Long-Term Treatment With a Specific Rho-Kinase Inhibitor Suppresses Cardiac Allograft Vasculopathy in Mice

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Abstract—Cardiac allograft vasculopathy (CAV) continues to be a major cause of late graft failure after cardiac transplantation. We have demonstrated that Rho-kinase, an effector of the small GTPase Rho, plays an important role in the pathogenesis of arteriosclerosis. In this study, we examined whether the Rho-kinase–mediated pathway is also involved in the pathogenesis of CAV using a specific Rho-kinase inhibitor and a dominant-negative Rho-kinase. Hearts from AKR mice were heterotopically transplanted to C3H/He (allograft) or AKR mice (isograft), and the effects of long-term oral treatment with fasudil, which is metabolized to a specific Rho-kinase inhibitor hydroxyfasudil, on CAV were examined at 2 and 4 weeks after the transplantation. Coronary remodeling in the allografts characterized by intimal thickening and perivascular fibrosis was dose-dependently suppressed in the fasudil group compared with the control group ($P<0.01$, n=9 to 10). The inhibitory effects of hydroxyfasudil were mimicked by in vivo gene transfer of dominant-negative Rho-kinase ($P<0.05$, n=4). Among the proinflammatory cytokines examined, those of macrophage migration inhibitory factor, interferon-γ, and transforming growth factor-β1 were upregulated in the control group and were dose-dependently inhibited in the fasudil group ($P<0.01$, n=5). Vascular inflammation in the allografts, as evidenced by accumulation of inflammatory cells (macrophages and T cells), was also significantly inhibited in the fasudil group ($P<0.05$, n=5 to 10). These results indicate that long-term treatment with fasudil suppresses CAV in mice, suggesting that Rho-kinase is an important therapeutic target for the prevention of CAV. (*Circ Res.* 2004;94:46-52.)

Key Words: arteriosclerosis ■ cytokines ■ transplantation

Cardiac allograft vasculopathy (CAV) continues to be a serious problem for long-term survival of patients with cardiac transplantation, as it is a major cause of the graft failure after the first year of transplantation.[1-3] The coronary remodeling associated with CAV is characterized by progressive intimal thickening.[4,5] Although the cause of CAV is known to be autoimmunity, its pathogenesis, including the nature and sequence of cellular/molecular events leading to it, remains to be elucidated. To develop an effective preventive therapy for CAV, it is important to identify the key molecule(s) involved in this disorder.

Rho-kinase, an effector of small GTPase Rho, plays an important role in adhesion, migration, proliferation, and cytokinesis of vascular smooth muscle cells (VSMCs),[6-8] all of which may be involved in the pathogenesis of arteriosclerosis. We have recently demonstrated that Rho-kinase is substantially involved in the pathogenesis of cardiovascular remodeling.[6,9] Indeed, Rho-kinase is involved in migration of inflammatory cells, which may be involved in the pathogenesis of CAV.[6,9] Rho-kinase also is substantially involved in the downregulation of endothelial NO synthase (eNOS).[10] The present study was thus designed to examine whether Rho-kinase is involved in the pathogenesis of CAV in mice and, if so, what mechanisms are involved.

Materials and Methods

This study was reviewed by the Committee on Ethics in Animal Experiments of Kyushu University and was carried out according to the Guidelines for Animal Experiments of Kyushu University and the Japanese Government.

Animals

AKR female mice (H-2^k, aged 9 to 11 weeks) were used as heart donors, and C3H/He (H-2^h) female mice (allograft transplantation) and AKR female mice (isograft transplantation) of the same age were used as recipients. A total of 258 mice (Japan SLC Inc, Tokyo, Japan, or Seac Yoshitomi, Tokyo, Japan) were used in this study. The animals were housed to have free access to food and drink and were maintained at 23±2°C with 12-hour light and dark cycle.

Cardiac Transplantation and Drug Administrations

Heterotopic cervical cardiac transplantation was performed by the standard technique.[11] A day before cardiac transplantation, recipients

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were randomized into the following 3 groups and pharmacological treatment with fasudil was started: recipients transplanted with allografts with (fasudil group) or without (control group) oral treatment with fasudil (Asahi Kasei Corp, Tokyo, Japan; 10 and 30 mg/kg per day in drinking water) and recipients with isografts without any drug (isograft group). In a preliminary study, we checked the volume of daily water intake of recipient animals for 4 weeks after the transplantation. The recipients were able to freely access the water in which fasudil was dissolved. The amount of fasudil with which the animals were treated was calculated with recipient weight and drinking water volume. We have previously confirmed that fasudil is metabolized to hydroxyfasudil, a specific inhibitor of Rho-kinase, after oral administration.[12] We have previously confirmed that the inhibitory effect of hydroxyfasudil on Rho-kinase is 100 times higher than for protein kinase C (PKC) and 1000 times higher than for myosin light-chain kinase.[12] Furthermore, the inhibitory effect of hydroxyfasudil on 16 kinases, including Rho-kinase, has recently been examined. Among the kinases tested, hydroxyfasudil at 10 μmol/L showed more than 50% inhibition only for Rho-kinase (97.6%).[13] Thus, we consider that hydroxyfasudil is a reasonably selective inhibitor for Rho-kinase in the present study. Plasma concentrations of fasudil and hydroxyfasudil were measured by high-performance liquid chromatography at 4 weeks after the transplantation.[14]

Adenovirus-Mediated In Vivo Gene Transfer

Adenovirus vectors encoding a mutant (NK1036→TT) Rho-binding (RB) domain of Rho-kinase plus a pleckstrin homology domain (RB/PH[TT]; 2.2×10^9 pfu/mL in 0.15 mL),[15] which is a dominant-negative form of Rho-kinase (DN-Rho-kinase), and those with LacZ (2.3×10^9 pfu/mL in 0.15 mL as a control) were transfected to allografts as previously described.[16] In a preliminary study, we confirmed the expression of the LacZ gene throughout the heart by X-gal staining 1 week after the transplantation. After 4 weeks, the grafts were harvested and the extent of CAV was analyzed histologically.

Histology and Morphology

All grafts were perfused with sodium nitroprusside (10^{-5} mol/L) as a vasodilator and fixed with paraformaldehyde under 180 cm H_2O before embedding in paraffin. The grafts were cut transversely into 3 blocks, fixed in 4% phosphate-buffered paraformaldehyde, and embedded in paraffin. Three sections (5 μm thick) were made from each block and stained with Masson's trichrome. The intima, media, and perivascular fibrosis areas were measured at a magnification of ×200 (BX50F-3, Olympus Optical Co, Tokyo, Japan). The ratio of the intimal area to total vascular area and that of the perivascular fibrosis area to total vascular area were calculated.

Western Blot Analysis

Four weeks after the transplantation, cardiac grafts were isolated and total protein was extracted from each graft. The extent of phosphorylation of ezrin, radixin, and moesin (ERM), the substrates of Rho-kinase, was measured as described previously to examine the Rho-kinase activity in vivo.[15] We loaded an equal amount of protein on each well of polyacrylamide gel for the electrophoresis. The amount of proteins derived from vascular wall cells is different among samples because each sample, especially allografts, contains extracellular matrix (ECM). Therefore, each band intensity of the ERM was normalized by a corresponding value of total actin.[17] We used an antibody to phosphorylated ERM and that to total ERM that we developed ourselves[18] and rabbit anti-total actin antibody (A2066, Sigma, St Louis, Mo).

