Long-Term Treatment With a Rho-Kinase Inhibitor Improves Monocrotaline-Induced Fatal Pulmonary Hypertension in Rats

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Abstract—Primary pulmonary hypertension is a fatal disease characterized by endothelial dysfunction, hypercontraction and proliferation of vascular smooth muscle cells (VSMCs), and migration of inflammatory cells, for which no satisfactory treatment has yet been developed. We have recently demonstrated that intracellular signaling pathway mediated by Rho-kinase, an effector of the small GTPase Rho, is involved in the pathogenesis of arteriosclerosis. In the present study, we examined whether the Rho-kinase–mediated pathway is also involved in the pathogenesis of fatal pulmonary hypertension in rats. Animals received a subcutaneous injection of monocrotaline, which resulted in the development of severe pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular lesions in 3 weeks associated with subsequent high mortality rate. The long-term blockade of Rho-kinase with fasudil, which is metabolized to a specific Rho-kinase inhibitor hydroxyfasudil after oral administration, markedly improved survival when started concomitantly with monocrotaline and even when started after development of pulmonary hypertension. The fasudil treatment improved pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular lesions with suppression of VSMC proliferation and macrophage infiltration, enhanced VSMC apoptosis, and amelioration of endothelial dysfunction and VSMC hypercontraction. These results indicate that Rho-kinase–mediated pathway is substantially involved in the pathogenesis of pulmonary hypertension, suggesting that the molecule could be a novel therapeutic target for the fatal disorder. (Circ Res. 2004;94:385-393.)

Key Words: pulmonary hypertension ■ Rho-kinase ■ vascular smooth muscle cells ■ endothelial nitric oxide synthase ■ macrophages

Primary pulmonary hypertension (PPH) is a life-threatening disease characterized by a marked and sustained elevation of pulmonary artery pressure. The disease has no obvious causes and ultimately results in right ventricular (RV) failure and death. The pathological changes of hypertensive pulmonary arteries include endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells (VSMCs), and migration of macrophages.1–3 PPH continues to be a serious clinical problem with high morbidity and mortality.4

In 1990s, Rho-kinase/ROK/ROCK was identified as an effector of the small GTPase Rho,5–7 which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expression.8–10 In a series of experimental and clinical studies, we have demonstrated that Rho-kinase–mediated pathway is substantially involved in the pathogenesis of arteriosclerosis.11–17 These Rho-kinase–mediated alterations in blood vessels also may be involved in the pathogenesis of pulmonary hypertension (PH). In this study, we examined whether Rho-kinase–mediated pathway is involved in the pathogenesis of rat model of fatal PH in vivo.

Materials and Methods
The present study was approved by the Institutional Animal Care and Use Committee of the Kyushu University Graduate School of Medical Sciences.

Animal Model of Fatal PH
A total of 323 adult male Sprague-Dawley rats (Charles River, Yokohama, Japan; 250 to 300 g body weight) were used, including 156 for survival study, 83 for hemodynamic and histology study, 36
for immunohistochemistry. 25 for organ chamber experiments, 15 for Western blot analysis, and 8 for drug concentration measurement. They received a single subcutaneous injection of saline or monocrotaline (MCT, 60 mg/kg, Wako), which induces severe PH in 3 weeks with a subsequent high mortality rate in rats. 18 For the long-term inhibition of Rho-kinase, we confirmed that repetitive in vivo gene transfer of dominant-negative Rho-kinase to pulmonary arteries is technically difficult and that genetic disruption of Rho-kinase is embryonic lethal. Therefore, we used a long-term pharmacological inhibition with fasudil (Asahi Kasei), which we found is metabolized in the liver to a specific Rho-kinase inhibitor hydroxyfasudil after oral administration. 13 Hydroxyfasudil is a specific Rho-kinase inhibitor as its specificity for Rho-kinase is 100 times higher than for protein kinase C and 1000 times higher than for myosin light-chain kinase. 13 Furthermore, among the 16 kinases recently tested, only hydroxyfasudil (10−3 mol/L) showed more than 50% inhibition for Rho-kinase (98%). 19 Thus, we consider that hydroxyfasudil is a reasonably selective inhibitor for Rho-kinase.

