of MPAP in normoxic rats (Fig. 1A). Similarly, Y-27632 caused a remarkable reduction of TPR in chronically hypoxic rats but no reduction in normoxic rats (Fig. 1B). Pressor response to acute hypoxia was inhibited by Y-27632 in normoxic and chronically hypoxic rats (Fig. 1C). Intravenous Y-27632 also reduced systemic arterial pressure dose dependently in normoxic and chronically hypoxic rats (data not shown).

**Effects of Y-27632 on Baseline Perfusion Pressure in Blood-Perfused Lungs**

The baseline perfusion pressure of HL was higher than that of NL: 26.7 ± 3.0 vs. 16.1 ± 1.6 mmHg (P < 0.05). In contrast to no effect of nifedipine, Y-27632 caused a marked reduction of the baseline perfusion pressure in HL (Fig. 2). The highest concentration of Y-27632 (10 μM) decreased pressure to a nearly normal level: 19.1 ± 1.2 mmHg after Y-27632 in HL vs. 17.3 ± 1.7 mmHg before Y-27632 in NL (P > 0.05). The Rho kinase inhibitor slightly reduced the baseline perfusion pressure of NL, whereas nifedipine had no significant effect.

**Mechanism of l-NNA Vasosconstriction in PSS-Perfused HL**

l-NNA increased the perfusion pressure of HL by 12.7 ± 1.3 mmHg (n = 35) over 35 min. At 10 μM, Y-27632 completely reversed l-NNA vasosconstriction, whereas nifedi-
pine and the MLCK inhibitor ML-9 caused only partial reversals (Fig. 3). A chemically different Rho kinase inhibitor, HA-1077, was as effective as Y-27632 in reversing the L-NNA vasoconstriction. Whereas the PKC inhibitor GF-109203X and the PI 3-kinase inhibitor LY-294002 partially reversed L-NNA vasoconstriction, the TK inhibitor tyrphostin A23 did not cause any reversal of the response (Fig. 3). Although the TK inhibitor genistein markedly reversed L-NNA vasoconstriction, so did its TK inactive analog, daidzein (data not shown).

**Effects of Y-27632 on Baseline Perfusion Pressure in PSS-Perfused Lungs**

The baseline perfusion pressure of PSS-perfused HL (10.7 ± 0.5 mmHg, n = 12) was higher than that of NL (6.7 ± 0.2 mmHg, n = 10). Similar to its effect on baseline perfusion pressure in blood-perfused lungs, 10 μM Y-27632 caused greater vasodilation in HL (−1.8 ± 0.3, n = 4) than in NL (−1.0 ± 0.1, n = 5, P < 0.05).

**Vasoconstrictor Responsiveness to KCl in PSS-Perfused Lungs**

Vasoconstrictor responsiveness to KCl was augmented in HL, and the augmentation was eliminated by pretreatment with Y-27632 (Fig. 4). In contrast, the Rho kinase inhibitor had no significant effect on the smaller KCl response in NL.

**Effects of ML-9 on KCl Vasoconstriction in PSS-Perfused Lungs**

Sustained pressor responses to KCl were 9.5 ± 0.7 mmHg at 33 ± 3 mM in HL (n = 4) and 9.9 ± 0.9 mmHg at 35 ± 2 mM in NL (n = 4). ML-9 caused a greater dose-dependent reversal of KCl vasoconstriction in NL than in HL (Fig. 5).

**Contractile Sensitivity to KCl in PA**

As in the perfused lung experiments, Y-27632 caused greater relaxation of LPA and SPA from HL than from NL at 30 and 40 μM KCl, respectively (data not shown).

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![Fig. 3. Acute vasodilator effects of Y-27632, HA-1077, nifedipine (Nif), ML-9, GF-109203, LY-294002, and tyrphostin A23 on N-nitro-L-arginine-induced sustained vasoconstriction in salt solution-perfused hypertensive lungs. Vasodilator effects are expressed as percent reversal of N-nitro-L-arginine vasoconstriction by each of the inhibitors. Values are means ± SE; n = 5. *P < 0.05 vs. Before.](image-url)
optimal resting tensions (Fig. 6). Also, similar to perfused HL,
hypertensive PA had increased contractile sensitivity to KCl
that was blocked by Y-27632 (Fig. 7). In contrast to the result
in perfused NL, Y-27632 also caused significant inhibition of
KCl contraction in normotensive LPA and SPA.

