Long-Term Inhibition of Rho-Kinase Suppresses Left Ventricular Remodeling After Myocardial Infarction in Mice

Tsuyoshi Hattori, BS; Hiroaki Shimokawa, MD, PhD; Midoriko Higashi, MD; Junko Hiroki, MD; Yasushi Mukai, MD; Hiroyuki Tsutsui, MD, PhD; Kozo Kaibuchi, MD, PhD; Akira Takeshita, MD, PhD

Background—Rho-kinase has been implicated as an important regulator of inflammatory responses mediated by cytokines and chemokines. Because proinflammatory cytokines play a critical role in left ventricular (LV) remodeling after myocardial infarction (MI), we examined whether long-term blockade of Rho-kinase suppresses LV remodeling in a mouse model of MI in vivo.

Methods and Results—Mice underwent ligation of the left coronary artery and were treated with a Rho-kinase inhibitor, fasudil (100 mg·kg⁻¹·d⁻¹ in tap water), for 4 weeks, starting 1 day after the surgery. At 4 weeks, LV infarct size was histologically comparable between the 2 groups. LV cavity dilatation and dysfunction evaluated by echocardiography were significantly suppressed in the fasudil group (P<0.05, n=15 to 28). The beneficial effects of fasudil were accompanied by suppression of cardiomyocyte hypertrophy and interstitial fibrosis (both P<0.01, n=6). The expression of inflammatory cytokines, including transforming growth factor (TGF)-β, TGF-β, and macrophage migration inhibitory factor, was upregulated in the noninfarcted LV in the control group and was significantly suppressed in the fasudil group (both P<0.05, n=10 to 11). Rho-kinase activity as evaluated by the extent of phosphorylation of the ERM family, a substrate of Rho-kinase, was significantly increased in the noninfarcted LV in the control group and was significantly suppressed in the fasudil group (P<0.05, n=5).

Conclusions—These results indicate that Rho-kinase is substantially involved in the pathogenesis of LV remodeling after MI associated with upregulation of proinflammatory cytokines, suggesting a therapeutic importance of the molecule for the prevention of post-MI heart failure. (Circulation. 2004;109:2234-2239.)

Key Words: myocardial infarction | heart failure | myocardium | remodeling | signal transduction

Myocardial infarction (MI) frequently causes left ventricular (LV) dilatation associated with cardiomyocyte hypertrophy and interstitial fibrosis in the noninfarcted myocardium. These changes in LV geometry, referred to as LV remodeling, are involved in the development of heart failure. ACE inhibitors have been shown to attenuate LV remodeling and improve the quality of life and the mortality and morbidity of patients with MI and heart failure. Although a number of clinical trials have demonstrated the effectiveness of ACE inhibitors in heart failure, they may not sufficiently antagonize the disease progression. Accordingly, it is important to develop a novel therapeutic strategy that effectively suppresses the development and progression of LV remodeling and failure after MI.

Rho-kinase, an effector of the small GTPase Rho, plays an important role in adhesion, migration, proliferation, and cytokinesis of vascular smooth muscle cells. Our previous studies demonstrated that Rho-kinase is substantially involved in the pathogenesis of vascular remodeling in vivo. Recent studies demonstrated that RhoA was also involved in the pathogenesis of cardiac hypertrophy. Thus, the present study was designed to examine whether Rho-kinase is involved in the pathogenesis of LV remodeling and dysfunction after MI in a mouse model in vivo.

Methods

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

Animal Preparation and Drug Administration

A total of 114 male CD-1 mice (age, 5 to 8 weeks; Kyudo Co Ltd, Saga, Japan) were used for the present study. MI was induced by ligating the left coronary artery under anesthesia with pentobarbital (50 mg/kg IP) (n=88). Sham operation was performed in the same manner but without coronary ligation (n=26). Animals with MI were
randomly divided into 2 groups with (n=41) or without (n=47) long-term oral treatment with fasudil, a Rho-kinase inhibitor (100 mg·kg⁻¹·d⁻¹ in the drinking water) for 4 weeks. The treatment with fasudil was started 1 day after the coronary ligation. To adjust the daily intake of fasudil, we measured the water intake and body weight on a daily basis. We have previously confirmed that fasudil is metabolized to hydroxyfasudil, an active major metabolite of fasudil and a specific inhibitor of Rho-kinase, after oral administration. Plasma concentrations of fasudil and hydroxyfasudil were measured by high-performance liquid chromatography at 4 weeks after the operation.17

**Echocardiographic and Hemodynamic Evaluation**

Four weeks after the coronary ligation, an echocardiographic study was performed with 12-MHz echocardiography (model Prosound SSD-5500, Aloka) under light anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% wt/vol, 8 µL/g IP) and spontaneous respiration as described previously. A 1.4F micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery and then advanced into the LV to measure LV pressures. We did not measure brain natriuretic peptide levels in the present study.