In the first prevention protocol, animals were injected with MCT or with concomitant oral treatment with a low-dose (30 mg/kg per day) or a high-dose (100 mg/kg per day) of fasudil. 12 In the second treatment protocol, animals received the two different doses of fasudil, starting at day 21 after MCT injection when severe PH had already been established. In this treatment protocol, hemodynamic parameters were also measured at day 35 in additional animals of the control and the fasudil groups in order to examine those variables before they died.

**Hemodynamic Measurements**

After the animals were anesthetized with sodium pentobarbital (30 mg/kg, IP), polyethylene catheters were inserted into the RV through the jugular vein and the carotid artery for hemodynamic measurements. RV systolic pressure and systemic blood pressure were measured with a polygraph system (AP-601G, Nihon Kohden).

**RV Hypertrophy**

The RV was dissected from the left ventricle (LV) and the septum (S) and weighed to determine the extent of RV hypertrophy (RVH) as follows: RV/(LV+S).

**Survival Analysis**

We examined the effects of fasudil on the survival of MCT-injected rats. The day of MCT injection was defined as day 0. This survival analysis covered the entire experimental period to day 63.

**Morphometric Analysis of Pulmonary Arteries**

After the hemodynamic measurements, lung tissue was prepared for morphometric analysis by using the barium injection method. 18 All barium-filled arteries of 15 to 50 µm in diameter were evaluated for muscularization of pulmonary microvessels. 18 Arteries of more than 50 µm in diameter were evaluated for measurement of medial wall thickness at a magnification of 400×. For each artery, the median wall thickness was expressed as follows: percent wall thickness=[(medial thickness×2)/external diameter]×100. 18

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed at day 21 in the saline-treated control group and the high-dose fasudil group in the prevention protocol. Proliferating cells were evaluated by proliferating cell nuclear antigen (PCNA) staining (Dako) and apoptotic cells by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method (apoptosis detection kit, Wako). Inflammatory cells were evaluated by ED-1 (analogue of CD68) staining (Santa Cruz Biotechnology). The number of PCNA- and TUNEL-positive cells in 10 fields for each section was quantitatively evaluated as a percent of that of total cells at a magnification of 400× in a blind manner. 18,20 The number of ED-1-positive cells was counted in 30 fields. 4

**Organ Chamber Experiments**

Organ chamber experiments were performed at day 21 in the control and the high-dose fasudil groups in the prevention protocol, when MCT-induced PH was established. The extrapulmonary arteries were carefully isolated and cleaned of any connective tissue in physiological salt solution (PSS). 21 The rings from each pulmonary artery (~1 mm in length) were mounted vertically between two hooks in organ chamber myographs (Medical Supply), which were filled with PSS and kept at 37°C. Isometric tension was measured with force transducers (Nihon Kohden). Each preparation was stretched in a stepwise manner to an optimal length where the force induced by 118 mmol/L KCl became maximal and constant. After equilibration for 30 minutes, endothelium-dependent relaxation to acetylcholine (ACh, 10−6 to 10−3 mol/L) was examined during a contraction to prostaglandin F2α (3×10−6 to 10−3 mol/L) in the presence of indomethacin (10−3 mol/L) with or without L-nitro-arginine (L-NNA, 10−4 mol/L). 22 Endothelium-independent contractions to serotonin (10−5 to 10−3 mol/L) and sodium nitroprusside (SNP, 10−10 to 10−3 mol/L) were also examined in rings without endothelium. The inhibitory effect of acute administration of hydroxyfasudil (10−3 mol/L) on the serotonin-induced VSMC hypercontraction was also examined.

