Involvement of RhoA/Rho kinase signaling in pulmonary hypertension of the fawn-hooded rat

Tetsutaro Nagaoka, Sarah A. Gebb, Vijaya Karoor, Noriyuki Homma, Kenneth G. Morris, Ivan F. McMurtry, and Masahiko Oka

Cardiovascular Pulmonary Research Laboratory, Department of Medicine, University of Colorado at Denver and Health Sciences Center, Denver, Colorado

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Nagaoka, Tetsutaro, Sarah A. Gebb, Vijaya Karoor, Noriyuki Homma, Kenneth G. Morris, Ivan F. McMurtry, and Masahiko Oka. Involvement of RhoA/Rho kinase signaling in pulmonary hypertension of the fawn-hooded rat. J Appl Physiol 100: 996–1002, 2006. First published December 1, 2005; doi:10.1152/japplphysiol.01028.2005.—The fawn-hooded rat (FHR) develops severe pulmonary hypertension (PH) when raised for the first 3–4 wk of life in the mild hypoxia of Denver’s altitude (5,280 ft.). The PH is associated with sustained pulmonary vasoconstriction and pulmonary artery remodeling. Furthermore, lung alveolarization and vascularization are reduced in the Denver FHR. We have recently shown that RhoA/Rho kinase signaling is involved in both vasoconstriction and vascular remodeling in animal models of hypoxic PH. In this study, we investigated the role of RhoA/Rho kinase signaling in the PH of Denver FHR. In α-toxin permeabilized pulmonary arteries from Denver FHR, the contractile sensitivity to Ca$^{2+}$ was increased compared with those from sea-level FHR. RhoA activity and Rho kinase I protein expression in pulmonary arteries of Denver FHR (10-wk-old) were higher than in those of sea-level FHR. Acute inhalation of the Rho kinase inhibitor fasudil selectively reduced the elevated pulmonary arterial pressure in Denver FHR in vivo. Chronic fasudil treatment (30 mg·kg$^{-1}$·day$^{-1}$, from birth to 10 wk old) markedly reduced the development of PH and improved lung alveolarization and vascularization in Denver FHR. These results suggest that Rho kinase-mediated sustained vasoconstriction, through increased Ca$^{2+}$ sensitivity, plays an important role in the established PH and that RhoA/Rho kinase signaling contributes significantly to the development of PH and lung dysplasia in mild hypoxia-exposed FHR.

Ca$^{2+}$ sensitivity; fasudil; vascular remodeling; lung dysplasia

THE FAWN-HOODED RAT (FHR) is a genetic strain that develops severe pulmonary hypertension (PH) when raised for the first 3–4 wk of life in the mild hypoxia of Denver’s altitude (5,280 ft., $P_{O_2}$ ~ 120 Torr) but not in normoxia (sea level, $P_{O_2}$ ~ 150 Torr) (15, 20, 25). The PH is associated with marked pulmonary artery (PA) medial thickening, vascular rarefaction, and sustained pulmonary vasoconstriction (15, 25, 32). Previous studies suggest several factors are involved in the development of severe PH in FHR, including increased endothelin-1 (ET-1) production (20, 30) and serotonin blood levels due to platelet storage-pool deficiency (31), reduction in lung endothelial nitric oxide synthase expression (16, 32), and decreased pulmonary vascular density associated with reduced alveolarization (15, 16, 32), all of which have been implicated in the pathogenesis of human PH.

