Beneficial Effect of Hydroxyfasudil, a Specific Rho-Kinase Inhibitor, on Ischemia/Reperfusion Injury in Canine Coronary Microcirculation In Vivo

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OBJECTIVES

We examined whether hydroxyfasudil, a specific Rho-kinase inhibitor, exerts cardioprotective effect on coronary ischemia/reperfusion (I/R) injury and, if so, whether nitric oxide (NO) is involved.

BACKGROUND

Recent studies have demonstrated that Rho-kinase is substantially involved in the pathogenesis of cardiovascular diseases; however, it remains to be examined whether it is also involved in ischemia/reperfusion (I/R) injury.

METHODS

Canine subepicardial small arteries (SA, ≥100 µm) and arterioles (A, <100 µm) were observed by a charge-coupled device intravital microscope during I/R. Coronary vascular responses to endothelium-dependent (acetylcholine, intracoronary [IC]) and -independent (papaverine, IC) vasodilators were examined after I/R under the following four conditions: control (n = 7), NO synthase inhibitor alone (NG-monomethyl-L-arginine [L-NMMA], IC, n = 4), hydroxyfasudil alone (IC, n = 7), and hydroxyfasudil plus L-NMMA (n = 7).

RESULTS

Hydroxyfasudil significantly attenuated serotonin (IC)-induced vasoconstriction of SA (−7 ± 1% vs. 2 ± 1%, p < 0.01). Coronary I/R significantly impaired coronary vasodilation to acetylcholine after I/R (SA, p < 0.05; and A, p < 0.01 vs. before I/R) and L-NMMA further reduced the vasodilation, whereas hydroxyfasudil completely preserved the responses. The vasoconstriction by L-NMMA after I/R was significantly improved by hydroxyfasudil in both-sized arteries (both p < 0.01). Expression of endothelial nitric oxide synthase (eNOS) protein in the ischemic endocardium of left anterior descending coronary artery area (as determined by Western blotting) significantly decreased (79 ± 4%) compared with the nonischemic endocardium of LCX area (100 ± 7%), which was improved by hydroxyfasudil (105 ± 6%, p < 0.01). Hydroxyfasudil significantly reduced myocardial infarct size, and hydroxyfasudil with L-NMMA also reduced the infarct size compared with L-NMMA alone.

CONCLUSIONS

Hydroxyfasudil exerts cardioprotective effects on coronary I/R injury in vivo, in which NO-mediated mechanism may be involved through preservation of eNOS expression. (J Am Coll Cardiol 2005;45:599–607) © 2005 by the American College of Cardiology Foundation

Ischemia-reperfusion (I/R) injury attenuates endothelium-dependent dilation of large coronary arteries both in vitro (1,2) and in vivo (3,4). Endothelial dysfunction causes adverse outcome in the coronary circulation (5). Reperfusion injury is caused by direct myocardial injury through coronary vasospasm, free radicals, and inflammatory responses (6,7). Furthermore, local coronary vasoconstrictions in response to vasoconstrictors (e.g., serotonin) are enhanced (8,9). However, the mechanism of I/R-induced vascular injury remains to be clarified.

Recent studies have demonstrated that Rho-kinase, an effector of the small guanosine triphosphatase Rho, is substantially involved in the pathogenesis of cardiovascular diseases (10). Shimokawa et al. (10,11) have recently found that hydroxyfasudil is a potent and specific inhibitor of Rho-kinase and markedly inhibits coronary hyperconstriction and macrophage migration. They also demonstrated that intracoronary serotonin induces coronary hypercontractions at the inflammatory coronary lesions both in vitro and in vivo, in which up-regulated Rho-kinase is substantially
involved (12). Recent studies demonstrated that endothelial expression and activity of Rho-kinase are enhanced by hypoxia, with a resultant down-regulation of endothelial nitric oxide synthase (eNOS) expression and reduced nitric oxide (NO) production (13), and that Rho-kinase is also involved in a canine model of cerebral infarction associated with superoxide production and neutrophil infiltration (14).

It is conceivable that Rho-kinase is involved in the mechanisms of I/R injury associated with reduced endothelial NO production. In this study, we thus examined whether hydroxysafudil exerts protective effect on coronary I/R injury in vivo and, if so, whether NO is involved.

METHODS

Animal preparation. This study conformed to the Guidelines on Animal Experiments of Kawasaki Medical School and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Mongrel dogs (15 to 25 kg, n = 31) of either gender were anesthetized with morphine (3 mg/kg, intramuscular) and sodium pentobarbital (25 mg/kg, intravenous). After intubation, each animal was ventilated with a high-frequency jet ventilator (model VS600, IDC, Pittsburgh, Pennsylvania) with room air supplemented by 100% oxygen. Aortic pressure and left ventricular pressure were continuously monitored with an 8-F pigtail double manometer catheter (SPC-784A, Millar, Texas). The proximal portion of the left anterior descending coronary artery (LAD) was isolated and a transonic flow probe (T206, Transonic Systems, Ithaca, New York) was placed around the vessel.

Needle-probe intravital microscope. The needle-probe (4.5 mm in diameter, VMS 1210, Nihon Kohden, Tokyo, Japan) contains a gradient index lens (with a magnification of 200) surrounded by light guide fibers and a double lumen sheath. A doughnut-shaped balloon on the tip avoids direct compression of the vessels by the needle tip (15).