Isolation of RNA and Ribonuclease Protection Assay

Cardiac grafts were homogenized in 0.8 mL of Isogen (Wako Pure Chemical Ind, Osaka, Japan), and total RNA was extracted according to the manufacturer's protocol. The RNase protection assay was performed using a multipurpose assay system (PharMingen, San Diego, Calif) for cytokines, chemokines, and adhesion molecules, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), transforming growth factor-β1 (TGF-β1), interleukin-6 (IL-6), macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin. Isotope-labeled hybridization reactions were electrophoresed on 5% acrylamide gel, and this gel was exposed to scientific imaging film (Kodak Inc, Rochester, NY). Areas of the respective transcript bands were measured and were normalized against that of GAPDH.

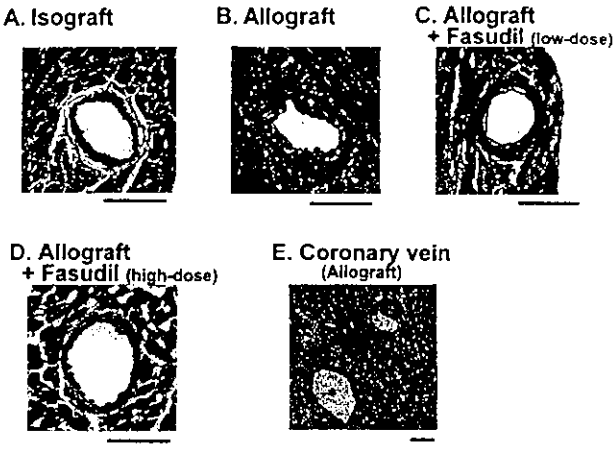

Figure 1. Representative photomicrographs of a mouse coronary artery from an isograft (A) and from an allograft in the control (B), the low-dose (C) and the high-dose fasudil group (D) at 4 weeks after the transplantation (Masson's trichrome staining, ×200). In the control allograft group, intimal thickening and perivascular fibrosis were noted, both of which were dose-dependently suppressed by fasudil treatment. Coronary vein, indicated by an asterisk, in the allograft is shown for comparison with coronary arteries (E). Bars=100 μm.

Immunohistochemistry

Four weeks after the transplantation, cardiac grafts were cut horizontally into 4 blocks, embedded in OTC compound (Sakura Finetechnical Co, Tokyo, Japan) and kept at −80°C until staining. Nine slices (3 slices from 3 blocks from the apex) were made for immunostaining with a kit (Histofine SAB-PO kit, Nichirei Co, Tokyo, Japan) for macrophages (MOMA-2) and CD4- and CD8-positive T cells, as previously described.[19] Three fields where a coronary artery was recognized in the center were selected from each animal to count the number of macrophages and calculate a percent-positive area in a blind manner at a magnification of ×200.

Statistical Analysis

All results are expressed as the mean±SEM. Data were analyzed either by unpaired t test or by ANOVA followed by Fisher's post hoc test for multiple comparisons. Values of $P<0.05$ were considered to be statistically significant.

Results

Cardiac Allograft Vasculopathy

Mice treated with fasudil were well tolerated and showed no side effects, such as weight loss, hair loss, or diarrhea. A total of 2369 coronary arteries were evaluated by computer-assisted analysis in terms of the severity of CAV. Four weeks after the cardiac transplantation from AKR to C3H/He mice, neointima formation (evaluated by intima/vascular area ratio) and perivascular fibrosis of coronary arteries were markedly enhanced in the control allograft group compared with the isograft group (Figures 1 and 2). By contrast, coronary veins

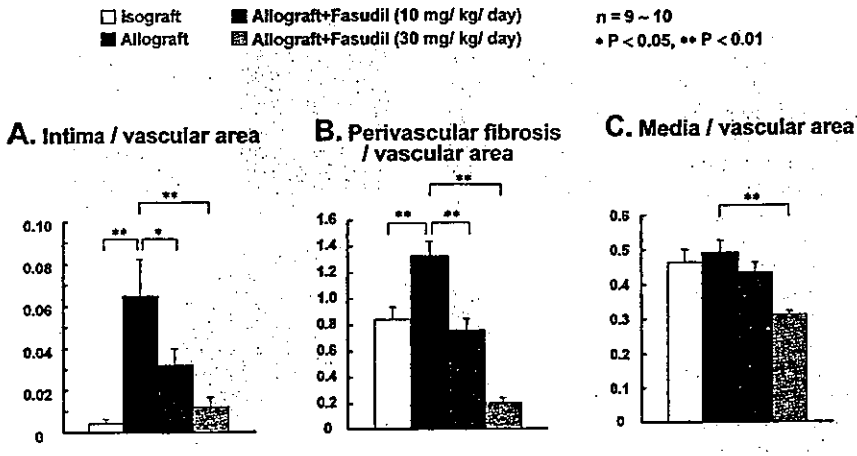

Figure 2. Long-term treatment with hydroxyfasudil inhibits the development of CAV in mice. In the control allograft group, intimal thickening (A, as expressed by intima/vascular area ratio) and perivascular fibrosis (B) developed at 4 weeks after the transplantation, both of which were dose-dependently suppressed by the fasudil treatment. By contrast, medial thickness was reduced only in the high-dose fasudil group (C).

were resistant to those changes (Figure 1E). In the isograft group, perivascular fibrosis was also developed probably due to a reperfusion injury alone (Figure 2B). Both neointima formation and perivascular fibrosis of the allografts were dose-dependently attenuated in the fasudil groups (Figures 1 and 2). The high dose of fasudil inhibited the perivascular fibrosis to the level seen in the native hearts (Figure 2B). The medial area of the coronary artery was reduced only in the high-dose fasudil group compared with the control group (Figure 2C). The medial area in the high-dose fasudil group (0.31 ± 0.01, n=9) was equal to that seen in the native hearts (0.36 ± 0.01, n=10). At 4 weeks after the treatment with fasudil, plasma concentrations of hydroxyfasudil (ng/mL) increased from 0 (control animals) to 5.53 ± 2.08 and 37.24 ± 19.12 in the low-dose (n=6) and the high-dose (n=5) fasudil groups, respectively, specific therapeutic ranges of the Rho-kinase inhibitor.[12] By contrast, fasudil was not detected in any groups.

Rho-Kinase Activity

The extent of ERM phosphorylation, as normalized by that of total actin, was significantly enhanced in the control allograft group compared with the isograft group (Figure 3). The long-term treatment with fasudil dose-dependently suppressed the increase in Rho-kinase activity in the allograft group (Figure 3). The total amount of ERM did not change among the 4 groups (Figure 3). The actin density was significantly less in the allograft group than any other groups because of the abundant ECM in the equal amount of the sample.

