Rho-Kinase Inhibitor Improves Increased Vascular Resistance and Impaired Vasodilation of the Forearm in Patients With Heart Failure

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Background—Rho-kinase is suggested to have an important role in enhanced vasoconstriction in animal models of heart failure (HF). Patients with HF are characterized by increased vasoconstriction and reduced vasodilator responses to reactive hyperemia and exercise. The aim of the present study was to examine whether Rho-kinase is involved in the peripheral circulation abnormalities of HF in humans with the Rho-kinase inhibitor fasudil.

Methods and Results—Studies were performed in patients with HF (HF group, n=26) and an age-matched control group (n=26). Forearm blood flow was measured with a strain-gauge plethysmograph during intra-arterial infusion of graded doses of fasudil or sodium nitroprusside. Resting forearm vascular resistance (FVR) was significantly higher in the HF group than in the control group. The increase in forearm blood flow evoked by fasudil was significantly greater in the HF group than in the control group. By contrast, FVR evoked by sodium nitroprusside was comparable between the 2 groups. Fasudil significantly augmented the impaired ischemic vasodilation during reactive hyperemia after arterial occlusion of the forearm in the HF group but not in the control group. Fasudil did not augment the increased FVR evoked by phenylephrine in the control group significantly.

Conclusions—These results indicate that Rho-kinase is involved in increased FVR and impaired vasodilation of the forearm in patients with HF. (Circulation. 2005;111:2741-2747.)

Key Words: heart failure ■ blood flow ■ vasoconstriction ■ vasodilation

Increased peripheral vascular resistance and impaired vasodilation of the peripheral vasculature are characteristic in patients with heart failure (HF).1,2 These characteristics cause fatigue and exercise intolerance in patients with HF and are considered to be mainly due to enhanced sympathetic drive and activation of the renin-angiotensin system.1–3 The dysfunction of vasodilator factors such as nitric oxide and atrial natriuretic peptide is also involved.4–10 Ischemia- and exercise-induced vasodilation of the extremities of patients with HF is markedly attenuated, and decreased exercise tolerance in patients with HF is not only due to impaired pump function of the heart but also to inadequate increases in muscle blood flow as a result of impaired vasodilation during exercise.2,5 Increased peripheral resistance and impaired vasodilation are consistent findings in HF.1,2,5–8 The molecular mechanisms underlying impaired vasodilation in patients with HF, however, remain to be elucidated.

Myosin light chain (MLC) phosphorylation is a crucial step for vascular smooth muscle cell (VSMC) contraction, which is regulated in a dual manner by MLC kinase and MLC phosphatase.11 Inhibition of MLC phosphatase results in increased MLC phosphorylation and subsequent VSMC hypercontraction (Ca2+ sensitization).11,12 This mechanism of Ca2+ sensitization in VSMCs is enhanced in animal models of HF.13 Rho-kinase/ROKα/RockII, which is activated by the small GTPase Rho, inhibits MLC phosphatase activity and thus plays a key role in Ca2+ sensitization and hypercontraction of VSMCs.11,12,14 Y-27632, a Rho-kinase inhibitor, preferentially lowers arterial pressure in rat models of hypertension in vivo, which indirectly suggests an involvement of Rho-kinase in hypertension.15 We recently demonstrated that Rho-kinase is upregulated and plays a key role in VSMC contraction in a porcine model of coronary artery spasm16,17 and in spontaneously hypertensive rats18 and that Rho-kinase might be involved in the pathogenesis of increased peripheral vascular resistance in hypertension in humans.19 Previous reports suggest that the Rho/Rho-kinase pathway plays a critical role in Dahl salt-sensitive hypertensive rats with congestive HF20 and might be involved in the enhanced arterial vasoconstriction in tachycardia-induced HF in dogs.13
It is unknown, however, whether this is also the case in human patients with HF.

