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Involvement of Rho-kinase in vascular remodeling caused by long-term inhibition of nitric oxide synthesis in rats

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Abstract

Long-term inhibition of nitric oxide (NO) synthesis with N^{ω}-nitro-L-arginine methyl ester (L-NAME) induces coronary vascular remodeling in rats. To determine the pathogenic mechanism involved in vascular remodeling, we examined the effects of fasudil, a Rho-kinase inhibitor, on vascular lesion formation. In rats treated with L-NAME at 10 mg/kg/day, vascular remodeling was evident in both large and small coronary arteries at the fourth week. Fasudil (3 mg/kg, p.o., twice daily) markedly prevented the development of vascular remodeling in small coronary arteries. Coronary flow was measured in Langendorff perfused isolated heart preparations. Long-term treatment with L-NAME caused a significant decrease in coronary flow, which was significantly inhibited by fasudil. Fasudil suppressed the structural and functional changes in coronary arteries by chronic blockade of NO synthesis. Thus, the Rho-kinase pathway may be substantially involved in the pathogenesis of vascular remodeling in this rat model.

Keywords: Nitric oxide (NO); Remodeling; Fasudil; Hydroxyfasudil; Rho-kinase

1. Introduction

Nitric oxide (NO), an important endothelium-derived relaxing factor, is synthesized in endothelial cells from L-arginine through a metabolic pathway mediated by endothelial NO synthase. Cumulative evidence suggests that NO is substantially involved in controlling vascular tone, platelet aggregation and leukocyte adhesion, and inhibits the growth and/or proliferation of blood vessels (Palmer et al., 1988; Kubes et al., 1991; Von der Leyen et al., 1995; Shimokawa, 1999).

Endothelial dysfunction is a well-known feature of disorders such as hypercholesterolemia and atherosclerosis in humans and animals (Kuo et al., 1992; Zeiher et al., 1993). It has been reported that long-term treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, produces systemic arterial hypertension, microvascular structural changes with thickening of the media, luminal narrowing, perivascular fibrosis, and hypertrophy of the myocardium in rats and pigs (Ribeiro et al., 1992; Arnal et al., 1993; Ito et al., 1995; Takemoto et al., 1997). These observations suggest that the coronary vascular and myocardial structural changes could be induced by reduced NO production, leading to the activation of neurohumoral systems and growth-promoting factors.

Studies in vitro and in vivo have suggested that Rho-kinase plays an important role in various cellular functions, including smooth muscle contraction, reorganization of the actin cytoskeleton, cell adhesion and migration, and cell growth (Amano et al., 1997; Narumiya et al., 1997; Uehata et al., 1997; Seasholtz et al., 1999). These lines of evidence suggest that the Rho-kinase pathway may also play an important role in vascular lesion formation. Indeed, it has recently been demonstrated that Rho-kinase is involved in various models of vascular disease (Eto et al., 2000; Miyata et al., 2000; Sawada et al., 2000). However, the role of Rho-kinase in the coronary vascular and myocardial structural changes caused by chronic blockade of NO synthesis remains to be clarified.

Fasudil, an antivasospastic drug, has recently been shown to be a potent inhibitor of Rho-kinase with a K_i value of 0.33–0.40 μM (Uehata et al., 1997; Nagumo et

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al., 2000). We also demonstrated that hydroxyfasudil, an active metabolite of fasudil after oral administration, is a potent and specific inhibitor of Rho-kinase (Shimokawa et al., 1999). This study was thus designed to determine whether long-term oral treatment with fasudil could inhibit L-NAME-induced structural and functional changes in coronary arteries and myocardium in rats.

2. Materials and methods

These experiments were reviewed by the Committee of the Ethics of Animal Experiments at the Institute for Life Science Research, Asahi Kasei, and were carried out in accordance with the Guidelines for Animal Experiments at the Institute for Life Science Research, Asahi Kasei.

2.1. Animal preparation

Twenty-five male Wistar–Kyoto rats aged 20 weeks (Charles River, Japan) were used. Three groups of rats were studied. The control group (Control, $n = 8$) received no treatment. The second group (L-NAME, $n = 8$) received L-NAME (10 mg/kg/day) via an osmotic infusion pump (Model 2ML4, Alzet) implanted intraperitoneally (i.p.). The third group (L-NAME + Fasudil, $n = 9$) received L-NAME (10 mg/kg/day, i.p.) and fasudil (3 mg/kg, twice a day) orally.