In Vivo Gene Transfer of DN-Rho-Kinase

To confirm the specificity of the inhibitory effect of hydroxyfasudil on CAV, adenovirus-mediated gene transfer of DN-Rho-kinase was performed while LacZ transfection was used as a control. X-gal staining demonstrated that LacZ was expressed widely in the cardiac grafts (Figure 4A). Histological analysis showed that the gene transfer of DN-Rho-kinase suppressed both intimal thickening (evaluated by intima/vascular area ratio) and perivascular fibrosis compared with that of LacZ (Figures 4B and 4C). In this experiment, since the extent of myocardial fibrosis was too high to identify some small blood vessels, we examined only relatively larger arteries where intimal thickening was prominent while perivascular fibrosis was less prominent. Thus, compared with the results obtained in the fasudil protocol (Figure 2), the extent of intimal thickening was relatively greater while that of perivascular fibrosis was relatively smaller (Figure 4).

Expressions of Inflammatory Molecules

RNase protection assay demonstrated that the expression of MIF, IFN-γ, and TGF-β1 in the allografts was significantly upregulated in the control group and was dose-dependently inhibited in the fasudil group (Figure 5). Only the expression of MIF was inhibited by a low dose of fasudil (Figure 5A), which also was effective to suppress the development of CAV (Figure 2A). The expression of TNF-α, MCP-1, VCAM-1, ICAM-1, and E-selectin in the allografts also was upregulated in the control group but was not significantly suppressed in the fasudil group (data not shown). IL-6 was not detected in any group examined.

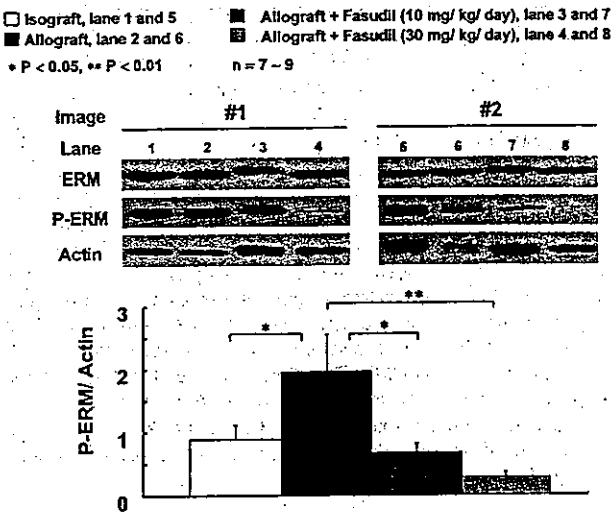

Figure 3. Representative images and quantified analysis of Western blotting for phosphorylated ERM, a marker of Rho-kinase activity, total ERM, and actin, in cardiac grafts at 4 weeks after cardiac transplantation. The Rho-kinase activity was increased in the control allograft group, which was dose-dependently suppressed by the fasudil treatment.

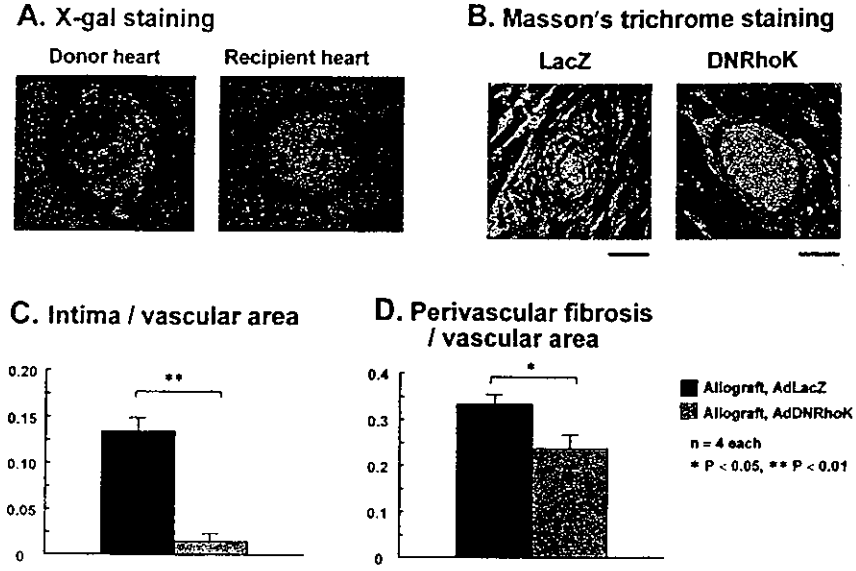

Figure 4. Inhibitory effects of in vivo gene transfer of DN-Rho-kinase on CAV in mice. DN-Rho-kinase and LacZ were transfected into allografts immediately after transplantation: A, X-gal staining performed 1 week after the transfection confirmed the expression of LacZ. B, Representative photomicrographs of a coronary artery in the allograft groups transfected with LacZ or DN-Rho-kinase. The intimal thickening (as expressed by the intima/vascular area ratio) (C) and perivascular fibrosis/vascular area ratio (D) were suppressed by in vivo gene transfer of DN-Rho-kinase. Bars=100 μm.

Inflammatory Cell Infiltration

Immunostaining demonstrated that a number of infiltrating macrophages (MOMA-2) and CD4- or CD8-positive T cells and their percent-positive area were minimal in the isografts (Figures 6 and 7). Both of them were significantly enhanced in the allograft group compared with the isograft group, which was significantly suppressed with the fasudil treatment (Figures 6 and 7).

Discussion

This study has revealed three novel findings as follows. First, the long-term treatment with fasudil dose-dependently suppressed the development of CAV, the effect of which was associated with the decrease in the Rho-kinase activity. Second, the beneficial effect of hydroxyfasudil was qualitatively mimicked by the in vivo gene transfer of DN-Rho-kinase. Third, the expression of several cytokines was upregulated in the allografts and was suppressed by the fasudil treatment, with the significant inhibitory effect noted for MIF, IFN-γ, and TGF-β1. These results suggest that Rho-kinase is substantially involved in the pathogenesis of CAV, implicating a potential usefulness of Rho-kinase inhibitors to prevent the disorder.

An effective strategy to suppress CAV has yet to be developed. In previous studies, angiotensin-converting enzyme inhibitor, angiotensin II receptor antagonist, and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors were shown to cause a 30% to 45% inhibition of the disorder.[20–22] The inhibitory effect of hydroxyfasudil (≈90%), at safe doses,[23] is more prominent than that of any of those drugs tested before, such as CGP53716, an inhibitor of the platelet-derived growth factor tyrosine kinase.[24] In the present study, the plasma level of hydroxyfasudil was 37.24±19.12 ng/mL (0.11±0.06 μmol/L), which is within its clinical therapeutic level,[12,14] suggesting that the oral treatment with fasudil is safe for both mice and humans. Thus, Rho-kinase could be regarded as an important molecular target for the prevention of CAV.