The present study was designed to determine whether Rho-kinase is involved in the increased peripheral vascular resistance in patients with HF by examining the vasodilator effect of a specific Rho-kinase inhibitor, fasudil. Fasudil is currently used in Japan for the treatment of cerebral vasospasm after subarachnoid hemorrhage. Fasudil is a specific Rho-kinase inhibitor, as is Y-27632, 15,16,22,23 although the latter is not yet approved for human use. We recently used fasudil to examine forearm vascular resistance in patients with hypertension, and there were no complications. Thus, fasudil is regarded as a specific Rho-kinase inhibitor that can be used safely in humans.

Methods

Patients
Twenty-six patients with HF (HF group; 17 men, 9 women; mean age 63 ± 3 years) and 26 control subjects (control group; 15 men, 11 women; mean age 60 ± 6 years) were enrolled in the present study. The HF group comprised 17 patients with ischemic heart disease, 8 patients with dilated cardiomyopathy, and 1 patient with valvular heart disease. All patients with HF were diagnosed according to the Framingham criteria.24 Physical activity was determined on the basis of the New York Heart Association (NYHA) functional class. There were 14 patients with NYHA class 3 and 12 patients with NYHA class 2 in the HF group. Systemic hypertension was defined as systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg. Hyperlipidemia was defined as serum total cholesterol > 220 mg/dL or serum triglyceride levels > 150 mg/dL. Diabetes mellitus was defined as fasting blood sugar > 110 mg/dL. Left ventricular ejection fraction was determined by the modified Simpson method or single-plane area-length method on echocardiogram. We defined the control group as a non-HF/nonhypertensive group. Subjects in the control group were admitted to our hospital because of atypical chest pain, fatigue, or palpitations. Careful examination was performed to rule out coronary artery disease (by coronary angiography) and other organic heart diseases (echocardiogram) or arrhythmia (Holter ECG or ECG monitoring during hospitalization and/or electrophysiological study). In the control group, 5 subjects had serum total cholesterol levels > 220 mg/dL, and 4 had serum triglyceride levels > 150 mg/dL. Serum total cholesterol levels were < 250 mg/dL, and serum triglyceride levels were < 180 mg/dL, even in the HF group. Six subjects had a history of smoking; however, they had quit smoking before admission. This was also true in the HF group. Some subjects in the control group transiently received some medications, such as ACE inhibitors, an angiotensin II (Ang II) receptor blocker, or a β-blocker, from general practitioners because of mild high blood pressure and/or palpitations after our careful interviews. The subjects, however, did not take those medications continuously, and we confirmed that they did not have HF, hypertension, or arrhythmias on admission. In contrast, patients in the HF group were given those medications because of HF. It was ethically difficult to discontinue those medications for the purpose of the study. Therefore, the medications were discontinued only on the day of the study, and restarted just after the study. The present study was approved by the ethics committee for human research in our institute, and written informed consent was obtained from each subject.

General Procedures
The present study was performed with subjects in a supine position and in a postabsorptive state with the room temperature at 25°C to 27°C. All medications were withheld on the day of the study. With the subjects under local anesthesia, the left brachial artery was cannulated with a 20-gauge intravascular cannula for drug infusion, and the cannula was connected to a pressure transducer for direct measurement of arterial pressure. The antecubital vein was cannulated, and blood samples were obtained for measurements of serum or plasma chemistry, including plasma brain natriuretic peptide (BNP), Ang II, and norepinephrine (NE).

Measurement of Forearm Blood Flow
Forearm blood flow (FBF) was measured with a strain-gauge plethysmograph with the venous-occlusion technique, as reported previously. A 19,25–27 FBF (mL·min⁻¹·100 mL⁻¹ of forearm volume) was calculated from the rate of increase in forearm volume while venous return from the forearm was prevented by inflation of a cuff on the upper arm. The pressure in the venous-occlusion or congesting cuff was 40 mm Hg. Circulation to the hand was arrested by inflation of a cuff around the wrist. An average of 4 measurements made at 15-second intervals was used for later analysis. Forearm vascular resistance (FVR) was calculated by dividing mean arterial pressure (diastolic pressure plus one third of pulse pressure in mm Hg) by FBF. FVR was expressed as units.