**Histological Analysis**

Histological analysis was performed as described previously with a partial modification. After 4 weeks, the heart was harvested and dissected into the right ventricle (RV) and the LV with the septum. The LV was cut into 3 transverse sections: apex, middle, and base sections before embedding in paraffin. From each section, 5-µm slices were cut and stained with Masson’s trichrome. Infarct size was calculated as a total infarct circumference divided by total LV circumference. Cardiomyocyte cross-sectional area and myocardial fibrosis were measured in a blind manner at a magnification of ×200 and ×400 (BX50F-3, Olympus Optical Co), respectively, using NIH Image software.

**Western Blot Analysis**

The extent of phosphorylation of the ERM (ezrin, radixin, and moesin) family, a substrate of Rho-kinase, was measured by Western blot analysis to determine the Rho-kinase activity in the noninfarcted LV in vivo as described previously. The amount of the ERM phosphorylation was normalized to that of total ERM. We used an antibody to phosphorylated ERM and one to total ERM that we developed ourselves.

**Ribonuclease Protection Assay**

The noninfarcted LV was harvested and was quickly frozen in liquid nitrogen. Total RNA was extracted from the specimens according to the manufacturer’s protocol and quantified by spectrophotometry. The RNase protection assay was performed on 10 to 20 µg of RNA per sample with a multiprobe RNase protection assay system (RiboQuant, PharMingen) for proinflammatory cytokines, including transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Protected mRNA samples with the 32P isotope were electrophoresed on 5% acrylamide gel and visualized by scientific imaging film (Kodak Inc). Areas of the respective transcript bands were measured and were normalized by the radioactivity of GAPDH mRNA.

**Statistical Analysis**

All results are expressed as the mean±SEM. Data were analyzed by 1-way ANOVA followed by Fisher’s post hoc test for multiple comparisons. Values of P<0.05 were considered to be statistically significant.

**Results**

**Mortality**

The survival rate of MI mice at 4 weeks was comparable between the control MI (77%) and the fasudil-treated MI (78%) groups. Postmortem analysis suggested that the cause of death was heart failure and/or arrhythmia. Two mice died of LV rupture in each group. No adverse effects of fasudil, such as weight loss (Table) or diarrhea (data not shown), were noted.

**Echocardiography and Hemodynamics**

LV chamber sizes, including LV end-diastolic and end-systolic dimensions, and LV posterior wall thickness (in the noninfarcted area) were significantly increased in the MI group compared with the sham-operated group (Table, Figure 1). Furthermore, LV anterior wall thickness (in the infarcted area) and LV fractional shortening were significantly reduced in the MI group compared with the sham-operated group (Table, Figure 1). Importantly, these LV remodelings and dysfunctions were all significantly suppressed in the fasudil-treated MI group (Table, Figure 1). Moreover, LV anterior wall thickness (in the infarcted area) and LV fractional shortening were significantly reduced in the MI group compared with the sham-operated group (Table, Figure 1). Importantly, these LV remodelings and dysfunctions were all significantly suppressed in the fasudil-treated MI group (Table). Mean aortic blood pressure and heart rate were comparable among the 3 groups (Table). LV end-diastolic pressure was increased in the MI group but was significantly suppressed in the fasudil-treated MI group (Table).

**Organ Weights and Histology**

The ratio of RV weight to body weight and that of lung weight to body weight were significantly increased in the MI group compared with the sham-operated group, both of which were significantly inhibited in the fasudil-treated MI group (Table). By contrast, the ratio of LV weight to body weight was comparable among the 3 groups (Table).
LV infarct size (% of LV) was comparable between the MI (58±3%) and the fasudil-treated MI (55±4%) groups at 4 weeks after coronary ligation. Cardiomyocyte cross-sectional area in the noninfarcted LV was significantly increased in the MI group compared with the sham-operated group and was again significantly attenuated in the fasudil-treated MI group (Figure 2). Interstitial fibrosis of noninfarcted LV was also significantly increased in the MI group compared with the sham-operated group and was significantly inhibited in the fasudil-treated MI group (Figure 3).