2.2. Isolated heart perfusion study

Four weeks after treatment, all animals were heparinized with sodium heparin (500 U/kg, i.p.) and anesthetized with sodium pentobarbital (40 mg/kg, i.p.). A midsternal thoracotomy was performed, and the heart was rapidly excised and placed into ice-cold Krebs–Henseleit buffer solution consisting of (in mmol/l) NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.5, $NaHCO_3$ 25, Na pyruvate 2.0, and glucose 11.1. Within 1 min, the heart was mounted in a Langendorff heart perfusion apparatus. The heart was perfused in a retrograde fashion via the aorta at a constant pressure of 55 mm Hg with oxygenated Krebs–Henseleit solution under an atmosphere of 95% O_2 and 5% CO_2 at 38 °C.

A latex balloon attached to a pressure transducer (P10EZ, Nihon Kohden) via rigid polyethylene tubing was inserted into the left ventricle to measure left ventricular pressure and its first derivative (LVdp/dt). Heart rate was monitored with a cardiotachometer (AT-601G, Nihon Kohden) triggered by left ventricular pressure. Coronary flow was measured via an extracorporeal type flow probe connected to an electromagnetic flowmeter (MFV-2100, Nihon Kohden). Coronary flow, heart rate, left ventricular pressure, and LVdp/dt were continuously monitored on a recticorder (RJG-4128, Nihon Kohden). Left ventricular developed pressure was calculated by subtracting endo-diastolic pressure from systolic pressure.

In two isolated hearts of the L-NAME group, a loss of cardiac function was observed, and therefore the heart function of these animals was not measured.

2.3. Histopathological and immunohistochemical study

After the functional study, the hearts were perfused at a constant pressure of 55 mm Hg with 10% formaldehyde solution for 10 min via retrograde infusion into the ascending aorta. After fixation, the left and right ventricles were separated from the atria and weighed. The left ventricle was sliced from the base (slice number 1) to the apex (slice number 5) into five sections. The sections were then fixed in 10% formaldehyde solution at room temperature for a minimum of 4 days. After fixation, slice numbers 2 and 3 were histopathologically examined. Paraffin slices from each heart were stained with Masson's trichrome and van Gieson elastin stain for light microscopy. The vessels with an internal elastic lamina were defined as arteries. Morphometric analyses were carried out using a computerized image analysis system (NIH-Image 1.61). To assess the thickening of the coronary arterial wall and perivascular fibrosis, cross-sectional images of small coronary arteries with external diameters 80 to 250 μm and large conduit arteries with external diameters ≥ 250 μm were studied. The wall-to-lumen ratio (the vessel wall area divided by the lumen area) was determined. The area of fibrosis immediately surrounding blood vessels was measured, and perivascular fibrosis was expressed as the ratio of the fibrotic area to the lumen area. In each heart, three to eight small coronary arteries and one to five large coronary arteries were studied, and the mean values of these arteries were used for subsequent analyses.

In addition, to identify proliferative cells and macrophages, immunostaining with antibodies against proliferating cell nuclear antigen (PCNA) and rat monocyte/macrophage (ED1) was performed in slice numbers 2 and 3, respectively. Each section stained by immunostaining for ED1 was scanned at ×400 magnification. The number of positive cells in each section was counted, and the average number of positive cells per section was used for analyses.

2.4. Pharmacokinetic study

Sixty male Wistar rats aged 9 weeks were used. Fasudil (10 mg/kg) was administered orally. Blood samples were taken from the abdominal artery before, and 5, 10, 20, 30, 45, 60, 90, 120 and 240 min after dosing.

Concentrations of fasudil or hydroxyfasudil in plasma were measured by high-performance liquid chromatography. The maximum plasma concentration, C_{max}, was derived from the average plasma concentration at each point. The area under the plasma-concentration curve was determined using the trapezoidal rule. The β-phase elimination half-life ($T1/2\beta$) was calculated by non-linear regression from the equation $T1/2\beta = \ln 2/\beta$.

