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 embryo 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^{-5} 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 with or without 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).[18]

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 the 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.[3]

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^{-9} to 10^{-5} mol/L) was examined during a contraction to prostaglandin $F_{2\alpha}$ (3×10^{-6} to 10^{-5} mol/L) in the presence of indomethacin (10^{-5} mol/L) with or without N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} mol/L).[21] Endothelium-independent contractions to serotonin (10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (SNP, 10^{-10} to 10^{-5} mol/L) were also examined in rings without endothelium. The inhibitory effect of acute administration of hydroxyfasudil (10^{-5} 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.[15] 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.[15] 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.[23–25]

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-

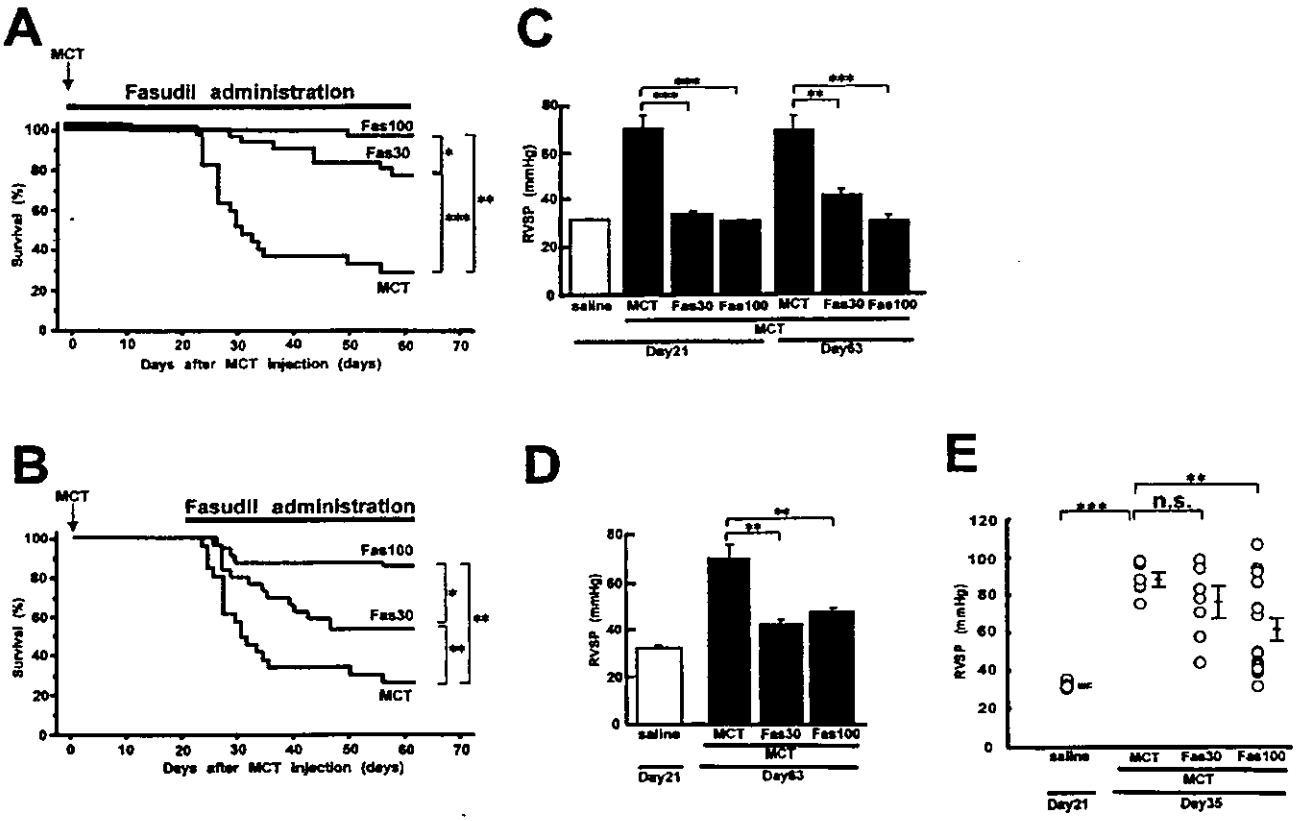

Figure 1. Fasudil improves survival of rats with MCT-induced PH. Compared with the saline-treated normal group (saline), in both the prevention (top) and treatment (bottom) protocols, the fasudil treatment markedly improved the survival (A and B) and RV systolic pressure (C and D). At day 35 (14 days after MCT injection), fasudil already started reducing RVSP in the treatment protocol in a dose-dependent manner (E). Fas 30 and 100; fasudil 30 and 100 mg/kg per day, orally, respectively. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$. n.s. indicates not statistically significant.

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 the treatment protocol, fasudil caused a marked regression of the MCT-induced PH at day 63 (Figure 1D). 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 21 (113±4 and 117±1, respectively, n=6 each) compared with the MCT alone group. In the treatment protocol, fasudil again improved the 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

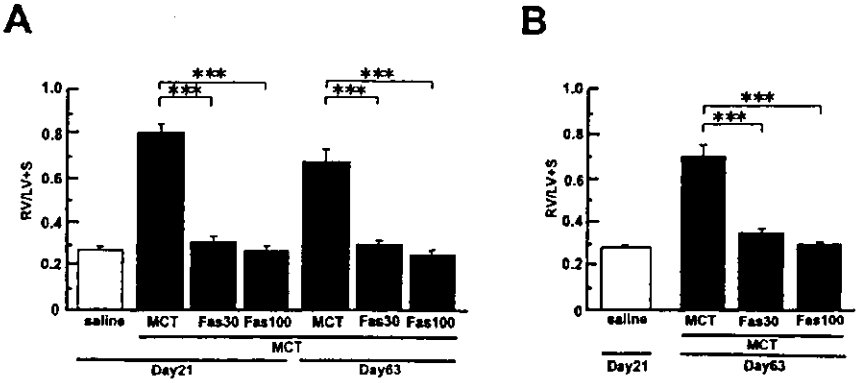

Figure 2. Fasudil improves RV hypertrophy in rats with MCT-induced PH. Compared with the saline-treated normal group (saline), in both the prevention (A) and treatment (B) protocols, the fasudil treatment markedly improved MCT-induced RV hypertrophy. Fas 30 and 100; fasudil 30 and 100 mg/kg per day, orally, respectively. ***$P<0.0001$.

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).

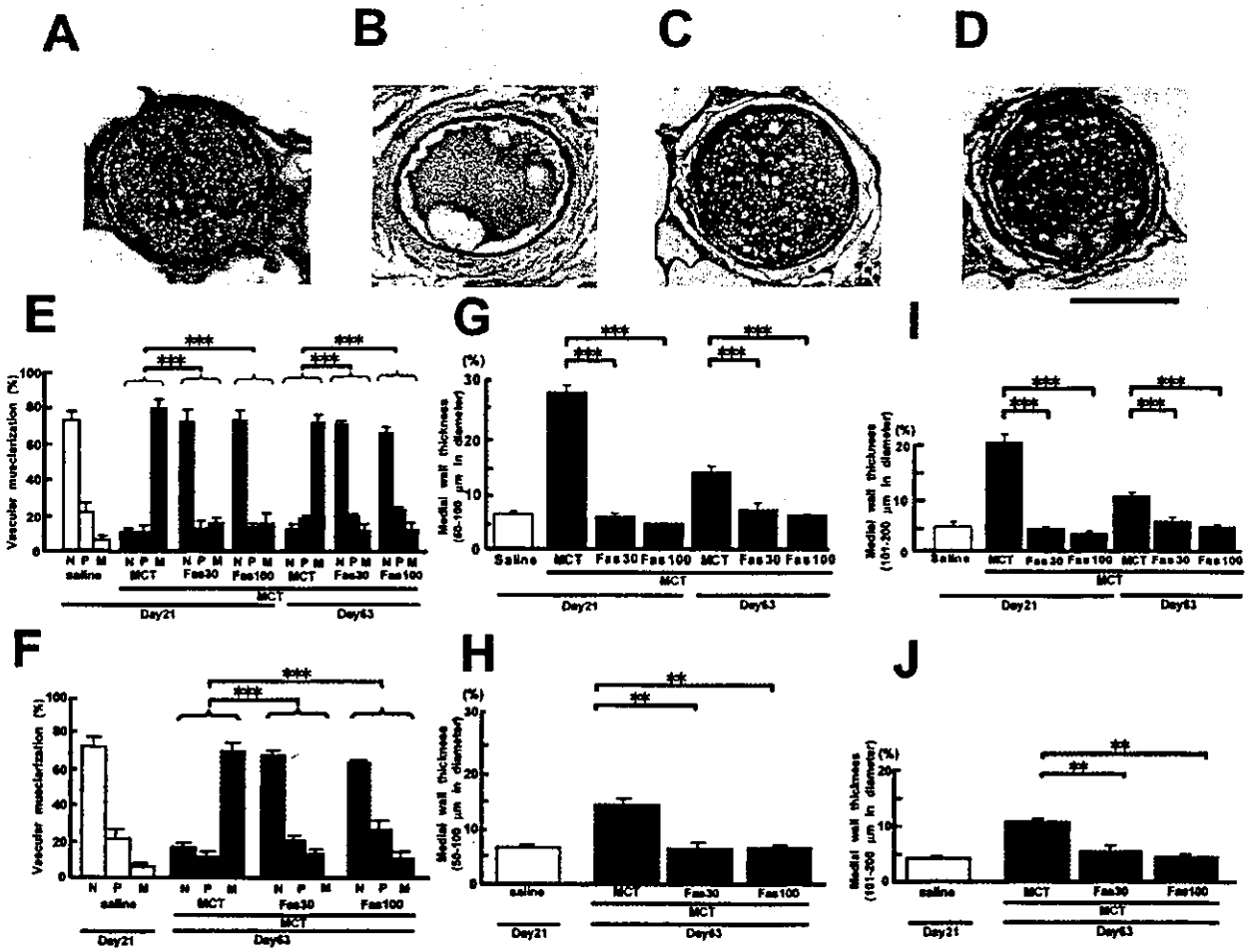

Figure 3. Fasudil suppresses medial thickening in rats with MCT-induced PH. Compared with the saline-treated normal group (A), medial wall thickening of the pulmonary artery was noted in the MCT group (B), whereas fasudil prevented (C) or markedly improved the medial thickening (D). Bar=50 μm. In the 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 (AUC_{0-24}, 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 al[36] 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.[13] In the present study, the mean value of the AUC_{0-24} 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 affects 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