Mouse Model of CAV

Although several studies were performed to elucidate the mechanisms of CAV, the pathogenesis of the disorder still remains unclear. Recently, a novel murine model of long-

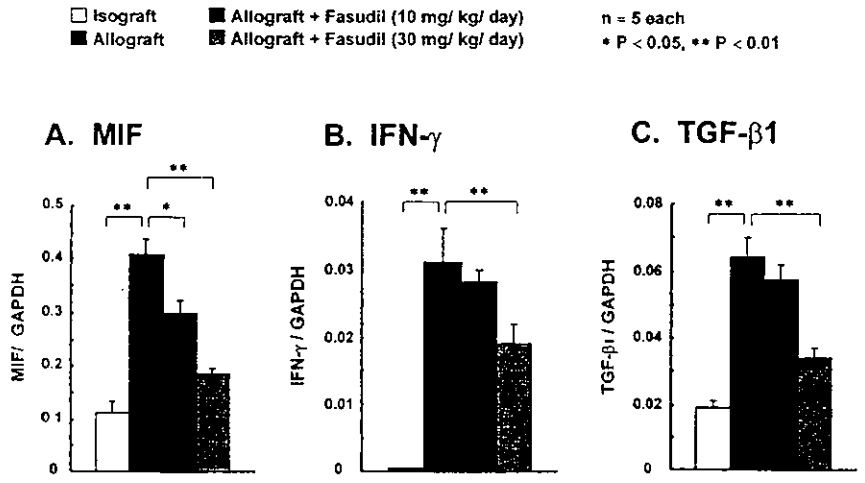

Figure 5. Inhibitory effects of the fasudil treatment on mRNA expression for MIF (A), IFN-γ (B), and TGF-β1 (C) in cardiac allograft at 2 weeks after the transplantation (RNase protection assay). In the control allograft group, the mRNA expressions for the 3 cytokines were all increased and were dose-dependently suppressed by the fasudil treatment.

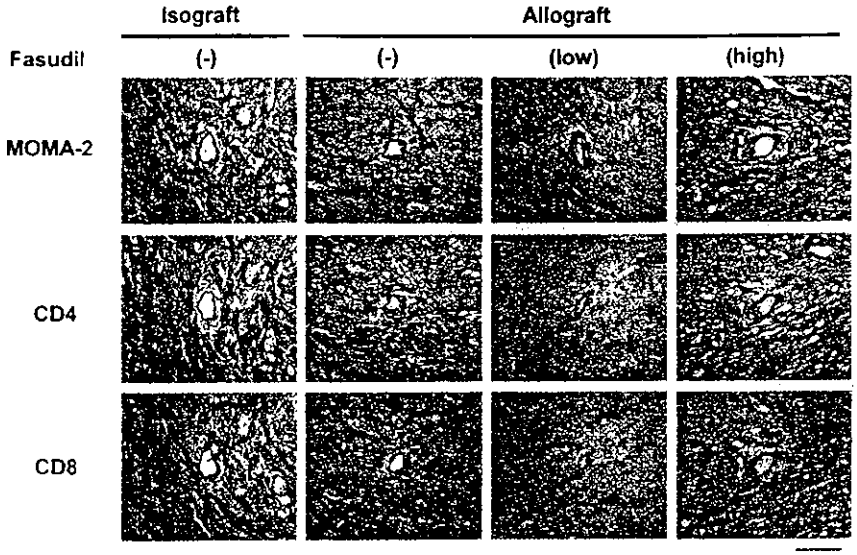

Figure 6. Representative photomicrographs of the mouse heart from the isograft group (A, E, and I) and from the allograft groups without (B, F, and J) and with a low dose (C, G, and K) and a high dose (D, H, and L) of fasudil at 4 weeks after the cardiac transplantation (×200). The accumulation of macrophages (MOMA-2) and CD4- and CD8-positive T cells, which are shown by arrows, was dose-dependently suppressed by the fasudil treatment. Bar=100 μm.

term CAV was developed using an H-2 identical combination, AKR ($H\text{-}2^k$) to C3H ($H\text{-}2^k$).[11,25] In this combination, because of a mismatch of minor antigens, the process of CAV is initiated as early as 2 weeks after grafting and is further developed at 4 weeks.[25] Since the coronary vascular lesions in this model have many similarities to those in humans,[25,26] the model has been used to examine the pathogenesis of CAV.[27,28]

Inhibitory Effects of Hydroxyfasudil on CAV

In the present study, intimal thickening of coronary arteries was suppressed by the long-term treatment with fasudil. To confirm the inhibitory effect of hydroxyfasudil on Rho-kinase, we also examined the effects of in vivo gene transfer of dominant-negative Rho-kinase. Compared with the results obtained in the drug protocol (Figure 2), the extent of intimal thickening was relatively greater while that of perivascular fibrosis was relatively smaller (Figure 4). This observation traces its source to the use of adenovirus as a vector. Since the extent of myocardial fibrosis was too high to identify some small blood vessels, we examined only relatively larger arteries where intimal thickening was prominent while perivascular fibrosis was less prominent.

In the present study, perivascular fibrosis in the allografts was enhanced in the control group compared with the isograft group and was markedly inhibited by long-term hydroxyfasudil. The extent of perivascular fibrosis in the high-dose fasudil group was equivalent to that in native hearts, a finding consistent with our previous study.[29] Since perivascular fibrosis in cardiac allografts is caused by both immune response and a reperfusion injury (as seen in isografts), hydroxyfasudil appears to inhibit both processes.

Regarding the medial thickening of coronary arteries, the value in the high-dose fasudil group was equal to that in native hearts. In the present study, a high dose of fasudil did not cause medial changes, which is in contrast to the previous report.[30] The discrepancy is probably due to some differences in experimental conditions between the present and the previous study. First, we examined mouse coronary arteries whereas rabbit carotid arteries were examined in the previous study. Second, we used a transplant model whereas a balloon injury model was used in the previous study.

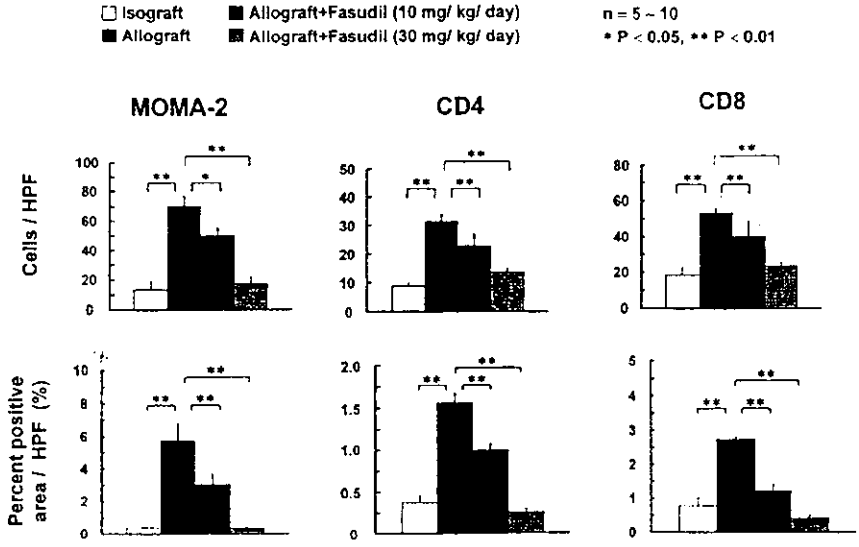

Figure 7. Quantitative analysis of the inhibitory effects of fasudil on the inflammatory cell accumulation in a cardiac allograft at 4 weeks after the transplantation in mice. The long-term treatment with hydroxyfasudil dose-dependently inhibited the accumulation of macrophages (MOMA-2) and CD4- and CD8-positive T cells. Top and bottom panels represent a number of those cells per high-power field (HPF) and a percent-positive area by those cells per HPF, respectively.