Study Protocol 1: Effect of Fasudil on FBF and FVR in Patients With HF and in Control Subjects
FBF, arterial pressure, and heart rate were measured at rest and during administration of graded doses of fasudil (3.2, 6.4, 12.8, and 25.6 μg/min) or sodium nitroprusside (SNP; 0.4, 0.8, 1.6, and 3.2 μg/min). Each dose of fasudil was infused for 15 minutes, and FBF was measured during the last minute of each infusion. Each dose of SNP was infused for 5 minutes, and FBF was measured after each infusion. Venous blood samples were drawn from the antecubital vein before and immediately after infusion of the peak dose of fasudil (25.6 μg/min) and at the end of the study for determination of the plasma fasudil concentration.

Study Protocol 2: Effects of Fasudil on FBF and FVR Responses to Reactive Hyperemia in Patients With HF and Control Subjects
We studied 8 patients with HF and 9 control subjects. FBF and FVR were measured as described in study protocol 1. To induce reactive hyperemia, FBF was occluded by inflation of a cuff placed over the left upper arm to a pressure of 200 mm Hg for 5 minutes. After the ischemic cuff occlusion was released, FBF was measured every 15 seconds for 3 minutes. Ten minutes after FBF returned to baseline values, fasudil (25.6 μg/min) was infused for 15 minutes, and the FBF responses to reactive hyperemia were measured by the same procedures described above.

Study Protocol 3: Effects of Fasudil on FBF and FVR During Infusion of Phenytoin in Control Subjects
To exclude the possibility that baseline differences in FBF might affect the results, we examined the FBF responses evoked by fasudil before and after infusion of phenytoin (400 mg/min) in control subjects (n = 5). FBF, arterial pressure, and heart rate were measured at rest and during administration of graded doses of fasudil (3.2, 6.4, 12.8, and 25.6 μg/min) or SNP (0.4, 0.8, 1.6, and 3.2 μg/min) as described in study protocol 1.

Drugs
The following drugs were used: fasudil hydrochloride hydrate (Eril; Asahi Kasei Pharmaceutical Corporation), SNP (Nitrop Inj; Maruishi Pharmaceutical Co), and phenytoin hydrochloride (Neosynesin; Kowa Company, Ltd). All drugs were dissolved in physiological saline immediately before use.

Statistical Analysis
All results are expressed as mean±SEM. Values at rest were compared by unpaired t test. Responses to graded doses of drugs in each group were examined by ANOVA for repeated measures. Two-way ANOVA was used to compare FBF, FVR, and reactive hyperemia responses in the 2 groups. The relationship between
plasma BNP or Ang II and FBF or FVR on the maximum dose of fasudil was examined with a linear regression analysis with Pearson correlation coefficients. A \( P \) value of <0.05 was considered to be statistically significant.

**Results**

**Baseline Characteristics**

There was no significant difference in mean blood pressure at rest between the HF and control group (Table). Resting heart rate was significantly higher in the HF group than in the control group (89±6 versus 68±8 bpm, \( P<0.01 \); Table). Basal FBF was significantly lower in the HF group than in the control group (3.8±0.4 versus 5.2±0.6 mL·min\(^{-1}·100\) mL\(^{-1}\), \( P<0.05 \); Table). Basal FVR was significantly higher in the HF group than in the control group (39±11 versus 20±4 U, \( P<0.05 \); Table). The 2 groups were comparable in age, gender, body mass index, serum total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, fasting blood sugar, and previous smoking habit (Table). Compared with the control group, the average left ventricular ejection fraction was significantly lower and the average plasma BNP level and plasma concentrations of Ang II and NE were significantly higher in the HF group (Table).

**Plasma Concentrations of Fasudil**

Just after the peak dose of fasudil, plasma fasudil concentrations significantly increased in both groups; the levels were comparable between groups (560±60 versus 526±86 nmol/L, respectively; Table).