**Western Blot Analysis**

Western blot analysis was performed at day 21 in the control and the high-dose fasudil groups in the prevention protocol. The bilateral pulmonary arteries were isolated and were stabilized in bubbling Krebs solution for 1 hour. These samples were immediately frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) cooled with dry ice, for Western blot analysis of phosphorylations of the ERM (ezrin, radixin, and moesin) family, a substrate of Rho-kinase. 13 ERM is phosphorylated by Rho-kinase at T567 (ezrin), T5648 (radixin), and T558 (moesin). 22 The frozen specimens were washed three times with acetone containing dithiothreitol (10 mmol/L) to remove the TCA and dried. The dried samples were cut into small pieces, exposed to 200 µL of SDS-PAGE sample buffer for protein extraction. The extracted samples (20 µg of protein) were subjected to SDS-PAGE/immunoblot analysis by using the specific ERM antibody. 13 The regions containing ERM family proteins were visualized by ECL Western blotting luminal reagent (Santa Cruz Biotechnology). The extent of the ERM phosphorylation was normalized by that of total ERM. The protein expression of endothelial nitric oxide synthase (eNOS) and β-actin as an internal control in lungs was also analyzed by Western blot analysis. 22–25

**Plasma Concentration of Hydroxyfasudil**

We measured plasma concentration of hydroxyfasudil every 6 hours a day in rats that received fasudil in drinking water. We obtained blood samples from carotid arteries in each rat. Plasma concentrations were measured by an HPLC method. 16

**Statistical Analysis**

All results are expressed as the mean±SEM. Survival curves were analyzed by the Kaplan-Meier method and analyzed by a log-rank test. Differences in all other parameters were evaluated by ANOVA followed by Fisher’s post hoc test. A value of P<0.05 was considered to be statistically significant.

**Results**

**Beneficial Effects of Fasudil on Survival**

In the control MCT group, survival rate at day 63 was only 27% (n=26) (Figures 1A and 1B). In the prevention protocol, the fasudil treatment markedly and dose-dependently improved the survival at day 63: 77% in the low-dose (n=30) and 94% in the high-dose (n=35) groups (Figure 1A). In the treatment protocol, fasudil again significantly and dose-
dependently improved the survival: 53% in the low-dose (n=30) and 86% in the high-dose (n=35) groups (Figure 1B).

**Improvement of PH and RVH by Fasudil**
The MCT group developed severe PH at day 21 with increased RV systolic pressure (a marker of systolic pulmonary pressure) compared with the sham-operated saline-treated group (Figures 1C and 1D). In the prevention protocol, fasudil markedly and dose-dependently suppressed the development of PH at day 21 in both the low-dose and the high-dose groups, the effects of which were maintained at day 63 (Figure 1C). In this protocol, we also measured RV systolic pressure at day 35 in the middle of the experiment in some animals separately before they died. The results showed that fasudil had started reducing RV systolic pressure in a dose-dependent manner (Figure 1E). Mean systemic arterial pressure (mm Hg) was significantly decreased in the MCT group (75±2, n=6) compared with the saline-treated group (115±2, n=6, P<0.0001). In the prevention protocol, fasudil prevented the reduction in systemic arterial pressure in the low-dose and the high-dose groups at day 63 (121±4 and 121±3, respectively, n=6 each). In the MCT group, a significant RVH was developed, and fasudil markedly suppressed the MCT-induced RVH in the prevention protocol (Figure 2A) and caused a marked regression of RVH in the treatment protocol (Figure 2B).

We also measured the extent of RVH in animals that died in the middle of the experiments. The measurement was performed within 12 hours after death in all animals. The extent of RVH in dead animals of the MCT group was 0.74±0.06 (n=7) with a pleural effusion and ascites. In the dead animals in the prevention protocol with fasudil, a similar extent of RVH was noted in both the low-dose (0.67±0.08, n=4) and the high-dose (0.72, n=1) groups with a pleural effusion and ascites. Similarly, the dead animals in the treatment protocol also showed marked RVH in both the low-dose (0.68±0.05, n=9) and the high-dose (0.71±0.04, n=3) groups with a pleural effusion and ascites.

**Inhibitory Effects of Fasudil on Medial Wall Thickening**
Medial thickness was markedly increased in the MCT group compared with the saline-treated group or the fasudil-treated groups (Figures 3A through 3D). We semiquantitatively evaluated the extent of muscularization of pulmonary microvessels (15 to 50 μm in diameter) because they are usually nonmuscular under normal conditions. In the prevention protocol, at both day
21 and day 63, fasudil prevented the muscularization at both a low-dose and a high-dose at day 21 and day 63 (Figure 3E). In the treatment protocol, fasudil markedly improved the muscularization at both doses at day 63 (Figure 3F).