2.5. Statistics

The results are expressed as means ± S.E.M. Statistical analysis was performed using analysis of variance, followed by Dunnett's multiple range test. P values of 0.05 or less were considered to represent statistically significant differences.

2.6. Drugs

The following drugs were used: fasudil (Asahi Kasei, Tokyo), N^{ω}-nitro-L-arginine methyl ester (Sigma, St. Louis), anti-proliferating cell nuclear antigen (Dako, Kyoto) and anti-ED1 (Serotec, Tokyo).

3. Results

None of the rats in any of the three groups died during the 4-week treatment period. Since the osmotic infusion pump weighed about 8 g both before and 4 weeks after the treatment in the L-NAME and the L-NAME + Fasudil groups, body weights at 1, 2, 3 and 4 weeks were calculated by subtracting the osmotic infusion pump weight from the measured body weight.

3.1. Body weight and heart weight

Body weights did not differ significantly among the three groups before treatment (Table 1). During the study, animals in the control group showed an increase in body weight, whereas those in the L-NAME and the L-NAME + Fasudil groups lost weight (about 20 g) 3 days after treatment ($P < 0.05$ vs. the control group) and thereafter gradually gained weight. Two weeks after treatment, there were no significant differences in body weight among the three groups. No significant differences were observed in absolute or relative heart or left ventricular weight among the three groups (Table 1).

Table 1
Body weight and heart weight

	Weeks	Control ($n = 8$)	L-NAME ($n = 8$)	L-NAME + Fasudil ($n = 9$)
Body weight (g)	0	344 ± 4	350 ± 3	350 ± 3
	1	350 ± 4	333 ± 4[a]	336 ± 4[a]
	2	354 ± 5	342 ± 5	344 ± 3
	3	363 ± 5	356 ± 4	359 ± 3
	4	374 ± 5	362 ± 3	366 ± 5
Heart weight (mg)	4	1327 ± 37	1312 ± 65	1243 ± 38
Left ventricular weight (mg)	4	975 ± 35	1000 ± 49	929 ± 37
Heart weight (mg)/body weight (g)	4	3.54 ± 0.08	3.62 ± 0.16	3.40 ± 0.12
Left ventricular weight (mg)/body weight (g)	4	2.60 ± 0.08	2.76 ± 0.13	2.54 ± 0.11

The results are expressed as means ± S.E.M.
L-NAME indicates N^{ω}-nitro-L-arginine methyl ester.
Body weights at 1, 2, 3 and 4 weeks were calculated by subtracting osmotic infusion pump weight from measured body weight.
[a] $P < 0.05$ vs. control group.