Forearm Vascular Responses to Fasudil and SNP

Fasudil evoked significant dose-dependent increases in FBF in the HF group but not in the control group (Figure 1). The increase in both the absolute FBF and percent change were apparent only in the HF group (Figure 1). In contrast, SNP induced comparable increases in FBF in the 2 groups (Figure 1). Fasudil evoked significantly greater decreases in both the absolute FVR value and percent change in the HF group compared with the control group, thus normalizing FVR in those patients (Figure 2). In contrast, SNP induced comparable decreases in FVR in both groups (Figure 2). Systemic arterial blood pressure and heart rate did not change significantly.
significantly during intra-arterial infusion of fasudil or SNP in either group. With the maximum dose of fasudil, there was a significant positive correlation between BNP and the increase in FBF ($r=0.6$, $P=0.002$; Figure 3A), and there was a significant negative correlation between BNP and the decrease in FVR ($r=-0.45$, $P=0.006$; Figure 3B). Moreover, with the maximum dose of fasudil, there was a significant positive correlation between plasma levels of Ang II and the increase in FBF ($r=0.54$, $P=0.001$; Figure 3C), and there was a significant negative correlation between plasma levels of Ang II and the decrease in FVR ($r=-0.58$, $P=0.005$; Figure 3D). There was, however, no significant correlation between baseline FBF and maximal forearm vasodilation in HF patients ($r=0.15$, $P=0.548$).

Effects of Fasudil on Response of FBF to Reactive Hyperemia
In the HF group, the response to reactive hyperemia was significantly lower than in the control group before the infusion of fasudil. After the infusion of fasudil, in the HF group, the response to reactive hyperemia was augmented to the levels of the control group (Figures 4A and 4C). In the control group, fasudil did not change the response to reactive hyperemia (Figures 4B and 4D).

Effects of Fasudil on FBF and FVR During Infusion of Phenylephrine in Control Group
Phenylephrine (400 ng/min) decreased FBF and increased FVR in the control group to levels comparable to those of the HF group. Fasudil did not significantly change FBF and FVR in the control subjects before or after infusion of phenylephrine (Figure 5). SNP induced comparable increases in FBF and decreases in FVR before and after infusion of phenylephrine (Figure 5).

Discussion
The major findings of the present study were that (1) the forearm vasodilator response to fasudil was significantly greater in the HF group than in the control group, whereas SNP induced a similar forearm vasodilator response in the 2 groups, and (2) fasudil augmented the impaired response to reactive hyperemia in the HF group. In a similar hypercontractile condition evoked by phenylephrine in the control group, fasudil did not induce the vasodilator response. These results suggest that activation of Rho-kinase is involved in the increased peripheral vascular resistance in patients with HF.
In the present study, basal FVR was significantly higher in the HF group than in the control group, and administration of fasudil into the forearm improved vasodilation and decreased FVR to the levels of the control group. These results suggest that activation of the Rho/Rho-kinase pathway is involved in the hyperconstriction of peripheral arteries in patients with HF. The preferential vasodilator effect of fasudil in the HF group was not due to structural changes in the arterial wall, because the response to SNP was comparable between the 2 groups. Previously, we demonstrated that SNP-induced vasodilation was similar between patients with HF and normal subjects, and the response to SNP in the present study was comparable. In the control group, the Rho/Rho-kinase pathway was not significantly activated, because fasudil did not significantly change FBF or FVR. These results are consistent with our previous report. Furthermore, after infusion of phenylephrine in the control group, which decreased FBF to levels similar to those in patients with HF, fasudil did not significantly change FBF and FVR. Thus, the difference in basal FBF does not account for our observation. We cannot exclude the possibility, however, that long-term adrenergic stimulation activates the Rho/Rho-kinase pathway, because NE might activate Rho-kinase. Together with the results of the effects of SNP, these findings suggest that the vasodilator effect of fasudil in the HF group was not due to the structural changes of the arterial wall and that Rho-kinase is involved in the pathogenesis of increased peripheral vascular resistance in patients with HF. Furthermore, we consider that the vascular effects of fasudil are independent of the basal FBF.