We next quantified medial wall thickness of pulmonary arteries in the ranges of 50 to 100 μm and 101 to 200 μm in diameter separately. In the prevention protocol, fasudil prevented the MCT-induced medial thickening of both-sized pulmonary arteries at both day 21 and day 63 (Figures 3G and 3I). In the treatment protocol, fasudil caused a marked improvement of the MCT-induced medial thickening of both-sized pulmonary arteries at day 63 (Figures 3H and 3J).

Figure 3. Fasudil suppresses medial thickening in rats with MCT-induced PH. Compared with the saline-treated normal group (A), medial wall thickening of the pulmonary artery was noted in the MCT group (B), whereas fasudil prevented (C) or markedly improved the medial thickening (D). Bar = 50 μm. In the prevention protocol (middle), the fasudil treatment markedly suppressed the MCT-induced muscularization of pulmonary microvessels (15 to 50 μm in diameter) (E) as well as percent medial wall thickening of pulmonary arteries at both 50 to 100 μm (G) and 101 to 200 μm levels (I). In the treatment protocol (bottom), the fasudil treatment induced a marked improvement of the vascular muscularization of pulmonary microvessels (15 to 50 μm diameter) (F) and percent medial wall thickening of pulmonary arteries at both 50 to 100 μm (H) and 101 to 200 μm levels (J). N indicates nonmuscular; P, partially muscular; M, muscular. Results are expressed as mean±SEM (n=3 each). **P<0.01, ***P<0.0001.
Mechanisms for the Beneficial Effects of Fasudil on PH
PCNA expression in VSMCs was increased in the MCT group at day 21, which was prevented by fasudil (Figures 4A through 4C and 4J). Fasudil also significantly enhanced VSMC apoptosis (Figures 4D through 4F and 4K). The percentage of TUNEL-positive cells was significantly increased in the fasudil group compared with the saline-treated normal group and the MCT group (Figure 4K). Macrophage recruitment was increased in the MCT group, which was also markedly suppressed by fasudil (Figures 4G through 4I and 4L).

Endothelium-dependent relaxation of isolated pulmonary arteries to ACh was markedly impaired in the MCT group, which was prevented by fasudil (Figure 5A). This beneficial effect of fasudil was abolished by L-NNA (Figure 5B). Serotonin caused hypercontractions of pulmonary VSMC from the MCT group, which was prevented by the fasudil treatment and also by the acute administration of hydroxyfasudil (Figure 5C). Endothelium-independent relaxation to SNP also was slightly but significantly impaired in the MCT group, which was again prevented by the fasudil treatment (Figure 5D).

The extent of ERM phosphorylation was significantly increased in the MCT group and was markedly inhibited by the fasudil treatment (Figure 6A). The expression of eNOS in the lungs was significantly increased by the fasudil treatment (Figure 6B).

Plasma Concentration of Hydroxyfasudil
The mean value of the daily plasma concentration of hydroxyfasudil (AUC0–24, ng/hr per mL) in rats that received fasudil in drinking water was 627 and 1450 for the low-dose (30 mg/kg per day) and the high-dose (100 mg/kg per day) groups, respectively (n=4 each).

Discussion
The novel findings of the present study were that the Rho-kinase–mediated pathway is substantially involved in the MCT-induced PH and that the long-term inhibition of Rho-kinase with fasudil prevents or even causes a marked improvement of the MCT-induced PH through multiple mechanisms, including (1) inhibition of VSMC proliferation with enhanced apoptosis, (2) reduced macrophage infiltration, and (3) improvement of endothelium-dependent relaxation and VSMC hypercontraction (Figure 7).