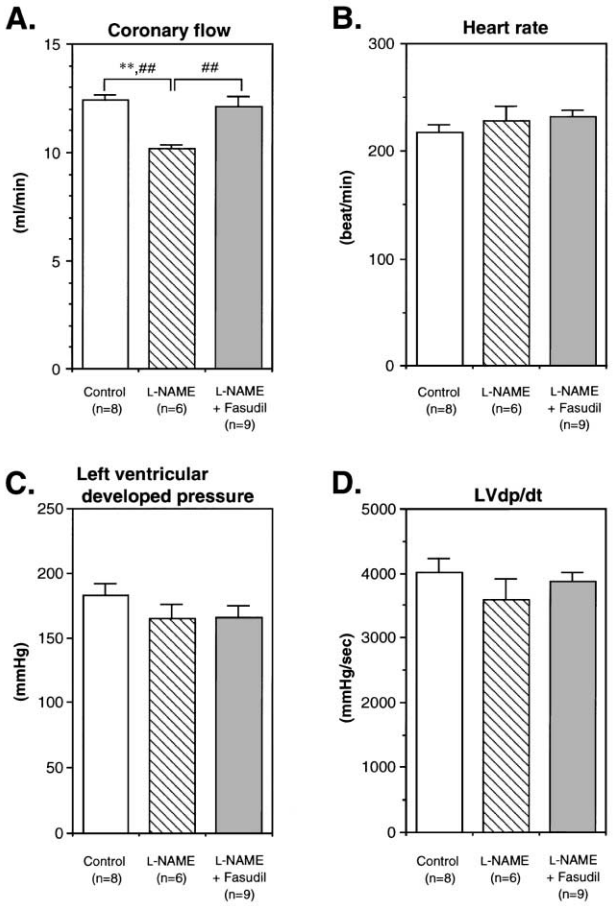

Fig. 1. Coronary flow (A), heart rate (B), left ventricular developed pressure (C) and LVdp/dt (D) of perfused rat hearts in the control group, the L-NAME group, and the L-NAME + Fasudil group. Coronary flow in the L-NAME group was significantly decreased, while there was no significant difference in coronary flow between the L-NAME + Fasudil group and the control group. Heart rate, left ventricular developed pressure or LVdP/dt was not significantly different among the three groups. The results are expressed as means ± S.E.M. $^{**}P < 0.01$ vs. control group. $\#\#P < 0.01$ vs. L-NAME group.

3.2. Isolated heart perfusion study

In isolated hearts of the L-NAME group, coronary flow was significantly decreased (Fig. 1). However, fasudil significantly inhibited the decrease in coronary flow and there was no significant difference between the control group and the L-NAME + Fasudil group. Left ventricular developed pressure, LVdp/dt or heart rate was not significantly different among the three groups (Fig. 1).

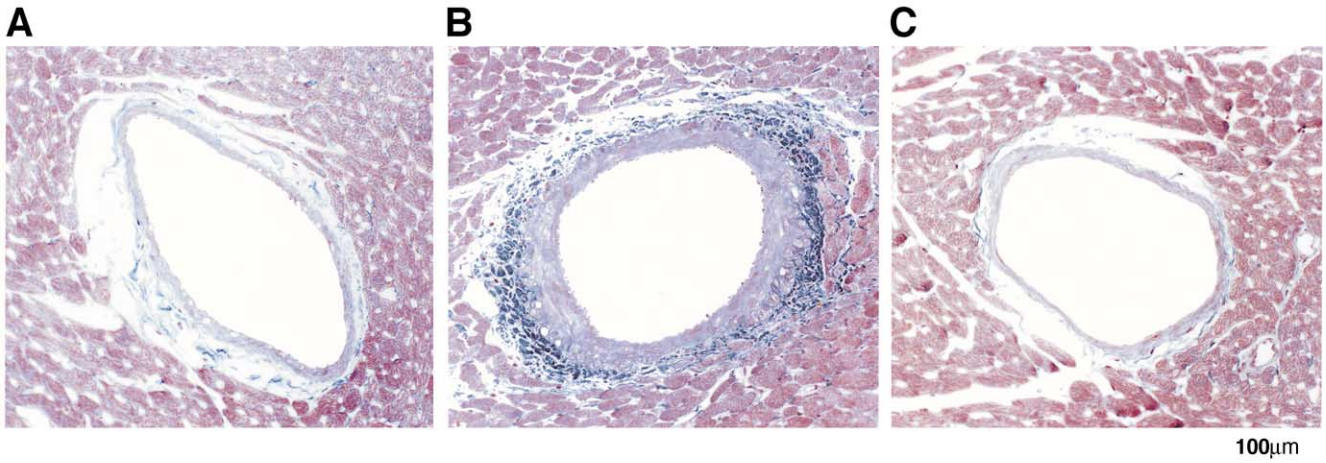

Fig. 2. Representative micrographs of small coronary arteries (Masson's trichrome staining) in the control group (A), the L-NAME group (B), and the L-NAME + Fasudil group (C). Compared with the control group, wall-to-lumen ratio and perivascular fibrosis were increased in the L-NAME group. Long-term oral treatment with fasudil inhibited the development of these vascular lesions.

3.3. Coronary vascular remodeling

Micrographs of the coronary arteries obtained from the control, L-NAME, and L-NAME + Fasudil groups are shown in Fig. 2. The total area of small and large coronary arteries did not differ significantly among the three groups (data not shown). Four weeks after treatment, the wall-to-lumen ratio in the coronary arteries was significantly in-