Mechanisms of Action of Hydroxyfasudil

Several mechanisms could be involved in the inhibitory effects of hydroxyfasudil. First, hydroxyfasudil could facilitate apoptotic cell death in the neointima as does Y-27632, another specific Rho-kinase inhibitor.[30,31] In addition, cytoplasmic translocation of ERM may be involved in an early phase of apoptosis.[32] Second, hydroxyfasudil could inhibit cell migration.[17,30,33] Third, hydroxyfasudil could suppress VSMC proliferation.[6]

Rho-kinase plays an important role in the pathogenesis of arteriosclerosis in vivo.[6] We have recently demonstrated that Rho-kinase–mediated phosphorylation of ERM and adducin is increased at arteriosclerotic coronary lesions in pigs and that long-term blockade of Rho-kinase by either hydroxyfasudil[34] or in vivo gene transfer of DN-Rho-kinase[15] induces a regression of the coronary lesions in vivo. Regarding the measurement of Rho-kinase activity in our study, we consider that the activity is significantly increased in our mouse model of CAV for the following reasons. First, we consider that a 2-fold increase in Rho-kinase activity is significant because this measurement only represents the whole level of Rho-kinase activity of the heart, and some population of vascular wall cells (eg, VSMCs and inflammatory cells) may have much higher activity of Rho-kinase. Second, the increased activity of Rho-kinase in CAV was normalized not only by pharmacological blockade of Rho-kinase with hydroxyfasudil but also by in vivo gene transfer of DN-Rho-kinase. Third, as shown in Figure 3, the total ERM level was unchanged in the present study.

Antiinflammatory Effects of Hydroxyfasudil

In the present study, hydroxyfasudil dose-dependently suppressed upregulated MIF, IFN-γ, and TGF-β1 expression in allografts (Figure 5). We consider that hydroxyfasudil inhibits the inflammatory responses mediated by those cytokines and resultant formation of CAV. We have no definite explanations why some cytokines were selectively upregulated in our CAV model in a Rho-kinase–dependent manner. One possible explanation is that the contribution of Rho-kinase to cytokine expression may be variable depending on the condition of CAV and/or animals used. It has been reported that Rho-kinase regulates gene expression of plasminogen activator inhibitor-1 (PAI-1) but not extracellular signal–regulated protein kinase.[35]

MIF may be involved in the pathogenesis of graft rejection[36] and atherosclerosis.[37] Although we did not examine the molecular mechanism for the connection between Rho-kinase and MIF in this study, we have recently demonstrated that Rho-kinase is substantially involved in the upregulation of inflammatory molecules, such as PAI-1[35] and the downregulation of eNOS.[10] It has been reported that MIF upregulates the expression of ICAM-1 on endothelial cells while it decreases redox- or stress-induced apoptosis.[37] It is important to note that a low dose of fasudil, which suppressed the development of CAV, inhibited the expression of MIF alone. Thus, it is conceivable that MIF plays an important role in the pathogenesis of CAV in the present model. IFN-γ also may be involved in the progression of CAV. The development of CAV is suppressed in the grafts from IFN-γ–deficient mice, suggesting an involvement of the cytokine in the pathogenesis of the disorder.[38,39] TGF-β1 is known to increase fibronectin and type 1 collagen expression by fibroblasts.[40] In addition, an increased expression of fibronectin and laminin in the early posttransplantation period precedes cellular infiltration.[41,42] Thus, it is also conceivable that Rho-kinase–mediated upregulation of TGF-β1 is involved in the pathogenesis of CAV. In the present study, the expression of TNF-α, MCP-1, VCAM-1, ICAM-1, and E-selectin in the allografts was also upregulated in the control group but was not significantly suppressed in the fasudil group. This may suggest that hydroxyfasudil does not directly suppress CAV but rather downregulates inflammation across the whole graft, including the vasculature.

Limitations of the Study

Several limitations of the present study should be mentioned. First, the present model may not completely represent clinical cardiac transplantation partly because heterotopic cardiac transplantation was performed in this study and partly because only minor tissue mismatches are carried in the allografts. However, as discussed above, the present model is useful for examining the mechanisms of CAV.[27,28] Second, the whole heart was used for molecular analyses since it is difficult to isolate a sufficient amount of coronary arteries from the mouse heart. This means that the relevant findings may not specifically relate to the pathogenesis of CAV. Third, the potential effects of immunosuppressive agents on the development of CAV were not examined in the present study.

In summary, the present study demonstrates that hydroxyfasudil, a metabolite of fasudil, may act on Rho-kinase and possibly may have other antiinflammatory properties. The suppression of CAV by hydroxyfasudil in mice suggests that Rho-kinase is an important therapeutic target for the prevention of the disorder.

Acknowledgments

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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

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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-