**Rho-Kinase Activity**

The extent of ERM phosphorylation in the noninfarcted LV as normalized by that of total ERM was significantly increased in the MI group compared with the sham-operated group (Figure 4). This Rho-kinase activation was significantly suppressed in the fasudil-treated MI group (Figure 4).

**Expression of Proinflammatory Cytokines**

RNase protection assay demonstrated that the expression of TGF-β1 to -β3, MIF, and IL-6 was significantly enhanced in the MI group compared with the sham-operated group (Figure 5). The upregulation of TGF-β2 and -β3 and MIF was significantly inhibited in the fasudil-treated MI group, whereas that of TGF-β1 and IL-6 tended to be suppressed in the same group (Figure 5). By contrast, the expression of TNF-α was not enhanced in the MI group compared with the sham-operated group, and that of IFN-γ was not detected in any groups (data not shown).

**Plasma Concentration of Fasudil/Hydroxyfasudil**

At 4 weeks after the oral treatment with fasudil, plasma concentration of hydroxyfasudil (μmol/L) increased from 0 (sham-operated group) to 0.37±0.12 in the fasudil-treated MI group (n=6), which is within the specific therapeutic ranges of the Rho-kinase inhibitor.20,22 By contrast, fasudil was not detected in any groups.

**Discussion**

The novel findings of the present study are that (1) Rho-kinase is upregulated in a mouse model of LV remodeling...
after MI, (2) the long-term blockade of Rho-kinase with fasudil significantly suppresses the development of LV remodeling and dysfunction after MI, and (3) the suppression of the expression of proinflammatory cytokines may be involved in the beneficial effects of fasudil. To the best of our knowledge, this is the first study that demonstrates the potential therapeutic importance of Rho-kinase for the prevention of LV remodeling and dysfunction after MI.

**LV Remodeling and Rho-Kinase**

In the present study, increased cardiomyocyte cross-sectional area in the control group was significantly attenuated in the fasudil group, which is consistent with the results of echocardiography. The fasudil treatment suppressed the increases in LV end-diastolic pressure, the ratio of RV weight to body weight, and the ratio of lung weight to body weight. The results with Western blotting also demonstrate that Rho-kinase activation is involved in the pathogenesis of LV remodeling. Thus, Rho-kinase signaling seems to be involved in the pathogenesis of LV remodeling and dysfunction after MI in the present model.

With regard to the downstream target of Rho-kinase, myosin light chain could be important in the pathogenesis of LV remodeling. We have previously demonstrated that fasudil/hydroxyfasudil suppresses myosin light chain phosphorylation. Mutation of the cardiac myosin regulatory light chain near the phosphorylation site causes significant cardiac hypertrophy in humans. These results suggest that Rho-kinase activation is involved in the pathogenesis of LV remodeling.

**LV Remodeling and Cytokines**

In the present study, the overexpression of TGF-β mRNA was significantly suppressed by the fasudil treatment. The
expression of TGF-β is increased during the post-MI period for several weeks.26 Because TGF-β expression is noted in most embryonic tissues, this cytokine seems to play an important role for the development of the heart in the fetus.27 Therefore, TGF-β2 may be involved in the induction of the fetal gene program in the myocardium, such as cardiac hypertrophy.26 By contrast, TGF-β3 has been shown to associate with collagen production26,28 and post-MI cardiomyocyte hypertrophy, because it is overexpressed in the heart after MI.28 The present results suggest that Rho-kinase signaling is involved in the expression of TGF-β23 in the post-MI LV remodeling.

Although the role of MIF in the myocardium remains to be elucidated, it has been demonstrated that MIF is expressed in the myocardium in response to redox stress, such as hypoxia and hydrogen peroxide.29 This suggests that MIF may play an important role in the pathogenesis of LV remodeling and dysfunction after MI.29 In the present study, the expression of MIF was enhanced in the noninfarcted LV and was inhibited by the fasudil treatment, which suggests its involvement in the pathogenesis of LV remodeling.