Previous studies suggested that maximal vasodilation is impaired in patients with HF. The findings of the present study are consistent with this suggestion. In the present study, submaximal vasodilation induced by ischemia (reactive hyperemia) was significantly impaired in the HF group, and fasudil increased the maximal FBF and decreased the minimal FVR. These responses were not observed in the control group. We evaluated forearm vasodilating responses induced by reactive hyperemia during infusion of the maximum dose of fasudil, which increased FBF and decreased FVR in the HF group, by examining the effect of fasudil-induced Rho-kinase inhibition. At this dose of fasudil, baseline FBF and FVR were comparable between the 2 groups (Figure 4). These results indicate that activation of the Rho/Rho-kinase pathway is involved in impaired vasodilation induced by metabolic stimulation in patients with HF.

In the present study, the plasma concentrations of fasudil were not significantly different between the 2 groups. We and others previously demonstrated that the IC50 value of fasudil is <1.9 μmol/L when tested in vitro, and the achieved concentration in patients in the present study was high enough to inhibit Rho-kinase activity. We previously demonstrated that fasudil augmented the impaired vasodilation in patients with hypertension, and we used the same dose in the present study. Fasudil prevents acetylcholine-induced coronary artery spasm and the resultant myocardial ischemia in patients with vasospastic angina and coronary microvascular spasm. Noma et al demonstrated that smoking activates Rho-kinase in forearm VSMCs but does not alter the vasodilating effect induced by exogenous nitric oxide in forearm VSMCs in healthy young men. In that study, graded doses of fasudil (3, 10, and 30 μg/min) were infused for 5 minutes, but the plasma fasudil concentration was not measured. We used a similar dose of fasudil administered for a longer period of time. These results suggest that the dose of fasudil selectively and specifically inhibited Rho-kinase.

Vasodilation evoked by fasudil correlated to the plasma BNP levels, which suggests that activation of the Rho/Rho-kinase pathway is related to the severity of HF. Plasma BNP levels are now widely accepted as a prognostic marker of HF. In contrast, the basic disorder of HF did not correlate to the extent of vasodilation evoked by fasudil, which suggests that activation of the Rho/Rho-kinase pathway in the forearm vasculature is associated with an HF state rather than basic disorders.

In the present study, 6 control subjects had a “previous” smoking habit. Chronic smoking activates Rho-kinase in forearm VSMCs in healthy young men. In the present study, there was no vasodilator response to fasudil in the control group, even in previous smokers. There was also no significant difference in the responses of FBF and FVR to fasudil between nonsmokers and the previous smokers in the HF and control groups. From these results, we consider that the previous smoking habit had no effects on activation of Rho-kinase in these patients.

Previous studies suggest that hypercholesterolemia impairs endothelial function. Creager et al reported that in humans with hypercholesterolemia, whose average serum cholesterol was 275 mg/dL, there is a decreased effect of nitrovasodilators, including endothelium-derived relaxing factor, on the vascular smooth muscle of resistance vessels. Casino et al reported that hypercholesterolemic patients, whose average serum cholesterol levels were 292 mg/dL, have impaired endothelium-dependent vascular relaxation. In the present study, in the control group, 5 subjects had serum total cholesterol levels >220 mg/dL, and 4 had serum triglyceride levels >150 mg/dL. Serum total cholesterol
levels were <250 mg/dL and serum triglyceride levels were <180 mg/dL, however, even in the HF group. Therefore, we consider that these levels of hypercholesterolemia are not enough to activate Rho-kinase.