DISCUSSION

The major findings of this study are as follows: 1) acute
intravenous administration of Y-27632, a selective Rho kinase
inhibitor, caused striking reductions of MPAP and TPR in
chronically hypoxic pulmonary-hypertensive rats; 2) in con-
trast to little effect of nifedipine, Y-27632 caused a marked
decrease in the elevated basal vascular tone of isolated blood-
perfused HL; 3) Y-27632 and another Rho kinase inhibitor,
HA-1077, completely reversed l-NNA-induced vasoconstric-
tion in PSS-perfused HL, whereas inhibitors of MLCK, TK,
PKC, and PI 3-kinase caused only partial or no reversal of the
response; and 4) vasoconstrictor responsiveness to KCl was
enhanced in hypertensive PSS-perfused lungs and PA rings
from chronically hypoxic rats, and the enhancement was elim-
inated by Y-27632. These results indicate that Rho/Rho kinase-
mediated Ca\(^{2+}\) sensitization of vasoconstriction contributes
significantly to the increased pulmonary vascular tone and
vasoreactivity of hypoxic PH.

Hypoxic PH contributes to the morbidity and mortality of
adult and pediatric patients with various lung and heart dis-
eases (8, 47, 49). In addition to structural remodeling of PA,
sustained abnormal pulmonary vasoonstriction plays an es-
sential role in the development of hypoxic PH. Several differ-
ent vasoconstrictors, e.g., ET-1, serotonin, thromboxane, and
angiotensin II, are implicated in the complex pathogenesis of
PH (5, 13, 17, 39, 40, 51). Recent studies suggest that sustained
vasoconstriction in various vascular beds by most agonists
substantially involves Ca\(^{2+}\) sensitization of VSMC contraction
Thus it is reasonable to hypothesize that Rho/Rho kinase-
mediated Ca\(^{2+}\) sensitization is involved as a common mecha-
nism of sustained vasoconstriction mediated by multiple ago-

Fig. 4. Concentration-response curves to KCl in salt solution-
perfused NL and HL with and without (vehicle) pretreatment
with 10 μM Y-27632. Values are means ± SE; n = 5 for NL
and 8 (vehicle) and 4 (Y-27632) for HL. *P < 0.05 vs. NL;
†P < 0.05 vs. vehicle.

Fig. 5. Acute vasodilator effects of ML-9 on sustained KCl vasoconstriction in
NL and HL. Vasodilator effects are expressed as percent reversal of KCl
vasoconstriction by ML-9. Values are means ± SE; n = 4. *P < 0.05 vs. NL.

Fig. 6. Effects of 10 μM Y-27632 on resting tone of isolated large and small
pulmonary arteries from normoxic and chronically hypoxic rats. Values are
means ± SE; n = 5. *P < 0.05 vs. normotensive.
TPR in chronically hypoxic pulmonary hypertensive rats and that Y-27632, but not nifedipine, markedly reduced the increased basal tone in chronically hypoxic HL and PA rings. These findings support the hypothesis that activation of Rho/Rho kinase signaling contributes significantly to the sustained vasoconstriction in hypoxic PH. In addition, it was unexpected that the elevated MPAP in chronically hypoxic rats and baseline perfusion pressure in HL would be nearly normalized by Y-27632. This finding suggests that although pulmonary vascular remodeling, i.e., medial thickening of PA and muscularization of pulmonary arterioles, is significant in 3- to 4-wk-hypoxic rats (31), vasoconstriction mediated by Rho kinase activation is a major component of the increased vascular resistance at this stage of PH.