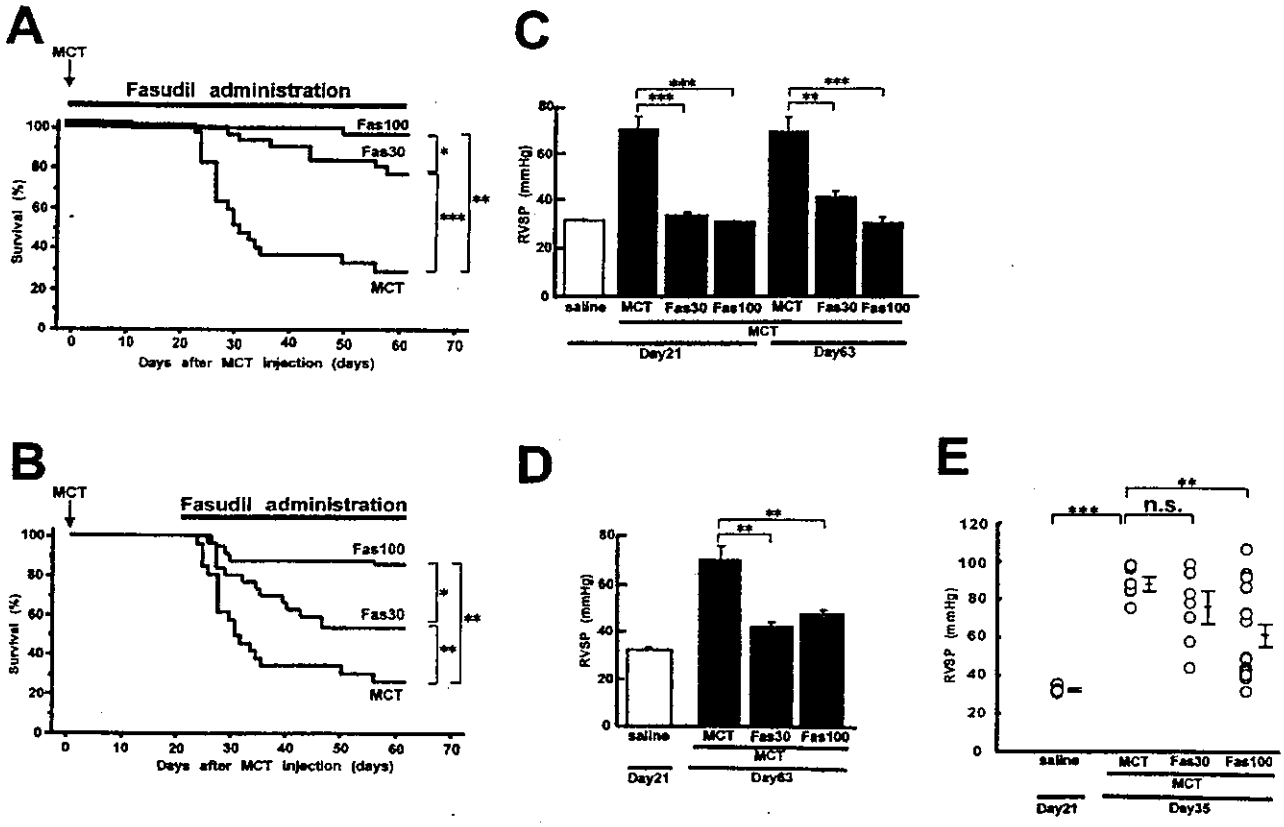

Figure 1. Fasudil improves survival of rats with MCT-induced PH. Compared with the saline-treated normal group (saline), in both the prevention (top) and treatment (bottom) protocols, the fasudil treatment markedly improved the survival (A and B) and RV systolic pressure (C and D). At day 35 (14 days 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

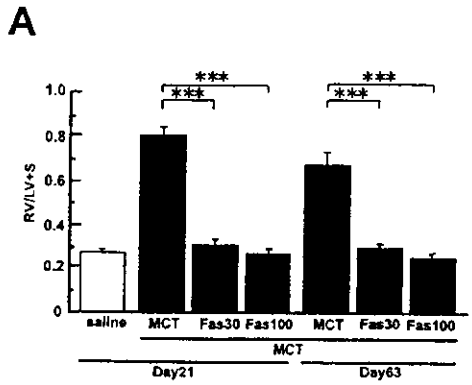

Figure 2. Fasudil improves RV hypertrophy in rats with MCT-induced PH. Compared with the saline-treated normal group (saline), in both the prevention (A) and treatment (B) protocols, the fasudil treatment markedly improved MCT-induced RV hypertrophy. Fas 30 and 100; fasudil 30 and 100 mg/kg per day,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

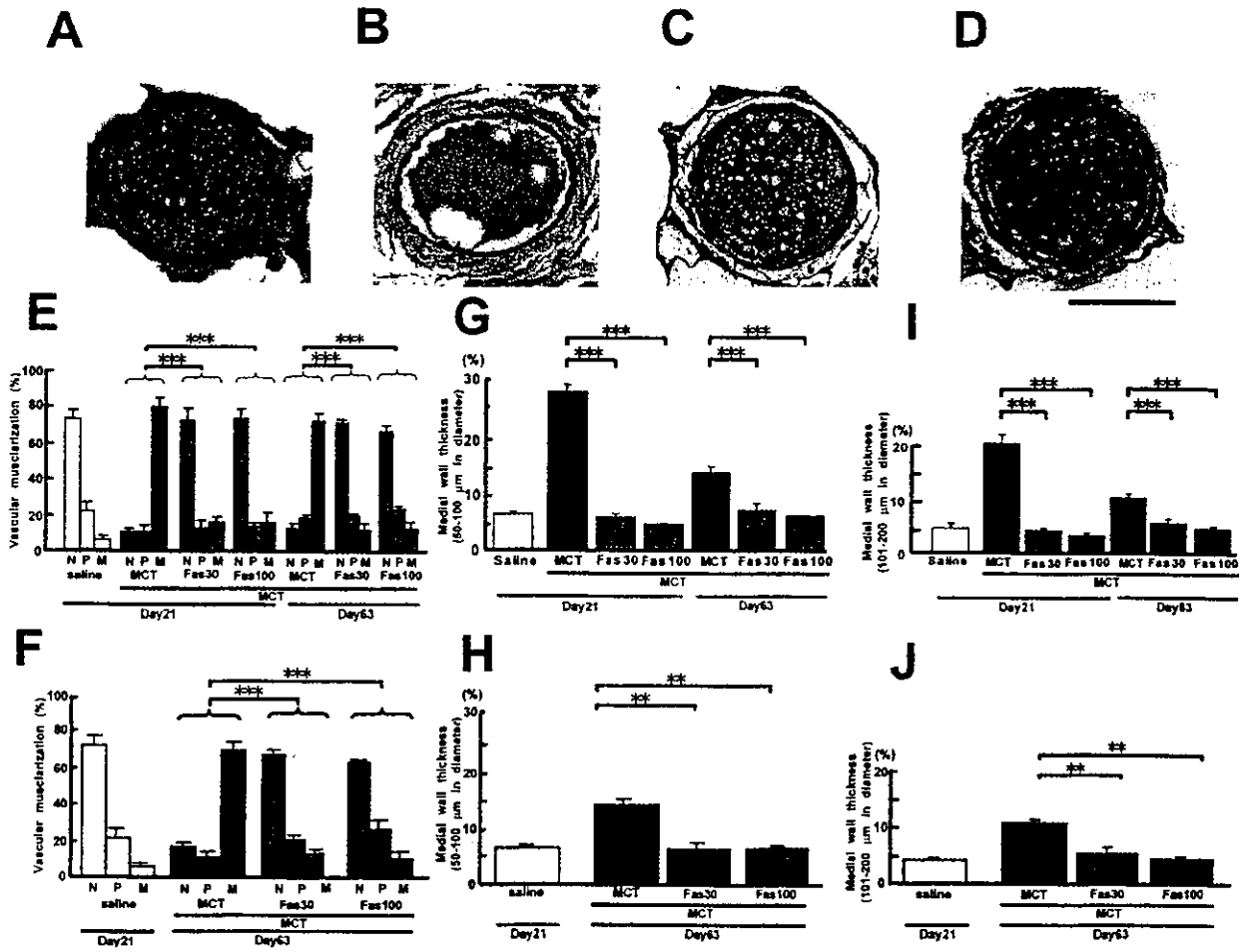

Figure 3. Fasudil suppresses medial thickening in rats with MCT-induced PH. Compared with the saline-treated normal group (A), medial wall thickening of the pulmonary artery was noted in the MCT group (B), whereas fasudil prevented (C) or markedly improved the medial thickening (D). Bar=50 μm. In the 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