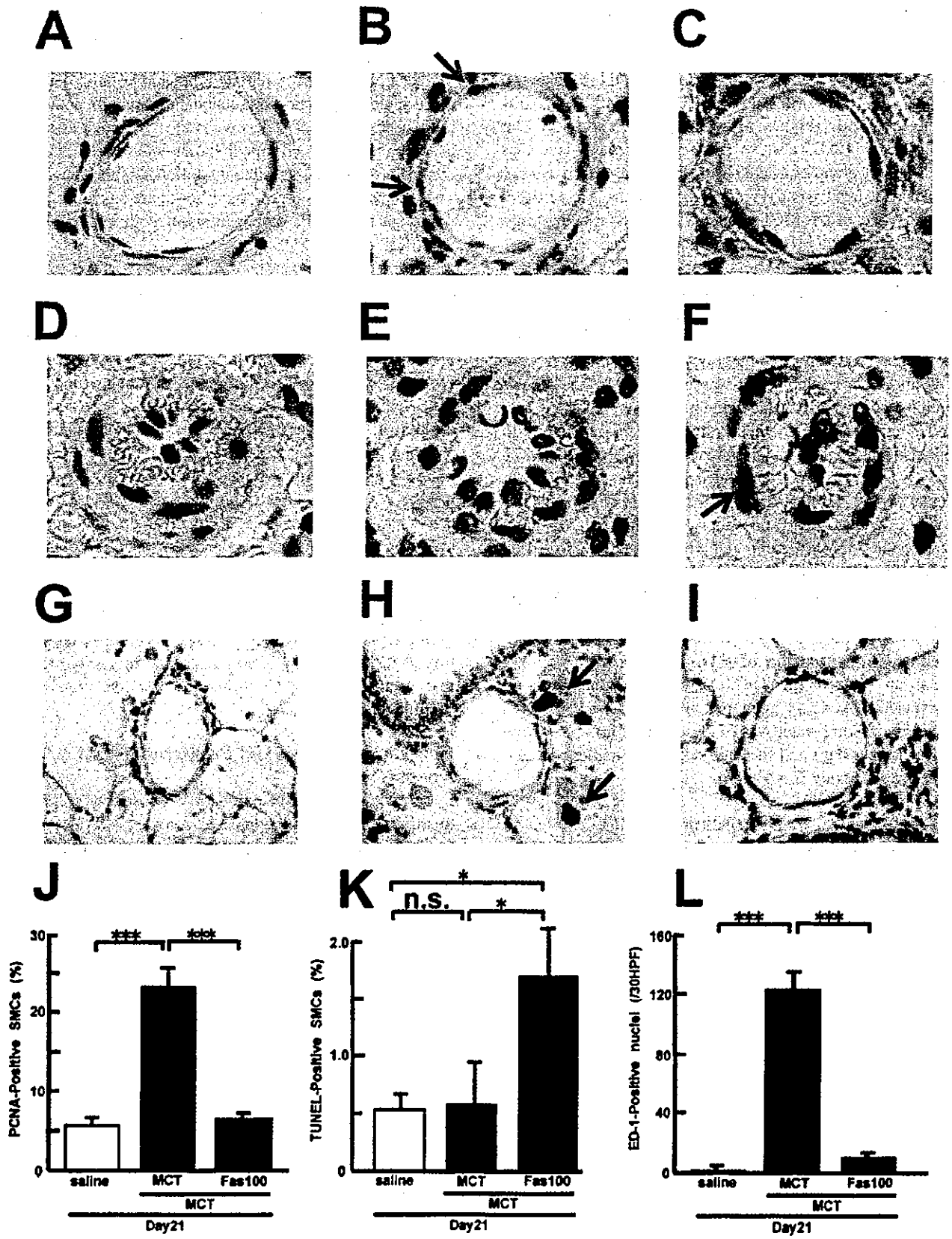

Figure 4. Mechanisms for the beneficial effects of fasudil on MCT-induced pulmonary remodeling. Histology of pulmonary arteries in the saline-treated normal group (A, D, and G), MCT group (B, E, and H), and high-dose fasudil group (C, F, and I) at day 21 in the prevention protocol. MCT-induced increase 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.

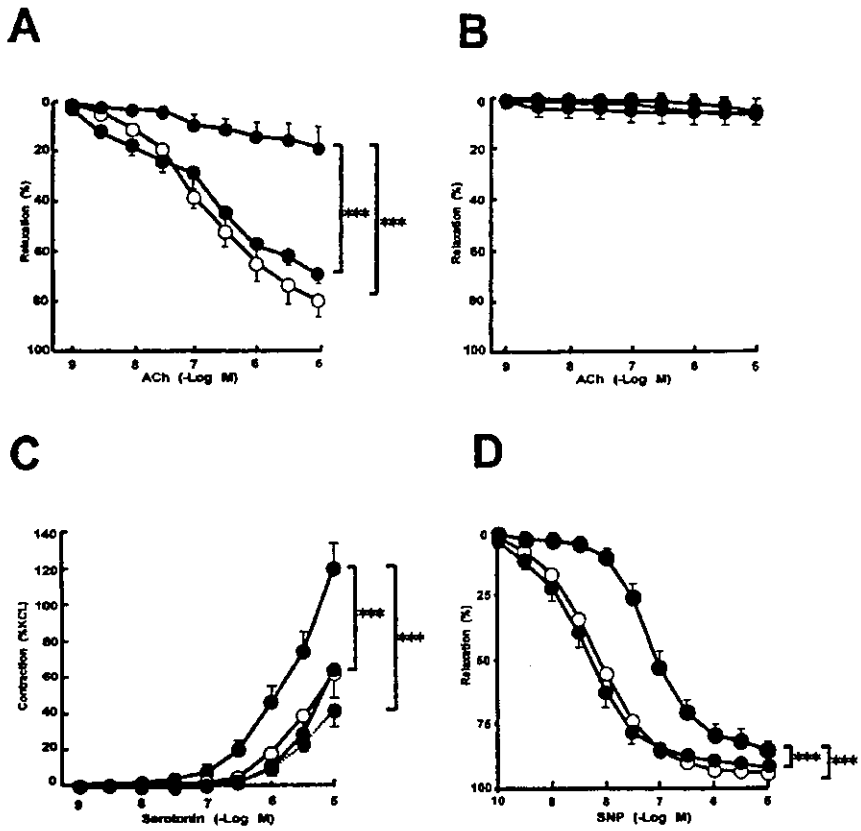

Figure 5. Fasudil improves endothelial and VSMC functions in rats with MCT-induced PH. A, MCT-induced endothelial dysfunction was markedly improved by the fasudil treatment at day 21 in the prevention protocol. B, Beneficial effect of fasudil was abolished by L-NNA (10^{-5} mol/L). C, Fasudil treatment significantly inhibited MCT-induced VSMC hypercontraction in response to serotonin (in rings without endothelium), as did acute administration of hydroxyfasudil (10^{-5} mol/L). D, Fasudil treatment improved the relaxation to sodium nitroprusside (SNP) in rings without endothelium compared with the MCT group. Open circle indicates saline-treated normal group; black circle, MCT-treated group; gray circle, fasudil-treated group; and black circle/dashed line, acute administration of hydroxyfasudil. Results are expressed as mean±SEM (n=6 to 7 each). ***$P<0.0001$.