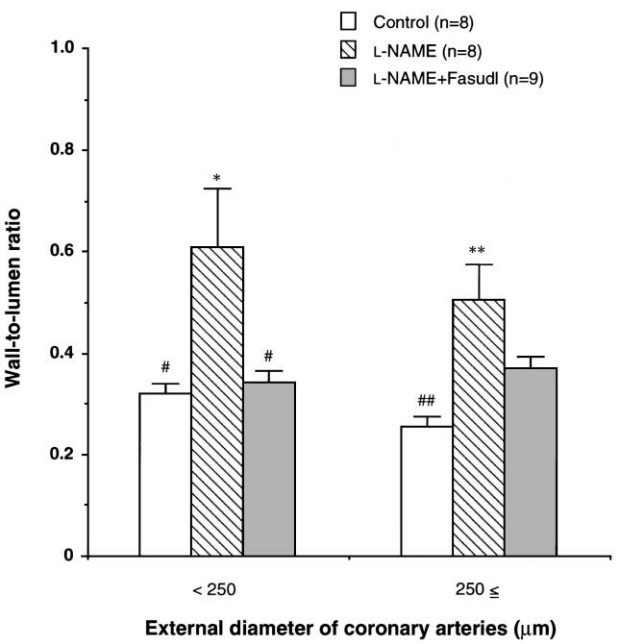

Fig. 3. Inhibitory effect of long-term oral treatment with fasudil on the increase in the wall-to-lumen ratio after 4 weeks of chronic administration of L-NAME. The wall-to-lumen ratio was significantly greater in the L-NAME group than in the control group. The vascular structural changes in small coronary arteries were significantly suppressed by the treatment with fasudil. The results are expressed as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group. $\#P < 0.05$, $\#\#P <$ 0.01 vs. L-NAME group.

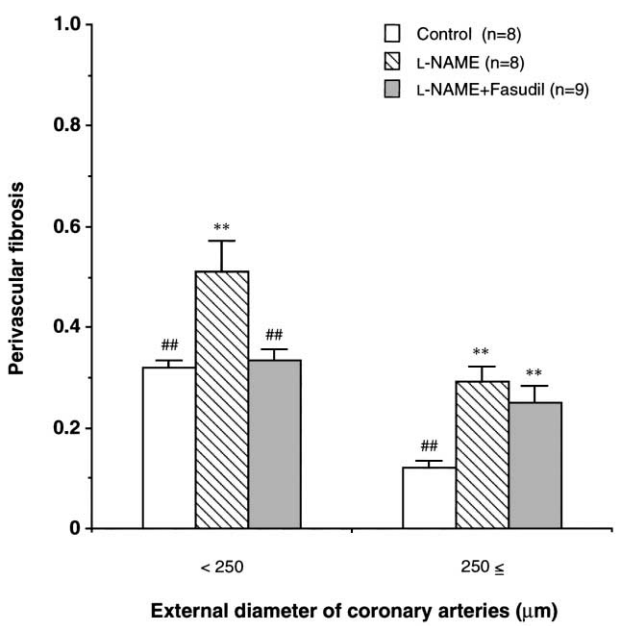

Fig. 4. Inhibitory effect of long-term oral treatment with fasudil on the increase in perivascular fibrosis after 4 weeks of chronic administration of L-NAME. The extent of perivascular fibrosis was significantly greater in the L-NAME group than in the control group. Perivascular fibrosis in small coronary arteries was significantly reduced by treatment with fasudil. The results are expressed as means ± S.E.M. $^{**}P < 0.01$ vs. control group. $\#\#P < 0.01$ vs. L-NAME group.

creased in the L-NAME group (Fig. 3). Perivascular fibrosis in the coronary arteries was also significantly greater in the L-NAME group than in the control group (Fig. 4). Both parameters of vascular remodeling were significantly ameliorated in small coronary arteries by treatment with fasudil (Figs. 3 and 4), whereas the beneficial effects of fasudil were relatively less pronounced in large coronary arteries (Figs. 3 and 4).

To elucidate the mechanism by which fasudil reduced coronary vascular remodeling, the proliferative activity of coronary vascular smooth muscle cells and the migration of macrophages were analyzed using immunohistochemical staining with anti-PCNA and anti-ED1, respectively. PCNA-positive cells were not detected in the vessel wall in either the L-NAME group or the L-NAME + Fasudil group 4 weeks after treatment. The number of ED1-positive cells was significantly greater in the myocardium from the L-NAME group than in that from the control group (Fig. 5). Long-term treatment with fasudil significantly inhibited the increase in ED1-positive cells, and there was no significant difference in the number of these cells between the control group and the L-NAME + Fasudil group (Fig. 5).