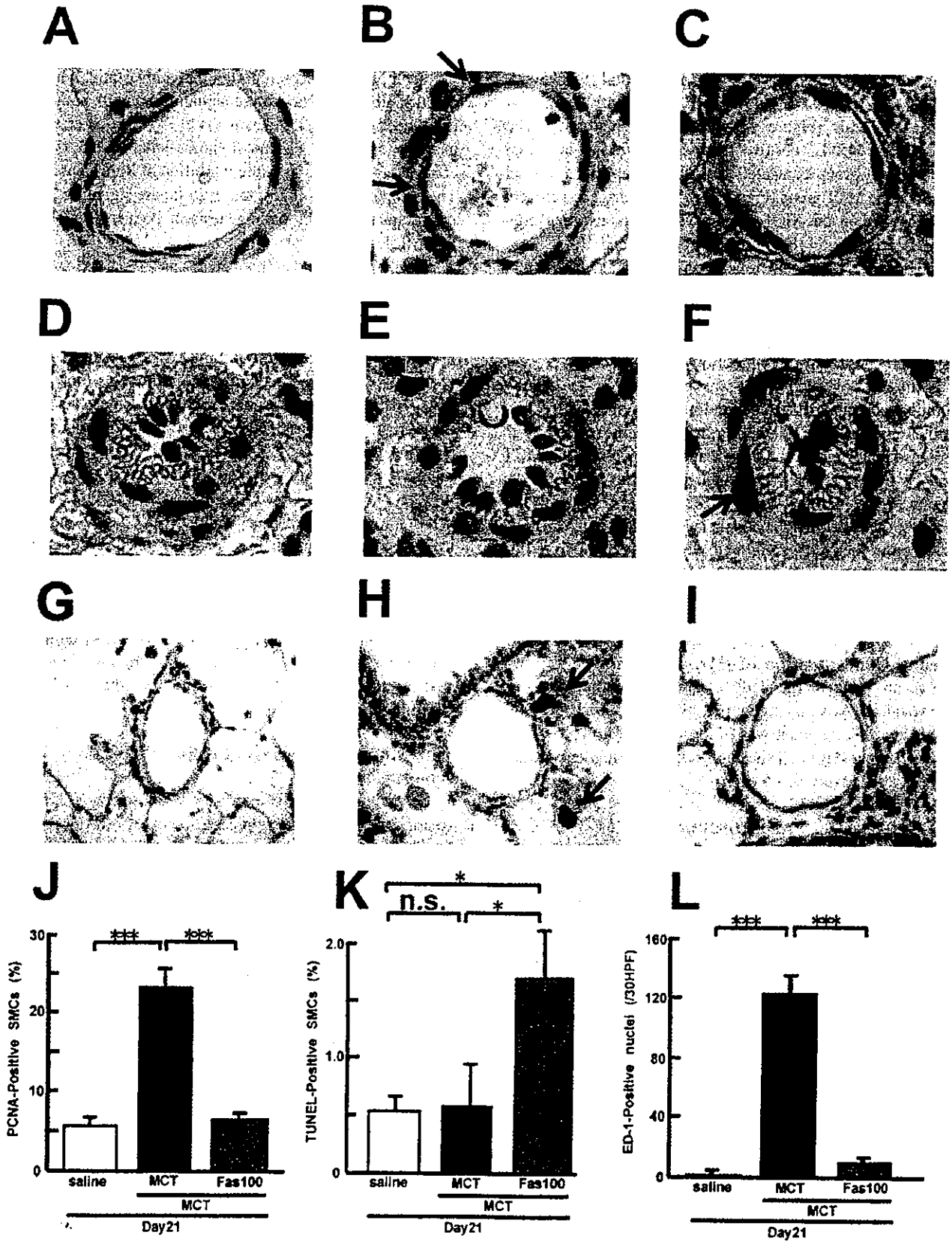

Figure 4. Mechanisms for the beneficial effects of fasudil on MCT-induced pulmonary remodeling. Histology of pulmonary arteries in the saline-treated normal group (A, D, and G), MCT group (B, E, and H), and high-dose fasudil group (C, F, and I) at day 21 in the prevention protocol. MCT-induced increase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

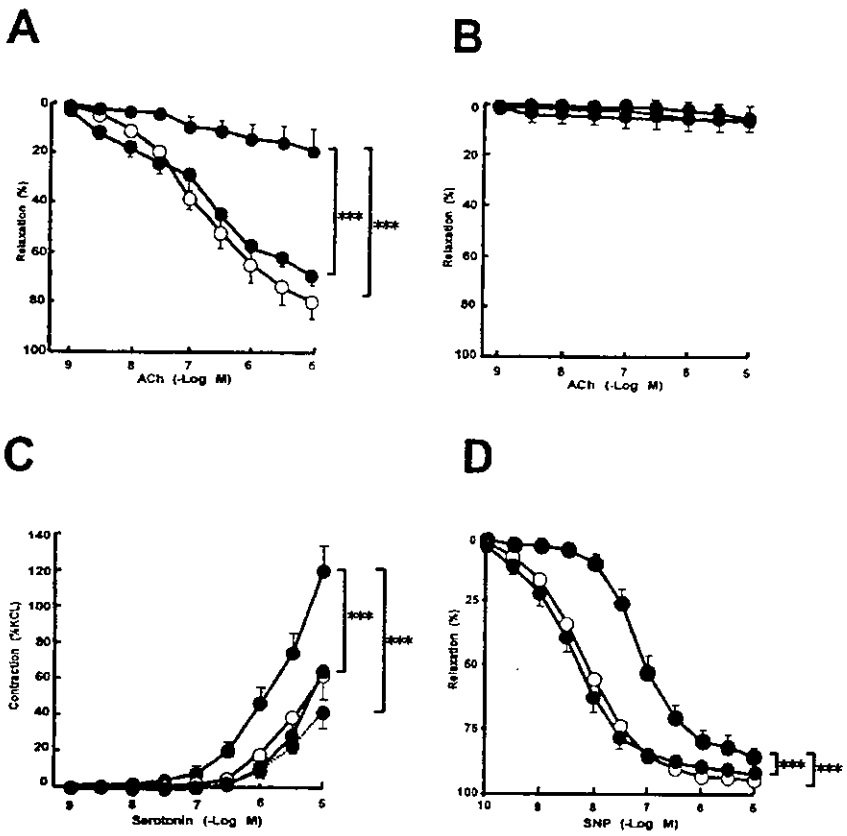

Figure 5. Fasudil improves endothelial and VSMC functions in rats with MCT-induced PH. A, MCT-induced endothelial dysfunction was markedly improved by the fasudil treatment at day 21 in the prevention protocol. B, Beneficial effect of fasudil was abolished by L-NNA (10^{-5} mol/L). C, Fasudil treatment 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,

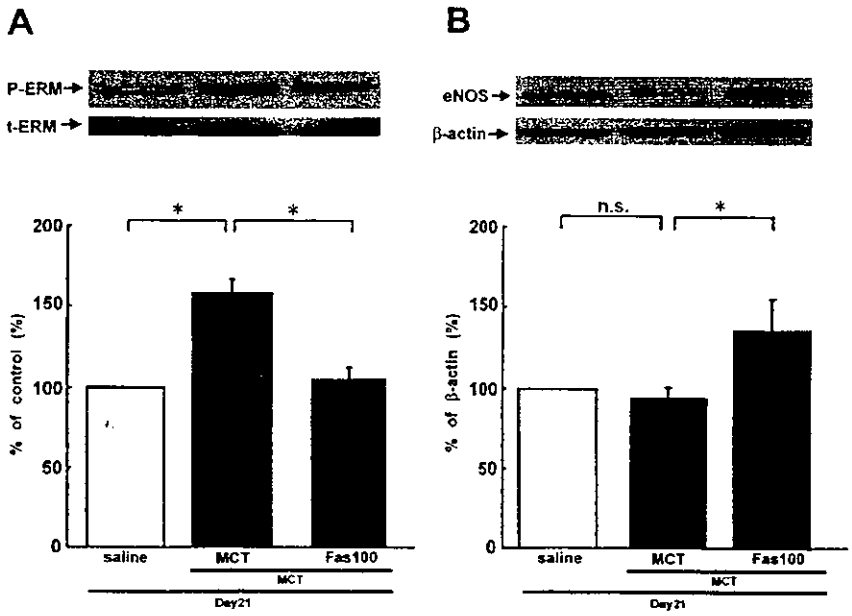

Figure 6. Effects of long-term treatment with fasudil on Rho-kinase activity eNOS expression in rats with MCT-induced PH. A, Compared with the saline-treated normal group, Rho-kinase activity, as evaluated by the extent of phosphorylation of the ERM family of pulmonary arteries, was significantly increased 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.