Possible Mechanisms of the Inhibitory Effects of Fasudil on LV Remodeling

Fasudil exerts various beneficial effects through inhibition of Rho-kinase both in vitro and in vivo.5,8 The plasma concentration of hydroxyfasudil noted in the present study is comparable to its effective concentration in humans.17 The mechanisms of the beneficial effects of Rho-kinase inhibitors on post-MI LV remodeling may be multiple. First, Rho-kinase inhibitors could inhibit the intracellular signal transduction of various vasoactive substances that are thought to be involved in the pathogenesis of LV remodeling, including angiotensin II, endothelin-1, thrombin, serotonin, norepinephrine, and platelet-derived growth factors.5,22 Second, Rho-kinase inhibitors could suppress the expression of TGF-β23 and MIF, in addition to that of proatherogenic molecules such as monocyte chemoattractant protein-1 and plasminogen activator inhibitor-1.5,30–32 Oral administration of fasudil suppresses cardiovascular lesion formation through suppression of the expression of several cytokines, including MIF, TGF-β2, IFN-γ, monocyte chemoattractant protein-1, and angiotensin II.20,22,32 These findings, together with the present results, suggest that fasudil could downregulate those inflammatory cytokines, resulting in the suppression of LV remodeling. Third, we were recently able to demonstrate that Rho-kinase inhibitors could ameliorate endothelial dysfunction through upregulation of endothelial NO synthase.33 It has been reported that endothelial NO synthase–derived NO effectively limits LV remodeling after MI in mice.34

Regarding the measurement of Rho-kinase activity, fasudil significantly suppressed the activity in the present study. Because this measurement represents only the whole cardiac activity of Rho-kinase of the LV, excluding the infarct area, it is highly possible that some population of cells (eg, myocytes near the infarcted area and inflammatory cells) have higher activity of Rho-kinase.20

In the present study, there was no significant difference in blood pressure or body weight between the control MI and the fasudil-treated MI groups. Thus, we consider that hemodynamic changes may not contribute directly, if at all, to the beneficial effect of fasudil. However, because we have demonstrated that fasudil improves vascular reactivity of the aorta, coronary artery, and mesenteric artery9,22 and renal dysfunction,35 it is highly possible that fasudil suppressed LV remodeling and dysfunction through these indirect effects.

Limitations of the Study

Several limitations of the present study should be mentioned. First, despite the beneficial effect of fasudil on LV remodeling, overall prognosis was unchanged. Because LV end-diastolic pressure in the MI group was slightly but significantly lower in the present study compared with our previous studies,18,19 the extent of heart failure may be less compared with that in those studies, and the development of heart failure may be slowly progressing. Thus, a longer period of observation would be needed to evaluate the prognostic effect of fasudil in the present model. Second, the beneficial effects of fasudil should be evaluated in comparison with other pharmacological agents that have already been shown to be
effective to prevent post-MI LV remodeling and dysfunction. Third, the beneficial effects of fasudil should be evaluated in large-animal models before its use in humans.

In summary, the present study demonstrates that upregulated Rho-kinase is substantially involved in the pathogenesis of post-MI LV remodeling, suggesting that Rho-kinase is an important molecular target for the prevention of the disorder.

Acknowledgments
This study was supported in part by grants-in-aid 12032215, 12470158, 12877114, 13307024, and 13557068 and a grant for the 21st Century COE Program from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan. We thank Prof S. Mohri at the Center of Biomedical Research, Kyushu University Graduate School of Medical Sciences, for his cooperation and Dr. S. Hayashidani and M. Sonoda for excellent technical assistance. We also thank Asahi Kasei Pharma Corporation, Tokyo, Japan, for providing fasudil.

References
Long-Term Inhibition of Rho-Kinase Suppresses Left Ventricular Remodeling After Myocardial Infarction in Mice

Tsuyoshi Hattori, Hiroaki Shimokawa, Midoriko Higashi, Junko Hiroki, Yasushi Mukai, Hiroyuki Tsutsui, Kozo Kaibuchi and Akira Takeshita

_Circulation._ 2004;109:2234-2239; originally published online April 19, 2004;
doi: 10.1161/01.CIR.0000127939.16111.58

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/18/2234

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/