3.4. Pharmacokinetic parameters

The pharmacokinetic parameters for fasudil and hydroxyfasudil are listed in Table 2. The plasma concentration of fasudil reached C_{max} about 10 min after administration and disappeared quickly. Hydroxyfasudil appeared simultaneously with fasudil and reached C_{max} 10 min after its administration. Hydroxyfasudil disappeared more gradually than fasudil. The C_{max} and AUC of hydroxyfasudil were 10–20 times higher than those of fasudil.

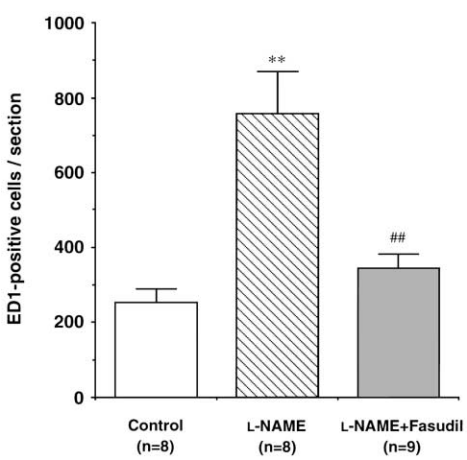

Fig. 5. Inhibitory effect of long-term oral treatment with fasudil on ED1-positive macrophage recruitment into the myocardium after 4 weeks of chronic administration of L-NAME. In the L-NAME group, recruitment of ED1-positive macrophages was observed in the myocardium, whereas fasudil significantly inhibited this recruitment. The results are expressed as means ± S.E.M. $^{**}P < 0.01$ vs. control group. $\#\#P < 0.01$ vs. L-NAME group.

Table 2
Pharmacokinetic parameters of fasudil and hydroxyfasudil following an oral administration of fasudil at 10 mg/kg

	C_{max} (μg/ml)	AUC (μg h/ml)	T1/2 (h)
Fasudil	0.287	0.094	0.15
Hydroxyfasudil	2.467	2.020	1.3

The results are expressed as means ($n = 6$).

4. Discussion

The present study demonstrated that long-term oral treatment with fasudil inhibited the L-NAME-induced structural and functional changes in the coronary artery in rats. To the best of our knowledge, this is the first report that demonstrates an important role of the Rho-kinase pathway in the pathogenesis of coronary remodeling induced by chronic blockade of NO synthesis in vivo.

It has been shown in rats that chronic treatment with L-NAME decreases NO synthesis and causes arterial hypertension and vascular and myocardial remodeling (Ribeiro et al., 1992; Arnal et al., 1993; Ito et al., 1995; Takemoto et al., 1997). Recently, it has been suggested that the Rho/Rho-kinase pathway may play an important role in vascular lesion formation (Eto et al., 2000; Miyata et al., 2000; Sawada et al., 2000). However, it is unknown whether Rho-kinase activation is involved in the L-NAME-induced vascular and myocardial structural changes.

In the present study, we demonstrated that fasudil significantly inhibited the decrease in coronary flow and markedly suppressed the development of vascular structural changes, including medial thickening and perivascular fibrosis, in small coronary arteries. Under physiological conditions, coronary vascular resistance at rest is regulated primarily by coronary microvessels (Chilian et al., 1988). Therefore, the decrease in coronary flow in the L-NAME group may be related to luminal narrowing in small coronary arteries. Fasudil may improve coronary flow by inhibiting the structural changes of small coronary arteries. Fasudil also reduced the vascular structural changes of large coronary arteries, albeit to a lesser extent. The reason for the difference in the inhibitory effects of fasudil on small and large coronary arteries remains to be examined in a future study. However, it is conceivable that the mechanism of coronary remodeling might be different in small and large coronary arteries.

Our finding that myocardial hypertrophy was not observed 4 weeks after L-NAME treatment was consistent with previous reports. Indeed, there is extensive evidence that structural changes of the coronary arteries occur by the fourth week, whereas myocardial hypertrophy occurs

by the eighth week of L-NAME administration (Ribeiro et al., 1992; Arnal et al., 1993; Ito et al., 1995; Takemoto et al., 1997). Further studies are required to elucidate the involvement of Rho-kinase in myocardial hypertrophy in this model.