Animal and human studies of systemic vascular diseases indicate that the small GTPase RhoA and one of its target proteins, Rho kinase (ROCK), contribute importantly not only to sustained vasoconstriction by inhibiting myosin light chain phosphatase and thereby increasing the Ca$^{2+}$ sensitivity of vascular smooth muscle cell contraction, but also to vascular remodeling by regulating various cell functions, including cell migration and proliferation and gene expression (9, 28). Accumulating evidence suggests that this signaling pathway is also involved in experimental pulmonary vascular disorders. For instance, our group and others have recently shown that RhoA/ROCK signaling is involved in sustained vasoconstriction and vascular remodeling in rodent models of hypoxic PH (4, 6, 10, 21, 23). Jernigan and colleagues (12) have shown that RhoA activity and ROCKII expression are increased in chronically hypoxic rat PA, and that inhibition of this pathway, rather than a decrease in cytosolic Ca$^{2+}$, mediates nitric oxide (NO)-induced dilation of these hypertensive vessels. Similarly, Weigand et al. (33) have found that ROCK activity contributes substantially to ET-1-induced Ca$^{2+}$ sensitization of hypoxia-induced hypertensive intrapulmonary arteries. Abe and colleagues (1) have reported that RhoA/ROCK signaling also plays a major role in the pathogenesis of monocrotaline-induced PH in rats. Although we have previously observed that acute administration of a ROCK inhibitor elicits pulmonary vasodilation in Denver FHR (21), the role of the RhoA/ROCK pathway in development of the severe PH in this model is uncertain. Therefore, the purpose of this study was to test whether RhoA/ROCK signaling contributes to the development of PH in Denver FHR. We examined Ca$^{2+}$ sensitivity, RhoA activity, and ROCK protein expression in FHR normoxic and hypertensive PA and the effects of acute and chronic ROCK inhibition on pulmonary hypertension in Denver FHR. Parts of this study have been previously presented in conference abstract (22) and proceedings (17) forms.

METHODS

All chemicals were purchased from Sigma unless specifically stated.

Animals. All experimental procedures were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center (Denver, CO). Animals were obtained from a breeding colony of FHR that was previously established at our institution (25). Experiments were performed with two groups of FHR (140–250 g); the mildly hypoxic, pulmonary hypertensive group was kept at Denver’s altitude (Denver FHR), and the normoxic, pulmonary nor-

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Received fasudil (30 mg·kg⁻¹·day⁻¹) was given to Denver FHR from immediately after birth to 10-wk-old (from birth to 4-wk-old through mother’s milk and thereafter by drinking water). After chronic treatment, hemodynamic measurements were made under anesthesia. We chose this dose on the basis of previous pharmacokinetic studies (11, 26) and a recent study examining its chronic effects on monocrotaline-induced PH (1). Although the exact concentration of fasudil the pups received from their mother [which received fasudil (30 mg·kg⁻¹·day⁻¹) by drinking water] was not determined, it has been preliminarily found that fasudil passes to mother’s milk at a concentration similar to that of blood when administered intravenously (personal communication with Dr. S.-I. Sato, Asahi Kasei). Measurements of PA medial wall thickness, arterial density, and alveolar size. Histological changes of PA were quantified by morphometry as previously described (7). After the hemodynamic measurement, the PA was cannulated and perfused with heparinized physiological salt solution (37°C, 20-cmH₂O pressure) to remove residual blood and then injected with a barium-gelatin mixture (60°C) centrifuged again (40,000 g) for 15 min) to generate the membrane fraction. Equal amounts of protein (15 μg) were loaded in wells. RhoA protein in membrane and cytosolic fractions was determined by standard Western blot analysis using a specific mouse monoclonal anti-RhoA antibody (1:250 dilution, Santa Cruz Biotechnology) and a peroxidase-labeled anti-mouse IgG antibody (Vector). Relative density of membrane to cytosolic RhoA was determined using National Institutes of Health Image software.

ROCK I and II expression. To examine total expression of ROCK I and II, isolated arteries were equilibrated and then homogenized in lysis buffer (10 mM HEPES, 2 mM EDTA, 1 mM MgCl₂) containing protease inhibitors (Pierce). Homogenate was centrifuged at 40,000 g for 30 min, and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in lysis buffer and centrifuged again (40,000 g for 15 min) to generate the membrane fraction. Equal amounts of protein (15 μg) were loaded in wells. RhoA protein in membrane and cytosolic fractions was determined by standard Western blot analysis using a specific mouse monoclonal anti-RhoA antibody (1:250 dilution, Santa Cruz Biotechnology) and a peroxidase-labeled anti-mouse IgG antibody (Vector). Relative density of membrane to cytosolic RhoA was determined using National Institutes of Health Image software.
RESULTS

**RV hypertrophy.** The presence of PH in the Denver FHR was reflected in RV/LV + S, which averaged 0.66 ± 0.01 (n = 31) vs. 0.29 ± 0.01 (n = 17) in sea-level FHR (P < 0.001).

**Ca²⁺ sensitivity of PA in FHR.** In α-toxin permeabilized PA, the Ca²⁺ concentration-contraction curve of Denver FHR was slightly but significantly left-shifted (pCa range from 6.0 to 6.5) compared with that of sea-level FHR (Fig. 1).