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.[41] 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.[42] Regarding the VSMC dysfunction, endothelium-independent relaxation of VSMC to SNP was slightly but significantly impaired in the control group. Importantly,

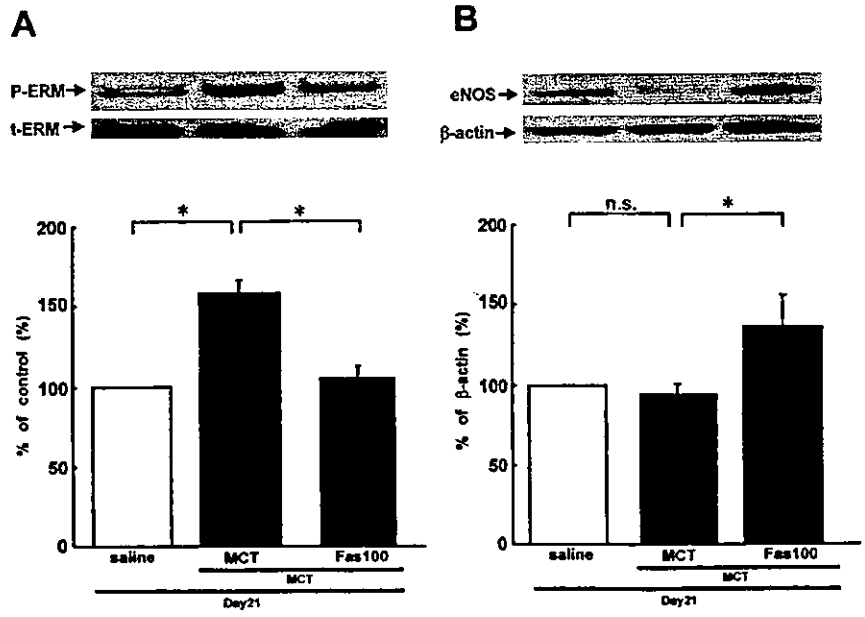

Figure 6. Effects of long-term treatment with fasudil on Rho-kinase activity eNOS expression in rats with MCT-induced PH. A, Compared with the saline-treated normal group, Rho-kinase activity, as evaluated by the extent of phosphorylation of the ERM family of pulmonary arteries, was significantly increased in the MCT group, which was significantly suppressed by the fasudil treatment at day 21 in the prevention protocol. P-ERM indicates phosphorylated ERM; t-ERM, total ERM. B, Fasudil treatment significantly increased eNOS expression of the lung at day 21 in the prevention protocol. eNOS level is shown as percent of the internal control β-actin level. Results are expressed as mean±SEM (n=3 to 5 each). *$P<0.05$. n.s. indicates not statistically significant.

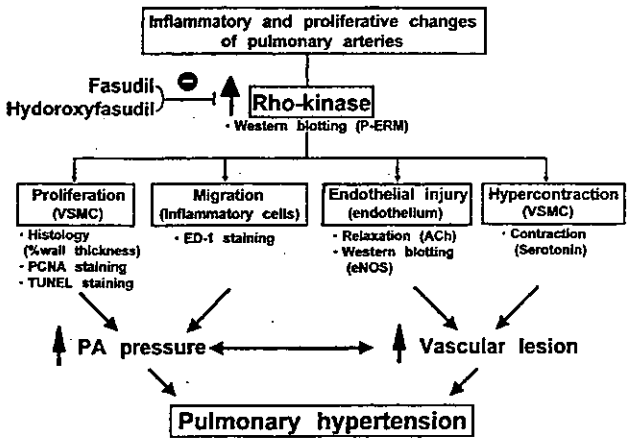

Figure 7. Summary of the present study. Multiple mechanisms appear to be involved in the pathogenesis of PH, all of which may be substantially mediated by Rho-kinase. Thus, the long-term blockade of Rho-kinase with fasudil or other Rho-kinase inhibitors may be useful for the treatment of PH.

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.[43] We also have recently demonstrated that hydroxyfasudil prevents hypoxia-induced downregulation of eNOS.[23] 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.[24,25,42]

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.[11,13,16] Robertson et al[44] also reported that Y-27632, another specific Rho-kinase inhibitor, suppresses hypoxia-induced vasoconstriction 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.[23] 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[45] 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.[46] We also have recently observed that long-term inhibition of Rho-kinase with fasudil suppresses hypoxia-induced PH in mice.[47] 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),[33,34,48] 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.[49] The present study suggests that Rho-kinase could be a novel therapeutic target for the treatment of PH in humans.

Acknowledgments

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Remnant Lipoproteins from Patients with Sudden Cardiac Death Enhance Coronary Vasospastic Activity Through Upregulation of Rho-Kinase

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Objective—Sudden cardiac death (SCD) still remains a serious problem. We have previously shown that remnant-like particles (RLP) are the major risk factor for SCD and that Rho-kinase plays a central role in the molecular mechanism of coronary vasospasm. In this study, we examined whether RLP from patients with SCD upregulate Rho-kinase associated with an enhanced coronary vasospastic activity.

Methods and Results—We isolated RLP and non-RLP in very-low-density lipoprotein (VLDL) fraction from SCD patients without coronary stenosis. We performed in vivo study in which we treated the coronary artery with RLP or non-RLP fraction at the adventitia in pigs. After 1 week, intracoronary serotonin caused marked coronary hyperconstriction at the segment treated with RLP fraction but not with non-RLP fraction ($P<0.001$, n=6), and hydroxyfasudil, a selective Rho-kinase inhibitor, dose-dependently inhibited the spasm in vivo. In organ chamber experiments, serotonin caused hypercontraction of vascular smooth muscle cells (VSMC) from RLP-treated segment, which was significantly inhibited by hydroxyfasudil ($P<0.001$, n=6). In cultured human coronary VSMC, the treatment with RLP significantly enhanced the expression and activity of Rho-kinase ($P<0.05$, n=6).

Conclusions—These results indicate that RLP from SCD patients upregulate Rho-kinase in coronary VSMC and markedly enhance coronary vasospastic activity. (*Arterioscler Thromb Vasc Biol.* 2004;24:918-922.)

Key Words: sudden cardiac death ■ lipoproteins ■ coronary vasospasm

Although a significant progress has been made in the treatment of ischemic heart disease, sudden cardiac death (SCD) still remains a serious problem.[1] Furthermore, there are many cases of out-hospital SCD without significant coronary stenosis.[2] Although coronary vasospasm has been postulated as one of the major causes of SCD,[3-5] the triggers for the spasm still remain to be elucidated.

Recently, a new method has been developed to isolate remnant-like particles (RLP), a major component of remnant lipoproteins mainly detected in very-low-density lipoprotein (VLDL) fraction, by using immunoaffinity gels coupled to anti-apoA-I and anti-apoB-100 antibodies.[6,7] With this method, it has been shown that plasma RLP level is an independent risk factor for coronary artery disease (CAD).[8,9] Furthermore, we have demonstrated that RLP are associated with severity of coronary atherosclerosis and also are the most significant risk factor for SCD without coronary stenosis in our postmortem studies.[2,10] RLP also are a major risk factor for myocardial infarction in patients with vasospastic angina with nearly normal coronary artery.[11] These findings suggest that RLP are substantially involved in the fatal events, such as coronary vasospasm and SCD.

Recent studies have shown the important role of small GTPase Rho and its effector, Rho-kinase, in Ca-independent regulation of smooth muscle contraction.[12] The Rho/Rho-kinase pathway modulates the phosphorylation level of myosin light chain (MLC) through inhibition of myosin phosphatase and contributes to the agonist-induced Ca-sensitization in smooth muscle contraction.[12] We have demonstrated that increased Rho-kinase activity in vascular sooth muscle cells (VSMC) plays a central role in the pathogenesis of coronary vasospasm in both animal models[13-16] and patients with vasospastic angina.[17] Thus, in this study, we examined whether RLP from patients with SCD without significant coronary stenosis upregulate Rho-kinase, resulting in enhanced coronary vasospastic activity in pigs.

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Serum Lipid Profiles in the SCD Patients and Controls.

	n	TC	TG	LDL	HDL	RLP-C	RLP-TG
SCD patients	15	223±15	211±27	139±9	40±4	23±3*	131±20†
Controls	5	177±27	148±30	159±6	41±7	13±4	29 ±8

TC indicates total cholesterol; TG, triglyceride; LDL, low-density lipoproteins; HDL, high-density lipoproteins; RLP-C and RLP-TG, remnant-like particles cholesterol and triglyceride, respectively. Results are expressed as mean±SEM (mg/dL).

*$P<0.05$, †$P<0.01$ vs controls.

Methods

All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in conformity with the institutional guidelines of the Kyushu University.

Animal Preparation

Twenty male domestic pigs (2- to 4 month-old and weighing 25 to 30 kg) were used. We anesthetized the animals with ketamine hydrochloride (15 mg/kg, intramuscular) and sodium pentobarbital (25 mg/kg, intravenous), ventilated with room air while oxygen was supplemented via a positive pressure respirator (Shinano, Tokyo, Japan). Under aseptic conditions, the proximal segments of the left anterior and the circumflex coronary arteries were carefully dissected and were gently wrapped with a cotton mesh after absorbing 0.1 mL of RLP or non-RLP in VLDL fraction in a randomized manner.[14]

Preparation of RLP and Non-RLP Fraction

The plasma was obtained from 15 SCD patients without significant coronary stenosis (13 males and 2 female, age 19 to 62 years.) and 5 healthy volunteers (5 males, age 49 to 76 years.). Among the 15 SCD patients, 14 had no obvious diseases and had not taken any medications before death. The remaining 1 patient was hypertensive but was without any antihypertensive medication. An informed consent was obtained from all the family of the patients. The mean elapsing time from SCD to the plasma collection was 8.5 hours. We have previously confirmed that there is no postmortem qualitative change in the plasma RLP within 12 hours after death.[2] RLP and non-RLP in VLDL fraction were isolated by the method by Nakajima et al with immunoaffinity chromatography using anti-apoA-I and anti-apoB-100 monoclonal antibodies.[6,7] Briefly, VLDL (d <1.006 kg/L) was isolated by density gradient ultracentrifugation from plasma samples. RLP (unbound fraction) were then isolated from VLDL fraction by immunoaffinity mixed gels containing 2 clones of monoclonal antibodies. Non-RLPs (bound fraction) were isolated from the gel with 5 mL of 3 mol/L sodium thiocyanate solution containing 0.1% bovine serum albumin (pH 7.4). RLP and non-RLP fractions were dialyzed against 5 L of PBS (pH 7.4) for 24 hours.[18] RLP and non-RLP in VLDL were concentrated by ultracentrifugation.