The present study did not address the precise mechanism(s) by which the Rho/Rho-kinase pathway is activated in patients with HF. In the animal models of HF, however, the Rho/Rho-kinase pathway is reported to be involved in the pathogenesis of HF.\textsuperscript{13,16,27} Rho-kinase is substantially involved in the pathogenesis of left ventricular remodeling after myocardial infarction associated with upregulation of proinflammatory cytokines.\textsuperscript{37} Differential activation of the Rho/Rho-kinase pathway plays a critical role in HF, and the Rho/Rho-kinase pathway is involved in the pathogenesis of cardiac dysfunction and cardiovascular remodeling.\textsuperscript{20} Hisaoka et al\textsuperscript{13} demonstrated that the Rho/Rho-kinase system is critically involved in the enhanced arterial vasoconstriction observed in HF. In that study, enhanced vasoconstriction was induced by a marked increase in Ca\textsuperscript{2+} sensitivity mediated through activation of the Rho/Rho-kinase pathway.\textsuperscript{13} Activation of the sympathetic nervous system and renin-angiotensin system occurs in HF, and this neurohumoral activation causes HF to deteriorate.\textsuperscript{2,3,8} This mechanism is also related to abnormal peripheral circulation in HF.\textsuperscript{2,3,9} Previous in vitro studies suggest that Rho-kinase is deeply involved in the Ang II–induced signaling pathway.\textsuperscript{40–42} Therefore, those studies suggest that activation of the renin-angiotensin system is upstream of the Rho/Rho-kinase pathway and that Ang II upregulates the Rho-kinase pathway in patients with HF. In fact, several neurohumoral factors such as Ang II, NE, and endothelin-1, which activate the Rho/Rho-kinase pathway, are increased in patients with HF.\textsuperscript{39,43} In the present study, we evaluated the correlation between plasma Ang II levels and the increases in FBF or the decreases in FVR caused by the infusion of fasudil. These results suggest that there is a correlation between plasma Ang II levels and Rho-kinase activity.

Furthermore, fasudil likely influences endothelial function. In fact, recent studies suggest an interaction between endothelial NO synthase activity and Rho-kinase.\textsuperscript{44} For example, it is suggested that NO induces vasodilation through inhibition of the Rho/Rho-kinase signaling pathway and that the Rho/Rho-kinase activity negatively regulates endothelial NO synthase phosphorylation.\textsuperscript{45} It is well established that NO activity is decreased in HF.\textsuperscript{6–10} In addition, we previously demonstrated that t-arginine supplementation improves both acetylcholine-induced and reactive hyperemia–induced forearm vasodilation in patients with HF, which suggests that the impaired vasodilation is caused by reduced NO activity in HF.\textsuperscript{5} Therefore, it is possible that inhibition of Rho-kinase improves NO activity in patients with HF. We did not address this issue in the present study. We do consider, however, that inhibition of Rho-kinase has multiple actions, including modulation of both endothelial and VSMC function. Because Rho-kinase causes VSMC hypercontraction via a Ca\textsuperscript{2+}-sensitization mechanism, it is difficult to exclude the possibility that fasudil acts directly on VSMCs. Also, in an in vitro study, activation of the Rho/Rho-kinase pathway was involved in arterial hypercontraction in an HF model through a Ca\textsuperscript{2+}-sensitization mechanism.\textsuperscript{13} Thus, we suggest that abnormalities of both endothelial and VSMC function are involved in the effects of fasudil on the increased vascular resistance in patients with HF. Further studies are needed to examine this issue.

In conclusion, the present study indicates that fasudil, a Rho-kinase inhibitor, improves the increased FVR and impaired vascular response to reactive hyperemia in the forearm of patients with HF and that these effects are not due to changes in the vascular structures.

**Clinical Implications**

We demonstrated that the Rho/Rho-kinase pathway is involved in the pathogenesis of HF. Thus, inhibition of the Rho-kinase pathway might be a potential therapeutic strategy for HF. Our results suggest that inhibition of Rho-kinase might improve abnormal peripheral circulation, thereby augmenting exercise tolerance in patients with HF.\textsuperscript{2,3,9} Furthermore, Rho-kinase is substantially involved in the pathogenesis of left ventricular remodeling after myocardial infarction associated with upregulation of proinflammatory cytokines,\textsuperscript{37} which suggests that these molecular mechanisms might be important targets for the prevention of post–myocardial infarction HF.

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