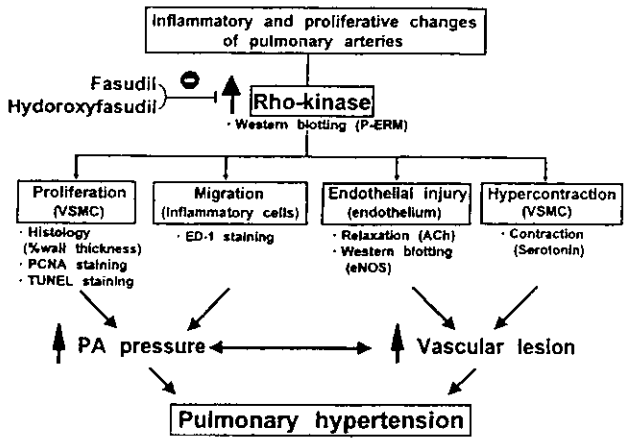

Figure 7. Summary of the present study. Multiple mechanisms appear to be involved in the pathogenesis of PH, all of which may be substantially mediated by Rho-kinase. Thus, the long-term blockade of Rho-kinase with fasudil or other Rho-kinase inhibitors may be useful for the treatment of PH.

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 Whittaker. 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-

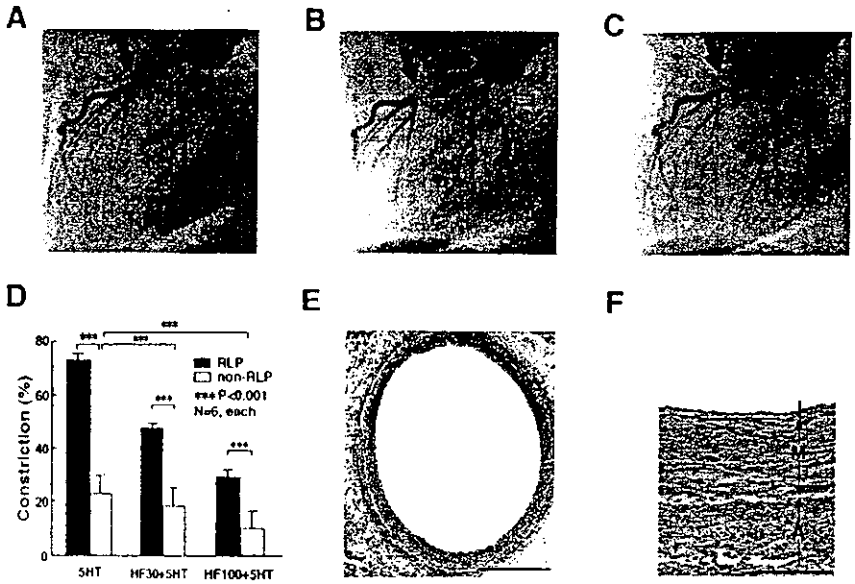

Figure 1. RLP from patients with SCD markedly enhance coronary vasospastic activity in pigs. A to C, Coronary angiograms before (A) and after intracoronary serotonin without (B) and with hydroxyfasudil (C). Black arrows indicate RLP site; white arrows, non-RLP site. D, Inhibitory effect of hydroxyfasudil 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,

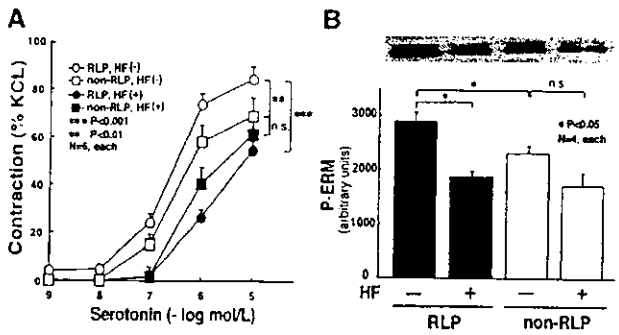

Figure 2. RLP enhance VSMC contractions and Rho-kinase activity. A, VSMC contractions to serotonin were significantly augmented at the RLP site (○) compared with the non-RLP site (□). Hydroxyfasudil significantly inhibited the VSMC hypercontractions only at RLP-site (●). B, The extent of the ERM phosphorylation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

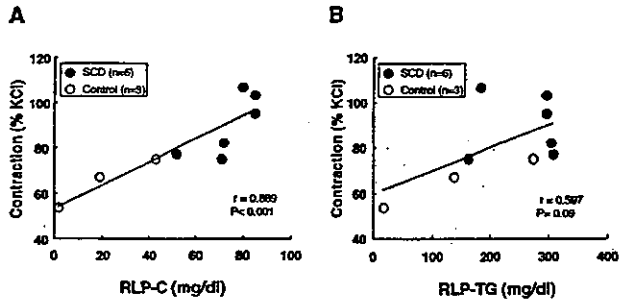

Figure 3. Correlation between RLP-C or RLP-TG level and that of serotonin-induced contractions (percent of contraction to 118 mmol/L KCl).

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.

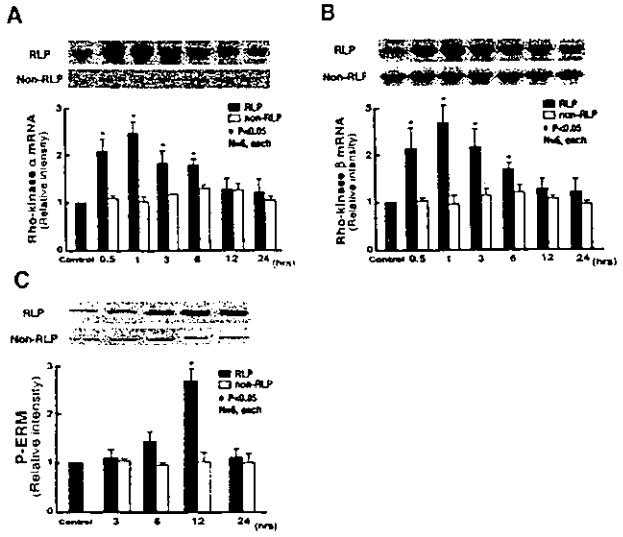

Figure 4. RLP enhance Rho-kinase expression and activation in human coronary VSMC. A to C, The treatment with RLP but not that with non-RLP significantly enhanced the mRNA expression of Rho-kinase α (ROCK2) (A) and Rho-kinase β (ROCK1) (B) (Northern blot). The blots are representative of 6 separate experiments. 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.

Acknowledgments

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