**RhoA translocation in PA.** We measured RhoA protein expression in cytosol and membrane fractions separately to assess RhoA activation. Expression of membrane RhoA was significantly higher in Denver FHR hypertensive PA than in sea-level FHR normotensive PA (Fig. 2, A and B). Membrane-to-cytosolic ratio of RhoA expression by densitometry was also higher in Denver FHR than in sea-level FHR (Fig. 2C).

**ROCKI and II expression in PA.** ROCKI protein expression in PA was greater in Denver FHR than in sea-level FHR (Fig. 3A). ROCKII expression was not significantly increased (P = 0.15) in PA of Denver FHR compared with that of sea-level FHR (Fig. 3B).

**Acute hemodynamic effects of inhaled fasudil in Denver FHR.** In Denver FHR with PH, acute inhaled fasudil caused a marked reduction in MPAP (from 70 ± 5 to 58 ± 4 and 54 ± 9 mmHg by 30 and 100 mM, respectively) with no effects on MSAP or cardiac output (Fig. 4). Calculated total pulmonary resistance was reduced significantly by inhaled fasudil (from 4,232 ± 552 to 2,923 ± 389 mmHg·l⁻¹·min⁻¹ by 100 mM).

**Chronic effects of fasudil in Denver FHR.** Denver FHR had much higher MPAP than sea-level FHR (49 ± 6 vs. 26 ± 1 mmHg, P < 0.05) (Fig. 5A). Chronic in vivo fasudil treatment
markedly reduced the development of PH in Denver FHR (MPAP; 49 ± 6 vs. 33 ± 2 mmHg, P < 0.05) (Fig. 5A) with no effect on MSAP (Fig. 5B). Similarly, RV/LV+S in Denver FHR was much higher than that in sea-level FHR, and this RV hypertrophy was markedly attenuated by chronic fasudil treatment (RV/LV+S ratio; 0.65 ± 0.03 vs. 0.43 ± 0.02%, P < 0.05) (Fig. 5C).

Consistent with previous studies (15, 19, 25), Denver FHR showed increased PA medial wall thickness (Fig. 6, A and C) and alveolar size (reflected by increased MLI) (Fig. 6, B and D) and decreased small barium-filled PA vessel count (Fig. 6, B and E) compared with sea-level FHR. These abnormalities were also significantly improved by the chronic fasudil treatment (Fig. 6, A–E).

**DISCUSSION**

The major findings of this study were that, in pulmonary hypertensive Denver FHR,

1) Ca$^{2+}$ sensitivity, membrane-to-cytosol ratio of RhoA (an indirect measure of RhoA activation), and ROCKI expression of PA were greater than those in sea-level FHR; and

2) acute inhalation of the Rho kinase inhibitor fasudil caused a dose-dependent selective pulmonary vasodilation; and

3) chronic fasudil treatment from the time of birth until 10 wk of age reduced the development of PH and improved alveolarization and vascularization.

Recent studies have indicated that RhoA/ROCK signaling contributes to the pathogenesis of chronic hypoxia- (4, 6, 10, 12, 21, 23, 33) and monocrotaline-induced (1) PH in rodents. In contrast to other strains of rat, the FHR develops severe PH in the mild hypoxia of Denver’s altitude but not in the normoxia of sea level (15, 20, 25). Although the exact mechanism of PH in Denver FHR is not fully understood, several factors that are closely related to the pathogenesis of human PH are implicated in the development and maintenance of severe PH in this strain. For example, mild hypoxia-exposed FHR lungs have elevated expression and production of ET-1 compared with normal Sprague-Dawley rat or sea-level FHR lungs (20, 30). Serotonin may play a role, since the FHR has increased plasma serotonin levels due to an inherited platelet storage-pool deficiency (31). Although a report by Nagaoka et al. questions whether platelet storage-pool deficiency alone is sufficient to cause the PH of this strain (20), a recent study has shown that expression of lung serotonin 1B receptor and serotonin-mediated PA contraction are greater in FHR than in
Sprague-Dawley rats (18), suggesting an increased pulmonary vascular responsiveness to serotonin in FHR.