Coronary Angiography

Coronary angiography was performed 1 week after the operation, using the quantitative cineangiography (QCA) system (Toshiba Medical). Coronary diameters at end-diastole were measured by computer-assisted QCA system in a blind manner. Coronary vasoconstrictor response to serotonin was expressed as a percent decrease in luminal diameter from the control level.[14] The inhibitory effect of hydroxyfasudil, a specific Rho-kinase inhibitor (Asahi Kasei),[13] was also examined.

Organ Chamber Experiments

The porcine coronary segments treated with either RLP or non-RLP fraction were carefully isolated in physiological salt solution. The rings without endothelium were then mounted vertically between 2 hooks in organ chamber myographs (Medical Supply). 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, contractions to serotonin (10^{-9} to 10^{-5} mol/L) were examined.[16] The acute inhibitory effect of hydroxyfasudil (10^{-5} mol/L) was also examined.

Western Blot Analysis

The ERM family, a substrate of Rho-kinase, is phosphorylated by the kinase at T567(ezrin), T5648(radixin), and T558(moesin).[19,20] The regions containing ERM family proteins were visualized by ECL Western blotting luminal reagent (Santa Cruz Biotechnology). Isolated coronary rings without endothelium and adventitial tissue were subjected to SDS-PAGE immunoblot analysis 1 week after the treatment. Phosphorylation of ERM was measured when the serotonin-induced (10^{-6} mol/L) contraction reached a maximum.[16]

Cell Culture

Human coronary VSMC (hcVSMC) were obtained from Bio Wittaker. The hcVSMC were grown to confluent, growth-arrested in DMEM with 0.1% BSA for 2 days, and used for the experiments. Passages 4 to 10 were used.

Northern Blot Analysis

Total RNA was isolated from cultured hcVSMC treated with either RLP or non-RLP in VLDL fraction from SCD patients for 30 minutes to 24 hours. The sequence of the primer for reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of human Rho-kinase α and β was amplified from a human blood cDNA library. We obtained direct purification products of DNA from these PCR amplifications used by the Wizard PCR Preps DNA purification system. A human Rho-kinase α/β cDNA was used as a probe. Northern blot analysis was performed as previously described.[21] For quantitative analysis, the density of the bands was measured by an NIH image analyzer, and the levels of Northern products for Rho-kinase α/β were normalized to those for α-actin.

Statistical Analysis

All results are expressed as the mean±SEM. Differences in all parameters were evaluated by ANOVA, followed by Fisher post-hoc test. A $P<0.05$ was considered to be statistically significant.

Results

Serum Lipid Profiles and Composition of Isolated RLP in the SCD Patients and Controls

Plasma concentrations of RLP, especially those of RLP-triglyceride, were significantly higher in the SCD patients compared with the healthy volunteers, whereas there was no difference in other lipid profiles between the 2 groups (Table). Isolated RLP from the SCD patients contained a significantly higher cholesterol level as compared with controls (58±9 versus 19±9 mg/dL, $P<0.05$) and tended to do so for triglyceride level (252±34 versus 126±52 mg/dL, $P=0.06$).

RLP from Patients with SCD Enhance Coronary Vasospastic Activity in Pigs

In the coronary angiography study 1 week after the treatment, intracoronary serotonin caused marked coronary hypercon-

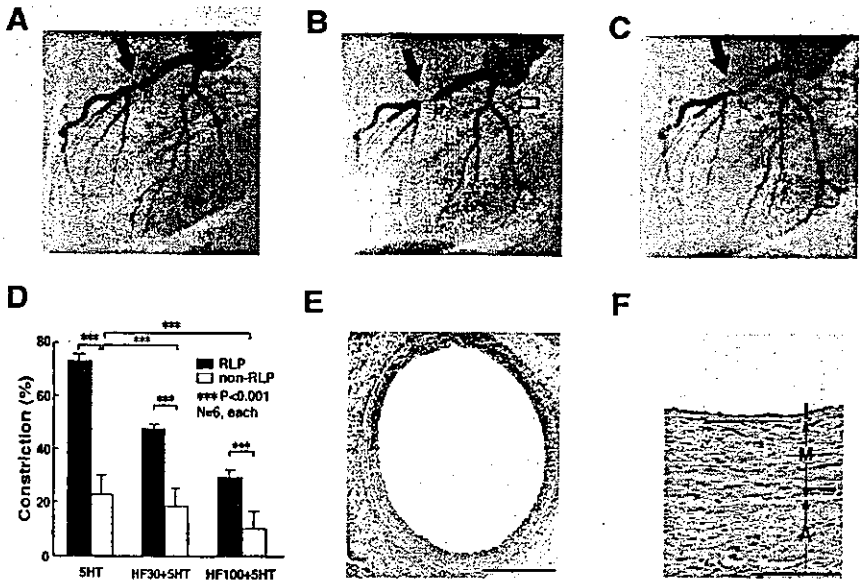

Figure 1. RLP from patients with SCD markedly enhance coronary vasospastic activity in pigs. A to C, Coronary angiograms before (A) and after intracoronary serotonin without (B) and with hydroxyfasudil (C). Black arrows indicate RLP site; white arrows, non-RLP site. D, Inhibitory effect of hydroxyfasudil on serotonin (5HT)-induced coronary hyperconstrictions. HF30 and HF100, hydroxyfasudil (30 and 100 μg/kg intracoronary). Results are expressed as mean±SEM. E and F, H&E staining of a coronary segment treated with RLP. The bar indicates 1 mm (E) and 100 μm (F). I indicates intima; M, media; A, adventitia.

striction at the segment treated with RLP but not at that with non-RLP fraction from the SCD patients without significant coronary stenosis (Figure 1A through 1D). The serotonin-induced coronary hyperconstrictions were dose-dependently inhibited by pretreatment with hydroxyfasudil, a specific Rho-kinase inhibitor (Figure 1C and 1D). Histological examination demonstrated that there was no obvious intimal thickening or mural thrombus formation at the spastic coronary segment treated with RLP, except for inflammatory cell infiltration at the adventitia (Figure 1E and 1F). By contrast, intracoronary serotonin caused a comparable extent of coronary vasoconstriction at the segments treated with RLP (31%±17%) and non-RLP (32%±28%) from the normal volunteers (n=3).

To examine the vasoconstrictor responses of VSMC, we performed organ chamber experiments at 1 week after the treatment with RLP and non-RLP from SCD patients. Serotonin (10^{-9} to 10^{-5} mol/L) caused concentration-dependent contractions of isolated coronary rings without endothelium. The serotonin-induced contractions were significantly augmented at the RLP-treated site as compared with the non-RLP–treated site and hydroxyfasudil significantly suppressed those contractions to serotonin only at the RLP-treated site (Figure 2A). To quantify the Rho-kinase activity of the porcine coronary arteries, we performed Western blot analysis for phosphorylated ERM (ezrin, radixin, and moesin) family, a substrate of Rho-kinase.[19,20] The extent of ERM phosphorylation was measured when the serotonin-induced contraction of each ring without endothelium reached maximum. The extent of ERM phosphorylation was significantly increased in the RLP-treated segment compared with non-RLP–treated segment and was again inhibited by hydroxyfasudil (Figure 2B).

There was a significant correlation between the extent of coronary vasoconstriction to serotonin in vitro and RLP-C ($P<0.001$), whereas such a tendency was noted between the former and RLP-TG (Figure 3).

RLP from Patients with SCD Upregulate Rho-Kinase in hcVSMC

We examined the effect of RLP and non-RLP in VLDL fraction from SCD patients on Rho-kinase expression and activity in cultured hcVSMC in vitro. Northern blot analysis revealed that mRNA expression of Rho-kinase α (ROCK2) and Rho-kinase β (ROCK1) was significantly increased in response to RLP but not to non-RLP in VLDL fraction (Figure 4A and 4B). Western blot analysis also revealed that the extent of phosphorylated ERM was significantly increased in response to RLP but not to non-RLP (Figure 4C). These results demonstrate that RLP, but not non-RLP in VLDL, exert a potent enhancing effect on the expression and activity of Rho-kinase.