Rho-Kinase in the MCT-Induced PH Model
MCT is known to cause endothelial injury of pulmonary arteries with subsequent proliferation of pulmonary VSMC and infiltration of inflammatory cells.3,18 Accumulating evidence indicates that Rho-kinase–mediated pathway is involved in the vascular effects of various vasoactive substances, including angiotensin II,26 endothelin-1,27 and serotonin,15 all of which may be involved in the pathogenesis of PH.28–30 We also have recently demonstrated that inflammatory stimuli (eg, angiotensin II and IL-β) upregulate Rho-kinase in human coronary VSMCs.31 Those inflammatory processes may activate Rho-kinase in this MCT-induced PH model. Thus, Rho-kinase may play an important role in the pathogenesis of PH both directly, by activating its substrates, and indirectly, by mediating the signal transduction of various inflammatory mediators.

Recently, it has been reported that simvastatin, which also could inhibit Rho/Rho-kinase signaling, inhibits both hypoxia-induced and MCT-induced PH.32–35 Nagaoka et al36 also have reported that chronic hypoxia-induced PH is almost completely reversed by acute inhibition of Rho-kinase in rats. These reports also suggest that Rho-kinase signaling plays an important role in the pathogenesis of both hypoxia-induced and MCT-induced PH.

Hydroxyfasudil as a Specific Rho-Kinase Inhibitor
Hydroxyfasudil, an oral metabolite of fasudil, is a specific Rho-kinase inhibitor.33 In the present study, the mean value of the AUC0–24 of hydroxyfasudil in the fasudil group was within its clinical therapeutic range in humans (unpublished data, 2003). In our series of experiments, the extent of the increase in Rho-kinase activity as evaluated by that of ERM phosphorylation was 1.5- to 2.0-fold.19,31,37,38 This Rho-kinase activity just represents the whole Rho-kinase activity in blood vessels, and it is highly possible that Rho-kinase activity may be much greater in activated cells (eg, inflammatory cells) but much less in others (eg, fibroblasts). We consider that Rho-kinase has multiple stimulatory effects on vascular lesion formation with this extent of activation (Figure 7), thus accelerating the process of PH.

In the present study, neither acute nor chronic administration of fasudil lowered systemic arterial pressure, indicating that the Rho-kinase inhibitor caused selective vasodilatation of pulmonary arteries.

Rho-Kinase and VSMC Proliferation and Apoptosis in PH
In our animal models of coronary arteriosclerosis, long-term treatment with fasudil suppressed coronary VSMC proliferation.11–17 Rho-kinase is involved in VSMC cytokinesis as well as gene expression of many atherogenic molecules that stimulate VSMC proliferation.8,10,11,37–39 Rho-kinase may affect various cyclin-dependent kinases.26,31 In this study, fasudil also significantly enhanced apoptosis, a finding consistent with our recent study.37 In the present study, established PH was improved to the normal level at day 63 with the fasudil treatment. Indeed, the long-term treatment with fasudil induced a marked improvement of medial wall thickening of pulmonary arteries partly due to its enhancing effect on VSMC apoptosis.

Rho-Kinase and Inflammatory Cell Migration in PH
Rho-kinase also is involved in inflammatory cell migration.11,40 We previously demonstrated that long-term treatment with fasudil suppresses chemokine-induced migration of macrophages in porcine coronary arteries in vivo.17 Macrophage recruitment has been implicated in the pathogenesis of PH because various vasoactive factors may be released from infiltrating inflammatory cells, especially macrophages, in pulmonary arteries.3 Macrophages may be the most impacted by fasudil, followed by VSMC and endothelial cells. The present
Figure 4. Mechanisms for the beneficial effects of fasudil on MCT-induced pulmonary remodeling. Histology of pulmonary arteries in the saline-treated normal group (A, D, and G), MCT group (B, E, and H), and high-dose fasudil group (C, F, and I) at day 21 in the prevention protocol. MCT-induced increase in PCNA-positive cells (arrows) was prevented in the fasudil group (A through C and J). TUNEL-positive cells (arrows) were increased in the fasudil group (D through F and K). MCT-induced increase in ED-1-positive macrophages (arrows) was prevented in the fasudil group (G through I and L). Results are expressed as mean±SEM (n=4 each). *P<0.05, ***P<0.0001. n.s. indicates not statistically significant.
study suggests that Rho-kinase–mediated macrophage recruitment also is involved in the pathogenesis of PH.