RhoA can be activated by various vasoconstrictors, including ET-1 and serotonin, whose receptors are coupled to G proteins, and plays a central role in the stimulation of ROCK and thereby inhibition of myosin light chain phosphatase activity and increased Ca\(^{2+}\) sensitization (24, 29). We found in this study that membrane-to-cytosol ratio of RhoA and Ca\(^{2+}\)

![Fig. 5. MPAP (A), MSAP (B), and right ventricle (RV)-to-left ventricle plus septum (LV + S) weight ratio (C) in SL, D, and fasudil-treated D (D + FAS) FHR. Values are means ± SE; n = 5–9. *P < 0.05.](image)

**Fig. 6.** Hematoxylin eosin stained representative barium-filled small pulmonary arteries (60–70 μm in external diameter) (A) and lung sections (B) from SL (left), D (center), and D + FAS (right) FHR. C: % medial wall thickness of ~50- to 100-μm-diameter pulmonary arteries. D: mean linear intercept. E: pulmonary arterial density. Values are means ± SE; n = 4 for SL; n = 4 for D; n = 5 for D + FAS. *P < 0.05.
sensitivity of PA in Denver FHR were higher than those in sea-level FHR. In addition, acute in vivo ROCK inhibition effectively reduced the sustained pulmonary vasconstriction in Denver FHR. These results suggested that RhoA was activated in PA, possibly by increased ET-1 and serotonin levels and/or responsiveness and deficient NO production (12, 32), which lead to ROCK-mediated increased Ca\(^{2+}\) sensitivity of contraction and sustained pulmonary vasconstriction in the mildly hypoxic pulmonary hypertensive FHR. The acute reduction in MAPAP by a ROCK inhibitor indicated that active vasconstriction, in addition to the pulmonary vascular remodeling and lung dysplasia (reduced vascularity), played a significant role in the elevated pulmonary artery pressure in 10-wk-old Denver FHR.

We also observed that ROCKI protein expression was greater in PA of Denver FHR than in those of sea-level FHR. This is similar to the findings in chronically hypoxia-exposed Sprague-Dawley rat PA in which both RhoA activity and ROCKII expression were increased compared with normoxic PA (6, 12). Although little is known about the regulation of ROCK expression, we speculate that in hypertensive FHR lungs increased stimulation of vascular smooth muscle by ET-1 and serotonin, and perhaps other unidentified inflammatory mediators, might activate nuclear factor-\(\kappa\)B (34), which leads to upregulation of ROCK expression (8).

We also examined whether chronic ROCK inhibition would attenuate the development of severe PH in Denver FHR. We found that 10-wk treatment of Denver FHR with fasudil (from birth to 10 wk old) markedly reduced the increases in MPAP, RV/LV + S, and PA medial wall thickness. These results indicated that, similar to the findings in the hypoxia- (4, 6, 10) and monocrotaline-induced (1) PH models, ROCK activity contributed importantly to the development of severe PH in the FHR model. The chronic effect of ROCK inhibitors on PH may be attributable to their ability to reduce sustained abnormal pulmonary vasconstriction, as shown in this and previous studies (10, 21, 23), and to attenuate PA remodeling by inhibiting proliferation and inducing apoptosis of pulmonary vascular smooth muscle cells (1).

In addition to inhibition of PH, chronic fasudil treatment improved the lung dysplasia in the Denver FHR (i.e., fasudil decreased alveolar size and increased pulmonary vascular density), suggesting that ROCK activation might also play a significant role in inhibiting lung development after birth. Further experiments are necessary to elucidate whether ROCK activation inhibits postnatal lung vascularization and alveolarization directly or through modulation of some other mediators, such as endothelial nitric oxide synthase (3, 14).

In summary, this study demonstrated that RhoA/ROCK signaling was involved in the elevated pulmonary vascular tone, development of PH, and lung dysplasia in mild hypoxia-exposed FHR. Results of this and previous studies (1, 4, 6, 10, 21, 23) indicate that RhoA/ROCK signaling plays key roles in the development of at least three different animal models of PH (i.e., Denver FHR, chronic hypoxia-induced, and monocrotaline-induced PH models). We thus propose that activation of this intracellular signaling pathway might be a common factor in the development of various forms of PH independent of their etiology and that RhoA/Rho kinase signaling might be a promising target for the treatment of human PH.

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REFERENCES