Discussion

The novel findings of this study were that RLP from SCD patients without significant coronary stenosis upregulate Rho-kinase, enhancing the coronary vasospastic activity both in vivo and in vitro, and a specific Rho-kinase inhibitor,

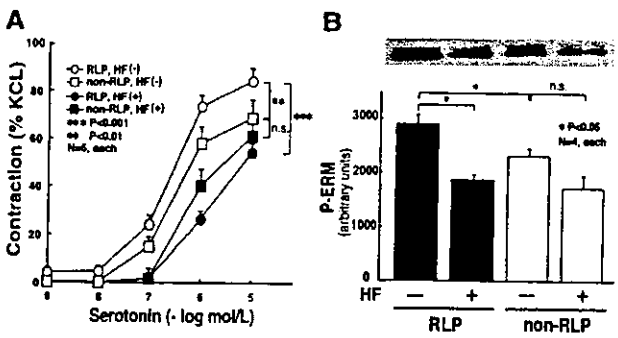

Figure 2. RLP enhance VSMC contractions and Rho-kinase activity. A, VSMC contractions to serotonin were significantly augmented at the RLP site (○) compared with the non-RLP site (□). Hydroxyfasudil significantly inhibited the VSMC hypercontractions only at RLP-site (●). B, The extent of the ERM phosphorylation of the coronary artery. Hydroxyfasudil significantly suppressed the enhanced ERM phosphorylation at RLP site. Results are expressed as mean±SEM.

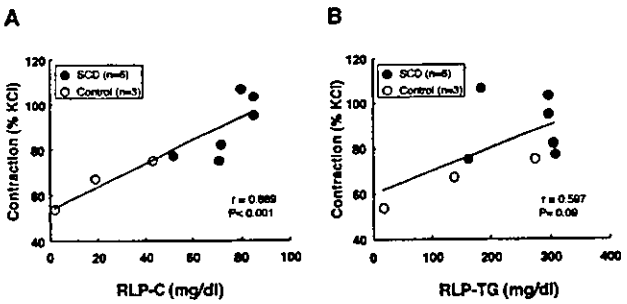

Figure 3. Correlation between RLP-C or RLP-TG level and that of serotonin-induced contractions (percent of contraction to 118 mmol/L KCl).

hydroxyfasudil, suppressed the coronary vasospastic activity both in vivo and in vitro. To the best of our knowledge, this is the first study that demonstrates the important role of RLP and coronary vasospasm in the pathogenesis of SCD.

Coronary vasospasm has been postulated to play an important role in SCD, although a direct demonstration for the hypothesis is still lacking. Likewise, although our previous postmortem studies demonstrated that RLP may be the major risk factor for SCD,[2,10] the mechanism for RLP-mediated SCD remains to be elucidated. In this study, we were able to demonstrate the close relation between RLP and coronary vasospasm that is mediated by upregulated Rho-kinase. We have previously shown that the expression and the activity of Rho-kinase are enhanced at the inflammatory coronary lesions in our porcine model with interleukin-1β.[14-16] The present study demonstrates that RLP from SCD patients also exert a potent upregulating effect on Rho-kinase in hcVSMC.

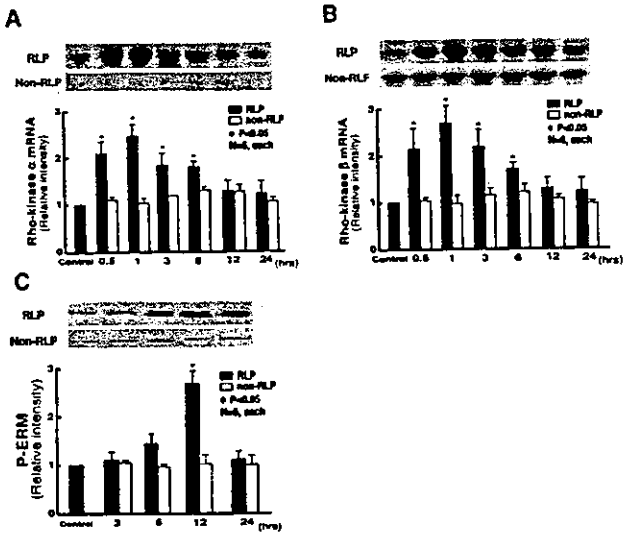

Figure 4. RLP enhance Rho-kinase expression and activation in human coronary VSMC. A to C, The treatment with RLP but not that with non-RLP significantly enhanced the mRNA expression of Rho-kinase α (ROCK2) (A) and Rho-kinase β (ROCK1) (B) (Northern blot). The blots are representative of 6 separate experiments. Bar graph shows the ratio of Rho-kinase mRNA expression normalized by that of α-actin under the control condition. Rho-kinase activity, as evaluated by the extent of ERM phosphorylation, was significantly enhanced (Western blot) (C). The blots are representative of 6 separate experiments. Bar graph shows the ratio of p-ERM expression to that under the control condition. Results are expressed as mean±SEM.

RLP exert several proinflammatory effects, including impairment of endothelium-dependent relaxation,[22] monocyte adhesion to the endothelium,[23] and VSMC proliferation.[24] It has been recently reported that postprandial increase in RLP is closely associated with postprandial inflammatory response.[25] RLPs are unique in dramatically increasing after a meal and remaining thereafter in the circulation for some time, although a wide individual variation appears to be present in the postprandial response.[26] Thus, it is conceivable that the adverse cardiovascular effects of RLP increase in the postprandial phase as compared with the fasting phase.[27] Indeed, clinical studies have demonstrated that postprandial increase in RLP is closely related to early atherosclerosis in healthy individuals.[28] In this study, no appreciable atherosclerotic lesion was noted at the RLP-treated site, indicating that functional alteration precedes the morphological one in coronary VSMC in response to RLP.

The present study also demonstrates the important proinflammatory effects of RLP to upregulate Rho-kinase. The important question arises as to whether Rho-kinase is upregulated by quantitative and/or qualitative alterations in RLP in SCD patients. The positive correlation between coronary vasoconstriction and RLP-C from the SCD patients and from the normal volunteers suggests that quantitative alteration in RLP-C is involved in the Rho-kinase upregulation. However, possible qualitative alteration in RLP in SCD patients remains to be examined. It has been recently reported that sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine, present in serum lipoproteins, behave as lipid mediator and cause vasoconstriction through upregulation of Rho/Rho-kinase pathway.[29-31] The possible role of S1P and sphingosylphosphorylcholine in RLP fraction remains to be elucidated in a future study.

In summary, this study provides the evidence that the elevated RLP level and the consequent upregulation of Rho-kinase are substantially involved in the pathogenesis of SCD. These results suggest that the detection of postprandial sustained increase in RLP is important to identify the subjects at high-risk for SCD and that the use of a Rho-kinase inhibitor could be a promising approach to prevent the fatal disorder.

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Long-Term Inhibition of Rho-Kinase Suppresses Neointimal Formation After Stent Implantation in Porcine Coronary Arteries: Involvement of Multiple Mechanisms

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Objective—We recently demonstrated that Rho-kinase, an effector of the small GTPase Rho, is substantially involved in the pathogenesis of arteriosclerosis. In this study, we examined whether Rho-kinase is also involved in in-stent restenosis and if so, what mechanism is involved.

Methods and Results—Pigs underwent stent implantation in the left coronary artery with or without administration of fasudil (30 mg/kg per day orally), a specific Rho-kinase inhibitor, starting 2 days before the procedure for a duration of 4 weeks. On day 28, reductions in coronary diameter and neointimal formation associated with macrophage accumulation, collagen deposition, and transforming growth factor (TGF)-β1 expression were noted at the stent site, and all were significantly suppressed by fasudil. On day 7, fasudil significantly increased the frequency of TUNEL-positive apoptotic cells, while it tended to reduce that of bromodeoxyuridine-positive proliferating cells in the neointima. Western blot analysis on day 7 demonstrated that phosphorylations of the ezrin/radixin/moesin family (a marker of Rho-kinase activity in vivo) and protein expression of monocyte chemoattractant protein-1and bcl-2 were upregulated at the stent site and were significantly suppressed by fasudil.

Conclusions—These results indicate that long-term inhibition of Rho-kinase suppresses in-stent neointimal formation by multiple mechanisms, including reduced vascular inflammation, enhanced apoptosis, and decreased collagen deposition. (*Arterioscler Thromb Vasc Biol.* 2004;24:181-186.)