**Rho-Kinase and Impaired Endothelium-Dependent Relaxation in PH**

MCT causes endothelial injury and subsequent endothelial dysfunction of pulmonary arteries. Impaired endothelium-dependent relaxation is caused by endothelial dysfunction and/or reduced VSMC vasodilator function. The present results demonstrate that both mechanisms are involved in the impaired endothelium-dependent relaxation in the MCT-induced PH. Regarding the endothelial dysfunction, a reduced NO bioactivity is involved as endothelium-dependent relaxation to ACh was totally mediated by NO in both the control and the fasudil-treated groups. Regarding the VSMC dysfunction, endothelium-independent relaxation of VSMC to SNP was slightly but significantly impaired in the control group. Importantly,
The fasudil treatment improved both endothelial and VSMC dysfunction.

Recently, it was shown that sildenafil may be useful for the treatment of PH for its enhancing effect on NO-mediated vasodilatation.\(^2\) We also have recently demonstrated that hydroxyfasudil prevents hypoxia-induced downregulation of eNOS.\(^2\) In the present study, fasudil significantly upregulated eNOS expression. It is important to note that any pharmacological treatment that is effective in this PH model is associated with upregulation of eNOS.\(^2\)\(^,\)\(^4\)\(^,\)\(^5\)

**Rho-Kinase and VSMC Hypercontraction in PH**

In the present study, VSMC contraction to serotonin was significantly enhanced in the MCT group, which may be involved in the increased pulmonary vascular resistance in the MCT-induced PH. We have demonstrated that Rho-kinase–mediated pathway plays a central role in the pathogenesis of VSMC hypercontraction or vasospasm in both porcine models and patients with vasospastic angina through inhibition of myosin phosphatase with subsequent enhancement of myosin light-chain phosphorylations.\(^1\)\(^1\)\(^,\)\(^3\)\(^,\)\(^6\) Robertson et al\(^4\) also reported that Y-27632, another specific Rho-kinase inhibitor, suppresses hypoxia-induced vasconstriction in rats. Fasudil may improve endothelial and VSMC function in a different way in the present study. In endothelial cells, fasudil improved NO-mediated endothelial vasodilator function partly through augmentation of endothelial eNOS expression.\(^2\) By contrast, in VSMCs, fasudil directly inhibited the Rho-kinase–mediated hypercontractions in a NO-independent manner as both acute and chronic treatment with fasudil abolished the VSMC hypercontractions. Recently, Sauzeau et al\(^5\) have reported that hypoxia-induced PH is associated with downregulation of RhoA expression and decreased contractility of conduit pulmonary arteries. It remains to be examined in future studies if and how RhoA expression and activity are altered in PH.

**Limitations of the Study**

Several limitations should be mentioned for the present study. First, MCT-induced PH model may not fully represent PPH in humans and thus the usefulness of Rho-kinase inhibitors should be examined in other PH models with different etiologies. However, it has been reported that Rho-kinase signaling also plays an important role in hypoxia-induced pulmonary vasoconstriction.\(^6\) We also have recently observed that long-term inhibition of Rho-kinase with fasudil suppresses hypoxia-induced PH in mice.\(^7\) These results suggest that Rho-kinase signaling is substantially involved in the pathogenesis of PH with different etiologies. However, like other drugs that have been reported to attenuate experimental PH (eg, statins, rapamycin),\(^3\)\(^3\)\(^,\)\(^4\)\(^,\)\(^8\) fasudil needs to be tested in the clinical setting. Second, some animals died in the fasudil groups. The cause of death appears to be RV failure due to PH even in the fasudil groups, suggesting that the fasudil treatment was not effective in all animals. It thus remains to be examined why fasudil was quite effective in some animals but not in others although the animals were genetically homogenous. Third, the mechanisms for the beneficial effects of fasudil were examined only in the prevention protocol due to the limited availability of the animals. However, it is conceivable that the same mechanisms of fasudil are involved in the treatment protocol.

**Clinical Implications**

PPH continues to be a serious clinical problem with high morbidity and mortality. We have recently confirmed the effectiveness and safety of oral administration of fasudil in patients with stable effort angina.\(^4\) The present study suggests that Rho-kinase could be a novel therapeutic target for the treatment of PH in humans.

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