Our results demonstrated that fasudil significantly inhibited the migration of macrophages into the myocardium. We and others previously reported that fasudil and hydroxyfasudil inhibited the migration of neutrophils, monocytes and smooth muscle cells (Negoro et al., 1999; Satoh et al., 1999; Miyata et al., 2000). Recent studies in vitro showed that Rho-kinase is involved in the adhesion and migration of various types of cells, including vascular smooth muscle cells and inflammatory cells (Narumiya, 1996; Amano et al., 1997). PCNA-positive cells were not detected 4 weeks after treatment in the vessel wall of the L-NAME group. It is possible that the proliferation of vascular smooth muscle cells does not contribute to the pathogenesis of vascular remodeling in our rat model. We did not examine the proliferation of vascular smooth muscle cells prior to 4 weeks of treatment in this study. However, fasudil and hydroxyfasudil did not suppress the proliferation of cultured vascular smooth muscle cells at 30 μM in vitro (data not shown), and it has also been reported that fasudil does not affect the proliferation of medial vascular smooth muscle cells following balloon injury (Negoro et al., 1999).

After oral administration of fasudil at 10 mg/kg, the C_{max} and T1/2 of fasudil were 0.287 μg/ml (approximately 0.9 μM) and about 10 min, respectively. The C_{max} and AUC of hydroxyfasudil were 10–20 times higher than those of fasudil. Hydroxyfasudil disappeared more gradually than fasudil. These pharmacokinetic data indicate that following oral administration in rats, fasudil is absorbed rapidly and metabolized to hydroxyfasudil. Hydroxyfasudil potently inhibits Rho-kinase (IC_{50} 0.9–1.8 μM), while its inhibitory effect is markedly (at least 50–100 times) less for myosin light chain kinase or protein kinase C (Shimokawa et al., 1999). Our present and previous findings suggest that hydroxyfasudil may play a major role in the pharmacological effects of fasudil as a Rho-kinase inhibitor in our rat model. Thus, it is possible that the Rho-kinase pathway may be involved in the pathogenesis of vascular remodeling induced by chronic blockade of NO synthesis. However, since we did not examine the effects of structural analogues of fasudil/hydroxyfasudil without Rho-kinase inhibitory activity or structurally dissimilar Rho-kinase inhibitors in our rat model, more evidence is required to establish the selective inhibitory effect of fasudil on Rho-kinase.

In the present rat model with L-NAME, it is suggested that increased local angiotensin I-converting enzyme expression may be important in the pathogenesis of cardiovascular remodeling (Negoro et al., 1999). The activation of angiotensin I-converting enzyme would increase the synthesis of angiotensin II, which directly induces the proliferation of smooth muscle cells and myocyte hypertrophy, and also induces the release of platelet-derived growth factor and transforming growth factor-β (Naftilan et al., 1989; Schunkert et al., 1990; Gibbons et al., 1992; Itoh et al., 1993). Interestingly, it has been demonstrated that angiotensin II activates the Rho/Rho-kinase pathway (Yamada et al., 2000). The present results, together with these lines of evidence, suggest that Rho-kinase may be important in the pathogenesis of the coronary and myocardial structural changes induced by chronic blockade of NO synthesis. Although the molecular mechanism for this observation remains to be examined, the present results suggest that Rho-kinase inhibitors may be useful for the treatment of vascular remodeling.

We did not measure blood pressure in the present study, but it is unlikely that the inhibition of structural changes by fasudil is due to its hypotensive effect because oral administration of fasudil at 3 mg/kg did not significantly decrease systemic blood pressure in conscious rats (unpublished observations). In addition, it has been suggested that L-NAME-induced structural changes are not due to elevated blood pressure (Takemoto et al., 1977).

In summary, we were able to demonstrate that long-term blockade of Rho-kinase with fasudil/hydroxyfasudil inhibited the coronary structural changes induced by chronic treatment with L-NAME in rats. Therefore, the Rho-kinase pathway may play an important role in the pathogenesis of vascular remodeling induced by chronic blockade of NO synthesis.

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