Key Words: Rho-kinase ■ stents ■ inflammation ■ apoptosis ■ collagen

Although the use of stents has dramatically increased in interventional cardiology, in-stent restenosis continues to be a serious problem and is more troublesome when it occurs.[1,2] Furthermore, pharmacological approaches have generally been unsuccessful in suppressing in-stent restenosis except for a recent promising outcome with a drug-eluting stent.[3,4] However, late neointimal catch-up remains a potential adverse outcome with the stent-based drug delivery.[5] More recently, extracellular matrix accumulation has been recognized as a very important component of in-stent restenosis in the chronic phase after stent implantation.[6] Since drug-eluting stents have several limitations for a defined period of time with kinetics and are expensive, there may become a need for systemic therapies to maintain neointimal inhibition, including matrix metabolism.[7,8]

Accumulating evidence has demonstrated that Rho-kinase, an effector of the small GTPase Rho, plays an important role in adhesion, migration, proliferation, and cytokinesis of vascular smooth muscle cells (VSMCs) and other vascular wall cells.[9–11] Rho-kinase is substantially involved in the signal transduction initiated by angiotensin II,[12] platelet-derived growth factor (PDGF),[13] thrombin,[14] and endothelin-1,[15] all of which may play an important role in the pathogenesis of restenosis,[16] especially in that of in-stent restenosis.[17–20]

We recently demonstrated that neointimal formation after balloon injury was significantly inhibited by in vivo gene transfer of dominant-negative Rho-kinase in porcine femoral arteries.[21] A similar finding was also noted in the rat carotid artery with Rho-kinase inhibitor Y-27632, although the relative contribution of inhibition of VSMC proliferation and enhancement of VSMC apoptosis to the inhibitory effect of a Rho-kinase inhibitor remains to be elucidated.[22,23] Furthermore, involvement of Rho-kinase in the extracellular matrix

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deposition, especially that of collagen, a major component of the neointima,[6] remains unknown. There are also some differences in the restenosis mechanisms between balloon angioplasty and stent implantation.[24-29]

In the present study, we examined whether long-term inhibition of Rho-kinase suppresses in-stent restenosis in porcine coronary arteries, and if so, what mechanism is involved. For this purpose, we used long-term oral treatment with fasudil, which we found is metabolized to hydroxyfasudil, a specific Rho-kinase inhibitor, after oral administration.[30]

Materials and Methods

This study was reviewed and approved by the Ethical Committee on the Animal Experiments of the Kyushu University Graduate School of Medical Sciences.

Animal Preparation

Thirty-four domestic male pigs (Kyudo, Tosu, Japan; aged 2 to 3 months and weighing 25 to 30 kg) were used. They were divided into 2 groups: the control group was treated with aspirin (325 mg/d) and ticlopidine (500 mg/d) alone (n=17) and the fasudil group with oral administration of fasudil (30 mg/kg per day, Asahi Kasei Company) in addition to the antiplatelet therapy (n=17). The oral administration of fasudil was started 2 days before the procedure and continued until the follow-up period. We performed coronary angiography, intravascular ultrasound (IVUS) imaging, and histological study at 4 weeks (n=6 each). We performed immunostaining for bromodeoxyuridine (BrdU) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) (n=5 each), and Western blot analysis for substrates of Rho-kinase at 1 week (n=6 each).

Stent Implantation

Nitroglycerin (10 μg/kg IC) was administered prior to angiography. A stainless-steel stent (3.0×18 mm, Multi-Link TRISTAR, Guidant) was implanted to either the left anterior descending (LAD) or the left circumflex coronary (LCx) arteries.[31] A segment with a mean coronary diameter of 2.3 mm was selected by using quantitative coronary angiography with a stent-to-artery ratio of approximately 1.3. A balloon catheter mounted with a stent was then advanced to the pre-selected coronary segments for deployment over a standard guide wire in a blind manner without knowledge about the fasudil treatment. The balloon catheter was inflated at 8 atm for 30 seconds once and was then slowly withdrawn, leaving the stent in place.[31]

Coronary Angiography

Left coronary angiography was performed before, immediately after, and 4 weeks after the stent implantation. Among the 12 animals that underwent angiography, 4 received a stent for LAD and 2 for LCx in the control group, while in the fasudil group, 3 received a stent for LAD and 3 for LCx. A preshaped Judkins catheter was inserted into the right or left carotid artery, and coronary angiography in a left anterior oblique view was performed.[31,32] Arterial pressure, heart rate, and ECG were continuously monitored and recorded on a recorder.

Left coronary angiography was performed in a left anterior oblique projection.[31,32] The measured coronary luminal diameters included mean reference diameter [(proximal + distal) reference diameters/2], mean diameter of the stent site at full expansion, stent-to-artery ratio [(2/1)], and minimal diameter of the stent site at follow-up.[31]

Coronary IVUS

To assess the extent of neointimal formation in vivo, we performed IVUS 4 weeks after the stent implantation, as previously described.[31]

Histopathological Study

For histological analysis, the heart was excised 4 weeks after stent implantation; the left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The dissected whole artery was embedded in methylmethacrylate, leaving the stent wires intact to minimize potential artifacts from cutting the wires.[8] After polymerization, stented segments were cut into 3 blocks (proximal, middle, and distal portion) using a rotating saw with diamond edge. The blocks were cut into thin sections (4 to 5 μm) with tungsten carbide blades using a microtome (Leica). The sections were then stained with van Gieson elastic stains. Morphometric analysis of the neointima from photomicrographs was performed for vessel injury score and neointimal area, which was determined by subtracting the lumen area from the area encircled by the internal elastic lamina.[31,33] Mean value of the injury score and neointimal area from the 3 blocks was used for analysis.

Immunohistochemistry

Immunostaining was performed with a specifically designed kit with a tyramide signal amplification (Dako). The antibodies used in this study included monoclonal antibodies to human macrophages (AM-3K, Transgenic) and human α-smooth muscle actin (1A4, Dako), polyclonal antibodies to transforming growth factor (TGF)-β1 (Santa Cruz Biotechnology), and Dolichos biflorus agglutinin for porcine endothelium (Sigma),[34] and nonimmune mouse IgG (Dako). We semi-quantitatively assessed the extent of macrophage accumulation using a conventional scale (0, no cells; 1, scattered cells; 2, focal deposits; and 3, diffuse intense infiltration)[26] and that of re-endothelialization as the percentage of circumference covered by the endothelium (1, <25%; 2, 25% to 75%; and 3, >75%).[4]

Proliferation and Apoptosis

A segment 2 to 3 mm long was cut from the midportion of the stented artery using fine scissors. Stent wires were carefully removed under a dissecting microscope before paraffin embedding.[31] The sections were subjected to BrdU and TUNEL stainings to identify proliferating and apoptotic cells, respectively. BrdU (50 mg/kg) was intravenously injected three times at 24, 16, and 8 hours before necropsy; BrdU-positive cells were detected by the LSAB method and counterstained with hematoxylin.[35] Apoptotic cells were detected with an apoptosis kit (Wako) with porcine small intestine as a positive control. A total cell number and a number of BrdU-positive and TUNEL-positive cells in high-power field were counted in 3 randomly selected fields of each section.[31] A number of BrdU-positive and TUNEL-positive cells were expressed as BrdU index and TUNEL index (BrdU- or TUNEL-positive cells/total cells ×100), respectively.[23]

Collagen Deposition

Collagen deposition was measured on the entire neointima 4 weeks after stent implantation when it became evident. To avoid color balance variation, sirius red staining of all sections was performed at the same time. Then, once a standard for the particular slide/section was set by polarization microscopy, all the sections from the different groups of animals were photographed with the same strength of light by digital image capture.[36]

Western Blot Analysis

Stented coronary segments were subjected to SDS-PAGE immunoblot analysis at 1 week, as described previously.[32] Phosphorylation of the ezrin/radixin/moesin (ERM) substrates of Rho-kinase was measured, using a rabbit polyclonal antibody to phosphorylated human moesin (Thr558), which also binds to phosphorylated ezrin (Thr567) and radixin (Thr564). Monocyte chemoattractant protein (MCP)-1 (R&D Systems) and bcl-2 (Roche Diagnostics) were also evaluated.

Statistical Analysis

Results are expressed as means±SEM. Throughout the text and figures, n represents the number of animals tested. Comparison

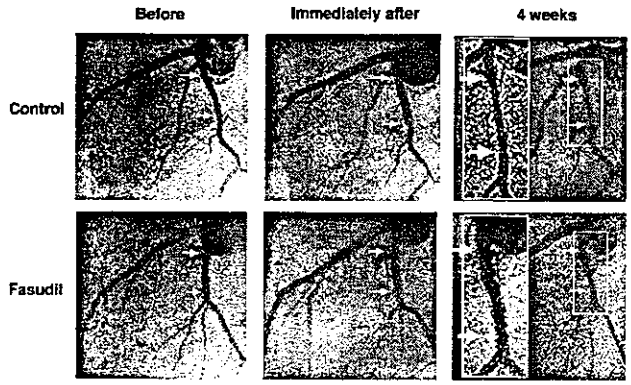

Figure 1. Coronary angiograms before, immediately after, and 4 weeks after stent implantation in the control and fasudil groups. The arrows indicate the proximal and distal portion of the stented coronary artery. High-resolution images are inserted.

between the control and the fasudil groups was performed by an unpaired, two-tailed t test. Multiple comparisons were made by analysis of variance followed by Scheffe post hoc test. A probability value of <0.05 was considered to be statistically significant.

Results

Coronary Angiography

Before stent implantation, there was no significant difference in coronary diameter (mm) between the control (2.33 ± 0.31) and the fasudil (2.31 ± 0.03) groups (Figure I and Figure I, available online at http://atvb.ahajournals.org). Similarly, there was no significant difference in the diameter (mm) at full stent expansion (2.95 ± 0.02 versus 2.97 ± 0.02) or stent-to-artery ratio (1.27 ± 0.02 versus 1.29 ± 0.02) between the 2 groups. Four weeks after the stent implantation, coronary diameter (mm) at the stent site was significantly decreased in the control group (1.65 ± 0.11) but remained unchanged in the fasudil group (2.23 ± 0.12) (Figure 1 and Figure I). There was no significant change in mean arterial pressure throughout the experiment in both groups (data not shown).