We previously observed that normoxia-ventilated HL from chronically hypoxic rats produce increased amounts of nitric oxide (NO) (23, 36) and that acute inhibition of NO synthesis by l-NNa unMASKS a marked and sustained vasoconstriction in HL that is not expressed in NL (24, 28). To further investigate the signaling mechanisms involved in the sustained vasoconstriction in hypoxic PH, we compared acute vasodilator effects of nifedipine with the effects of various kinase inhibitors on l-NNa-induced vasoconstriction in PSS-perfused HL. Similar to the different effects of Y-27632 and nifedipine on increased basal vascular tone in blood-perfused HL, the Rho kinase inhibitors Y-27632 and HA-1077 completely reversed l-NNa-induced vasoconstriction. In contrast, the L-type VDCC blocker and the MLCK inhibitor ML-9 caused only partial reversal of l-NNa-induced vasoconstriction. These results indicate that Rho kinase-mediated Ca\(^{2+}\) sensitization, rather than solely Ca\(^{2+}\)/calmodulin-induced activation of MLCK, is essential for the sustained vasoconstriction in response to inhibition of NO synthesis in HL. Activation of MLCK may be required for onset of the vasoconstriction, because we previously observed that Ca\(^{2+}\)-free perfusion prevented the response (28). Recent studies report that PKC (4, 6, 19), TK (12, 26), and PI 3-kinase (52) are involved in VSMC Ca\(^{2+}\) sensitization independently or in association with Rho/Rho kinase signaling. Our results that the PKC inhibitor GF-109203X and the PI 3-kinase inhibitor LY-294002 caused only partial reversal and the TK inhibitor tyrphostin A23 caused no reversal of l-NNa-induced vasoconstriction indicate minor roles for PKC (at least for conventional and novel isoforms) (10) and PI 3-kinase and no role for TK in the sustained vasoconstriction. A major role for Rho kinase-mediated Ca\(^{2+}\) sensitization in the vasoconstriction elicited by acute inhibition of NO synthesis is consistent with reports that NO/cGMP/cGMP-dependent protein kinase signaling suppresses Rho/Rho kinase activity (35, 37).

We found an augmentation of vasoconstrictor responsiveness to KCl in HL that was eliminated by Y-27632. If it is considered that KCl is a receptor-independent and voltage-gated Ca\(^{2+}\) influx-mediated vasoconstrictor and that Y-27632 had no effect on pressor response to KCl in NL, this result indicated that the enhanced vasoconstrictor response to KCl in HL was likely due to increased Rho kinase-mediated VSMC Ca\(^{2+}\) sensitivity. In other words, Rho kinase-mediated Ca\(^{2+}\) sensitization, rather than only Ca\(^{2+}\)-dependent MLCK activation, played a major role in KCl vasoconstriction of HL. This is supported by our finding that the MLCK inhibitor ML-9 caused greater reversal of KCl vasoconstriction in NL than in HL. Isolated PA ring experiments also showed that SPA and LPA rings from chronically hypoxic rats had increased contractile sensitivity to KCl that was blocked by Y-27632. These results further support the idea that there is Rho kinase-mediated increased Ca\(^{2+}\) sensitivity in the hypertensive pulmonary circulation. The normotensive perfused lungs and isolated arteries differed in that Y-27632 caused more inhibition of the KCl response in arteries than in lungs. We tested whether part of the Y-27632-sensitive contraction in KCl-stimulated isolated PA was due to release of catecholamines from nerve endings (14) but found that pretreatment with the \(\alpha\)- and \(\beta\)-adrenergic blockers phentolamine and propranolol had no effect on the KCl response in normotensive LPA rings (data not shown). It is apparent that there may be more basal Rho kinase activity in isolated LPA and conduit SPA than in situ perfused peripheral resistance PA, and additional work is required to identify an explanation.

In summary, this study showed that Rho kinase-mediated vasoconstriction contributed significantly to the sustained PH of chronically hypoxic rats reexposed to normoxia and to the increased basal vascular tone and vasoconstrictor reactivity in HL and PA isolated from chronically hypoxic rats. We propose that Rho kinase-mediated Ca\(^{2+}\) sensitization is a common major mechanism of abnormal sustained vasoconstriction mediated by multiple agonists in hypoxic PH. Further work is
required to identify the exact biochemical mechanisms by which the Rho kinase-mediated Ca\(^{2+}\) sensitization mediates the sustained vasoconstriction. Considering that Rho/Rho kinase signaling mediates not only agonist-induced sustained vasoconstriction but also vascular remodeling via cell migration and proliferation (16, 38, 50), we also speculate that Rho kinase inhibitors, such as fasudil (HA-1077) (41), might provide a new class of drugs for more effective treatment of hypoxic and other forms of PH. Recent preliminary reports from our (7) and other laboratories (1) indicate that chronic treatment with Rho kinase inhibitors inhibits development of hypoxic PH in mice and monocrotaline-induced PH in rats.

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