IVUS Analysis

IVUS analysis demonstrated that the extent of neointimal formation (as expressed by percentage of neointimal area to the area covered by stent) was significantly less in the fasudil group (36.6 ± 3.7) than in the control group (50.2 ± 4.7) ($P<0.05$).

Histology

Neointimal area (mm^2) was significantly less in the fasudil group (2.2 ± 0.2) than in the control group (3.1 ± 0.3) ($P<0.05$), while injury score was comparable between the control (1.13 ± 0.11) and the fasudil (1.20 ± 0.09) groups (Figure 2). Endothelialization score was also comparable between the control (2.8 ± 0.2) and the fasudil (2.8 ± 0.2) groups.

Rho-Kinase Activity

The extent of ERM family phosphorylation was significantly increased at the stent site in the control group and significantly suppressed in the fasudil group (Figure 3).

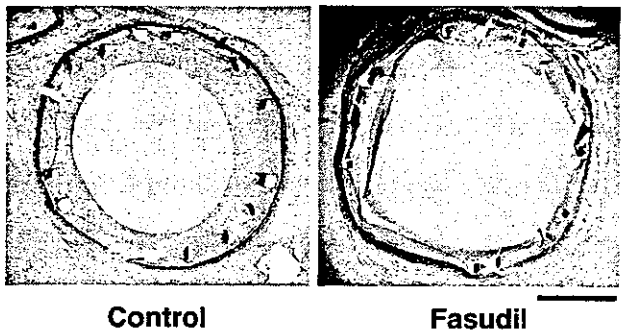

Figure 2. Photomicrographs (van Gieson elastic staining) of stented coronary segments 4 weeks after stent implantation in the control and the fasudil groups. Calibration, 1 mm.

Vascular Inflammation

At 4 weeks, macrophage accumulation was noted in the neointima and to a greater extent, in the adventitia in the control group and was significantly suppressed in the fasudil group (Figure 4 and Figure II, available online at http://atvb.ahajournals.org). At 1 week, MCP-1 protein expression increased and was again significantly reduced by fasudil (Figure III, available online at http://atvb.ahajournals.org).

Proliferation and Apoptosis

In the intact artery, neither BrdU-positive nor TUNEL-positive cells were detected in the intima or the media (data not shown). Although statistically insignificant, BrdU index (%) tended to be reduced in the fasudil group (23.2 ± 2.0) compared with the control group (33.2 ± 5.0) at 1 week ($P=0.10$). In contrast, TUNEL index at 1 week was significantly increased in the fasudil group (55.5 ± 5.4) compared with the control group (33.8 ± 4.6) ($P<0.05$) (Figure 5). Bcl-2 protein expression increased in the control group, which was significantly downregu-

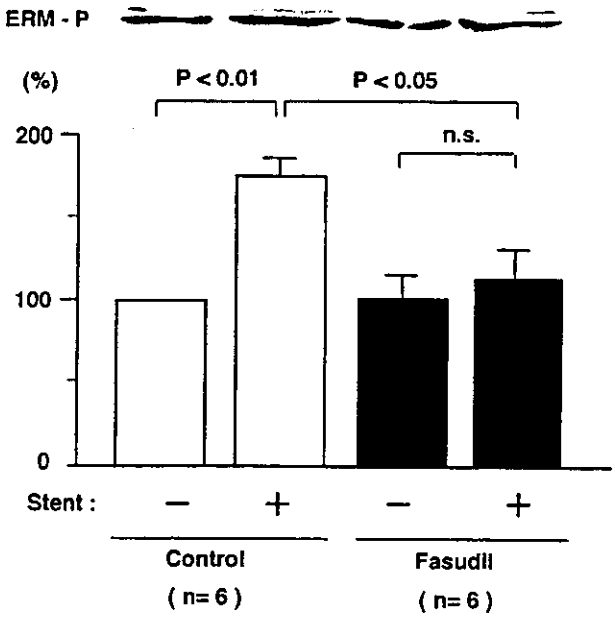

Figure 3. Western blot analysis for phosphorylated ERM (a marker of Rho-kinase activity) in porcine coronary segments with and without stent implantation in the control and the fasudil groups. Results are expressed as means±SEM.

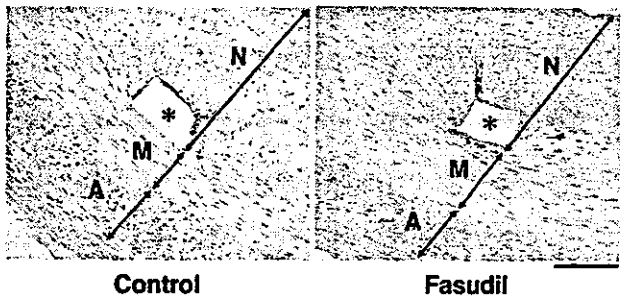

Figure 4. Inhibitory effects of fasudil on macrophage accumulation at the stented porcine coronary arteries. Photomicrographs show immunostaining for macrophages 4 weeks after stent implantation in the control and the fasudil groups. Calibration, 100 μm. N indicates neointima; M, media; A, adventitia. *Stent strut.

lated by the fasudil treatment (Figure IV, available online at http://atvb.ahajournals.org).

Collagen Deposition

Picrosirius red polarization showed that neointimal collagen content (%) was significantly less in the fasudil group (24.1±3.1) than in the control group (43.6±3.2) ($P<0.01$) (Figure 6). Immunostaining for TGF-β1 revealed reduced TGF-β1 immunoreactivity in the fasudil group rather than in the control group (Figure 6).

Side Effects

In the present study, no appreciable side effects, such as weight loss, diarrhea, or blood abnormalities, were noted in the fasudil group (data not shown).

Discussion

The novel findings of the present study were: (1) Rho-kinase activity was enhanced at the stent implantation site associated with neointimal formation and (2) long-term inhibition of Rho-kinase with fasudil significantly suppressed the neointimal formation by multiple mechanisms, including inhibition of vascular inflammation, enhanced apoptosis, and reduced collagen deposition (Figure V, available online at http://atvb.ahajournals.org). To the best of our knowledge, this is the first report that demonstrates the involvement of Rho-kinase in in-stent restenosis and

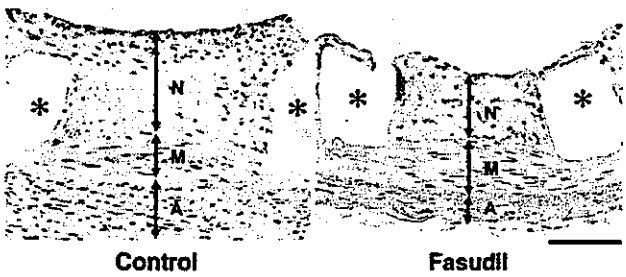

Figure 5. Proapoptotic effects of fasudil at the stented porcine coronary segments at 1 week after stent implantation. Photomicrographs show TUNEL-positive cells. More TUNEL-positive cells were noted in the neointima in the fasudil group than in the control group. Calibration, 100 μm. N indicates neointima; M, media; A, adventitia. *Stent strut.

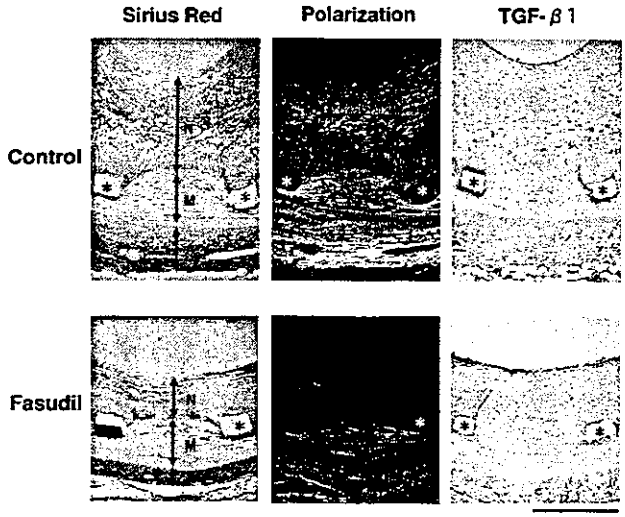

Figure 6. Inhibitory effects of fasudil on collagen deposition at the stented porcine coronary segments at 4 weeks after stent implantation. Sirius red stainings without (left) and with (middle) polarized light show more thickened neointima and abundant collagen deposition, respectively, in the control group compared with the fasudil group. Right, TGF-β1 immunoreactivity was detected in the neointima and the adventitia in the control group and was almost undetectable in the fasudil group. Calibration, 400 μm. N indicates neointima; M, media; A, adventitia. *Stent strut.

thereby the potential usefulness of a Rho-kinase inhibitor to prevent the disorder.

Increased Rho-Kinase Activity by Stent Implantation

We previously demonstrated that both expression and activity of Rho-kinase increase after balloon injury[21] and on stimulation by an inflammatory cytokine[9] in pigs in vivo. The present study also demonstrated that stent implantation increased Rho-kinase activity. Recent studies in vitro have shown that Rho-kinase and its substrates mediate actin cytoskeleton organization, cell adhesion and migration, and cytokinesis,[9-11] all of which may be involved in in-stent neointimal formation. Although a molecular mechanism for the upregulation of Rho-kinase by stent implantation remains to be elucidated, it has recently been demonstrated that various vasoactive factors enhance Rho-kinase activity in vitro.[9] Indeed, we have recently demonstrated that Rho-kinase plays an important role in angiotensin II-induced MCP-1 expression in cultured rat VSMCs.[12] Various growth factors (eg, PDGF) and cytokines, angiotensin-II, endothelin-1, and thrombin may all be involved in restenosis after angioplasty.[16-20] Importantly, all of them could upregulate Rho-kinase.[12-15] Thus, it is highly possible that Rho-kinase plays an important role in the pathogenesis of in-stent restenosis (Figure V).

Mechanism for the Inhibitory Effect of Fasudil on Neointimal Formation After Stent Implantation

The present study demonstrated that multiple mechanisms are involved in the inhibitory effect of fasudil on neointimal formation after stent implantation, including inhibition of

vascular inflammation, enhanced apoptosis, and reduced collagen deposition.

In-stent restenosis is characterized by prolonged and pronounced inflammation.[26,37–39] In this study, the long-term treatment with fasudil suppressed macrophage accumulation, not only around stent struts but also in the adventitia. We previously demonstrated that long-term treatment with fasudil significantly suppresses macrophage accumulation at the adventitia and subsequent coronary vascular lesion formation in porcine coronary arteries in vivo.[40] Two mechanisms may be involved for the anti-inflammatory effect of fasudil. First, fasudil may directly inhibit macrophage chemotaxis.[41] Second, fasudil may inhibit the expression of proinflammatory molecules such as MCP-1.[12] Importantly, the inhibitory effect of fasudil on the MCP-1 expression at the stent site (70% reduction) is equivalent to that of sirolimus-coated stent.[4] It is thus highly possible that reduced MCP-1 expression resulted in decreased macrophage accumulation at 4 weeks after stent implantation.

In the present study, fasudil significantly enhanced apoptosis, a consistent finding with a previous study.[23] However, the molecular mechanism for the proapoptotic effect of fasudil remains to be elucidated. In the present study, we demonstrated that downregulation of anti-apoptotic protein bcl-2 is involved in the proapoptotic effect of fasudil. Although statistically insignificant, fasudil also tended to reduce cellular proliferation. While it has been controversial whether an antiproliferative effect is involved in the antiatherogenic effect of a Rho-kinase inhibitor,[22,23] the present results suggest that such effect may not play a central role in the present porcine model, although this point remains to be examined in a future study. Fasudil did not affect the extent of re-endothelialization in vivo, a consistent finding with a previous report on Y-27632.[23] Thus, it is suggested that Rho-kinase is not involved in endothelial regeneration after vascular injury.

Finally, the importance of extracellular matrix formation is recognized as a key component of in-stent restenosis.[6] Stent implantation causes a significant increase in collagen synthesis and TGF-β expression compared with balloon angioplasty alone.[27,28] In the present study, we demonstrated the abundant collagen deposition associated with TGF-β1 expression in all layers of stented coronary arteries. TGF-β is one of the most potent stimuli for collagen synthesis and may contribute to the formation of restenotic lesions.[16] A substantial portion of the neointima consists of matrix rather than cells.[6] Thus, a strategy to inhibit TGF-β may be useful in preventing in-stent neointimal formation.[28] In this study, we were able to demonstrate for the first time that long-term treatment with fasudil significantly suppresses collagen deposition in the neointimal lesion after stent implantation due, at least in part, to the inhibition of TGF-β1 expression.

Possible Side Effects of Fasudil

In the present study, no appreciable side effects were observed in the fasudil group, and fasudil had no effects on arterial pressure or nonstented coronary artery. We have recently demonstrated that fasudil is well tolerated without any serious side effects in patients with angina.[42] Thus, fasudil may be a safe drug, although caution should be made when used clinically.

Limitations of the Study

Several limitations of the present study should be mentioned. First, the present study was performed in the normal porcine coronary artery without preexisting intimal thickening. Thus, the inhibitory effects of fasudil need to be confirmed in animal models with atherosclerotic coronary lesions. Second, cell-specific Rho-kinase expression was not examined. However, based on our recent findings[9,40] and the present results with stainings for BrdU, TUNEL and macrophages, we consider that Rho-kinase was expressed mainly in macrophages and VSMCs. Third, although we confirmed the inhibitory effect of fasudil (hydroxyfasudil) on Rho-kinase in the present study,[30] other unknown effects of this agent might be involved. Fourth, it remains to be examined how long fasudil should be continued to prevent neointimal formation after coronary stenting or whether lesion development is permanently suppressed by some period of treatment with fasudil. Finally, there are some differences in the mechanism between atherosclerosis and restenosis, including severity of injury, time course of the response, cellular/extracellular elements, and relation to lipids.[43] Thus, an agent that may have beneficial effects in atherosclerosis may not be equally effective in the prevention of in-stent restenosis.

In summary, the present results indicate that long-term inhibition of Rho-kinase suppresses in-stent restenosis by multiple mechanisms, suggesting the potential usefulness of a Rho-kinase inhibitor to prevent the disorder (Figure V).

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Rapid Communications

First Functionalized MRI Contrast Agent Recognizing Vascular Lesions

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A new MRI-contrast agent, EB-DTPA-Gd, that has an Evans Blue analogue as a sensing unit for endothelium lesions, was designed and synthesized. The agent also has diethylenetriamine-*N,N,N',N'',N''*-pentaacetic acid-Gd complex (Gadolinium-DTPA) units, which have been used as detection units for T1-weighted MRI. The EB-DTPA-Gd was able to recognize and adsorb to the vascular endothelium-denuded region of porcine aorta, and to decrease the relaxation time of circumferential water's protons, making possible MR imaging of the endothelium-denuded region. The compound can be employed as a contrast agent for the imaging of vascular lesions using MRI.

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Vascular endothelium plays a very important role in regulating vascular homeostasis. If the endothelium is damaged, this vascular lesion often leads to a cardiovascular disease, such as arteriosclerosis or spasms in the coronary artery.[1] Therefore, the detection and evaluation of vascular endothelium lesions in their early stage would be very important for effective diagnosis and therapy. However, no practical method for vascular lesion-specific detection has been reported as a practical diagnostic tool.

In this report, we describe a novel endothelium lesion-specific MRI (Magnetic Resonance Imaging) contrast agent, which has a dye component as a probing unit for the vascular lesion. In general, biomolecules such as antibodies, polysaccharides, or peptides have been used to recognize particular sites in biological systems.[2-4] However, such biomolecules are very expensive and are thus not practical for a contrast agent for blood vessels due to the large blood flow, in which a large amount of the agent would be needed. Therefore, we have looked for other less-expensive molecules that can recognize vascular endothelium lesions using the porcine aorta, and have at last found certain types of organic dyes to be useful for this purpose. In histochemistry, various dyes have been used for staining specific tissues and proteins.[5-7] These dyes can interact with their targets with high specificity. For example, Congo Red specifically binds to the amyloid beta protein.[7] In these dyes, Evans Blue has been used for staining a vascular endothelium injury.[8] Vascular endothelium forms a tight junction that regulates the molecular permeability into the vascular wall from the blood,[9] thereby providing a barrier in the vascular wall against blood flow. If the vascular endothelium is injured, many molecules begin to interact with the extracellular matrix and the vascular smooth muscle layer, which is located below the endothelium layer.

We have recently synthesized structural analogues of Evans Blue, and the dye unit was observed to selectively adsorb to a vascular endothelium-denuded region. We have therefore designed a new MRI-contrast agent that has Evans Blue analogue (Fig. 1). The compound can be used as a contrast agent for the imaging of vascular lesions using MRI.

Experimental

Synthesis of N-tert.butoxycarbonyl-2,2'-dimethylbenzidine

2,2'-Dimethylbenzidine (3.00 g, 14.1 mmol) was dissolved in dichloromethane (25 ml), and Boc-anhydride (3.08 g, 14.1 mmol) was added dropwise at r.t. while stirring. After overnight stirring, the remaining dimethylbenzidine was removed by washing with saturated aqueous tartaric acid. The organic phase was then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using ethylacetate-hexane (3:5) as an eluting solvent. The

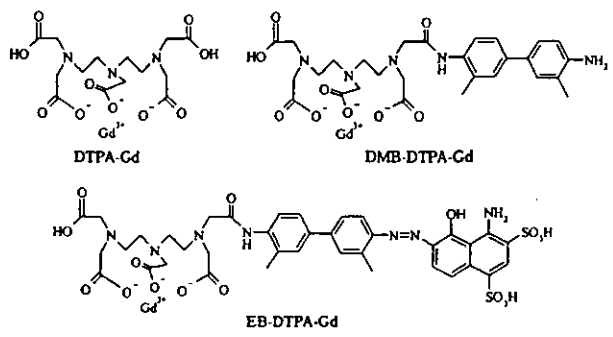

Fig. 1 Chemical structure of the MRI contrast agent.

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