Measurements of coronary diameters. We placed the needle probe gently on subepicardial microvessels. When a clear vascular image was obtained, end-diastolic vascular images were taken with 30 pictures/s (15).

Measurements of regional myocardial blood flow. Regional myocardial blood flow was determined by the nonradioactive microsphere (Sekisui Plastic Co, Ltd, Tokyo, Japan) technique, as previously described in detail (16).

Briefly, 1 ml of the microspheres suspension (2 to 4 × 10⁶ spheres) was injected into the left atrium 85 min after the onset of coronary occlusion. Just before microsphere administration, a reference blood flow sample was drawn from the femoral artery at a constant rate of 8 ml/min for 2 min. The X-ray fluorescence of the stable heavy elements was measured by a wavelength-dispersive spectrometer (model PW 1480, Phillips Co., Ltd., Eindhoven, the Netherlands) (16).

Myocardial blood flow was calculated according to the formula: time flow = tissue counts × (reference flow/ reference counts) and was expressed in ml/g per minute (16).

Western blotting. Proteins were separated on sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis as previously described (17). The tissues were homogenized in a sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% glycerol). The tissue lysate was centrifuged and the supernatant collected. Protein concentration was quantified by a bichinchoninate (BCA) protein assay kit (Pierce Chemical, Rockford, Illinois). An aliquot of 10 μg of protein from each sample was electrophoresed on a 7.5% SDS-polyacrylamide gel. Proteins were subsequently transferred to polyvinylidene difluoride membrane (Immobilon-P membrane, Millipore, Bedford, Massachusetts) electrophoretically (100 V for 1 h) and membranes were incubated with antibody. The antibodies used in this study were rabbit anti-phosphorylated ezrin/radixin/moesin (ERM) family, total ERM. The antibody against phosphorylated ERM recognizes human moesin (phosphorylated at Thr558), which also binds to the phosphorylated ezrin (Thr567) and radixin (Thr564). Therefore, we used the extent of phosphorylation of ERM as a marker of Rho-kinase activity.

The levels of Western blot for phosphorylated ERM were normalized to those for total ERM as a control. Membranes were then incubated with a horseradish peroxidase-conjugated horse anti-rabbit immunoglobulin G antibody (1:5,000). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit; Amersham Pharmacia Biotechnology, United Kingdom). The obtained samples were washed with ice-cold Tris-HCl buffer (pH 7.4), mixed with the sample buffer (4% sodium lauryl sulfate, 12% beta-mercaptoethanol, and 20% glycerol in 100 mM Tris–HCl [pH 6.8]), sonicated (1 min), boiled (3 min), and finally centrifuged (10,000 g, 60 min, 4°C). The resultant supernatant was stored at −80°C until use. The separation of proteins was carried out according to the previous study (18), with a minor modification. The relative intensity of immunoreactive bands was quantified by Image Master 1D Elite software (Amersham Biotech, Buckinghamshire, United Kingdom), and the data were estimated as percentage of each control.

Experimental protocols. After the surgical procedure and instrumentation, at least 30 min were allowed for stabilization while hemodynamic variables were monitored. The following protocols were examined.

1. We infused graded doses of hydroxysafudil (10, 30, and 100 μg/kg, IC), and coronary vascular responses were
analyzed for 4 min by measuring end-diastolic vascular diameters and flows of the LAD.

2. The arteriolar vasoconstrictor response to serotonin before and after hydroxyfasudil (100 μg/kg, IC) was examined with or without inhibition of NO synthase (L-NMMA, 2 μmol/min for 20 min, IC) (Fig. 1). Hydroxyfasudil or L-NMMA was administered at 5 min before infusion of serotonin. The time interval between L-NMMA and hydroxyfasudil was also 5 min.

3. The arteriolar vasodilator responses to endothelium-dependent (acetylcholine, 1 μg/kg IC) and -independent (papaverine, 1 mg IC) vasodilators were examined before and after coronary I (90 min)/R (60 min) under the following four conditions separately in different animals: 1) control conditions, 2) L-NMMA alone, 3) hydroxyfasudil alone (100 μg/kg IC), and 4) hydroxyfasudil plus L-NMMA (Fig. 1). The time interval between each treatment was also 5 min. The basal coronary diameter is before administration of acetylcholine or papaverine either before or after I/R. Hydroxyfasudil and L-NMMA were administered at 5 min after administration of acetylcholine or papaverine. Microspheres were administered at 85 min after the onset of coronary occlusion.

4. After 5 h of reperfusion, LAD and the left circumflex artery (LCX) and myocardial tissue of LAD and LCX area were obtained for Western blotting. We reoccluded the LAD and injected Evans blue dye into a systemic vein. Then myocardial slices (5 mm) were incubated in 1% 2,3,5-triphenyltetrazolium chloride (Sigma, Japan) solution to detect the infarct area. Infarct size was expressed as percentage of the infarct area that was contiguous with area at risk (19).

Drugs. We used the following drugs: hydroxyfasudil (Asahi Kasei Pharma, Tokyo, Japan), acetylcholine (Daiichi-Seiyaku, Tokyo, Japan), papaverine (Dainihon-Seiyaku, Tokyo, Japan), and NG-methyl-L-arginine (L-arginine)

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**Figure 1.** Experimental protocol. S = serotonin; L = L-NMMA; HF = hydroxyfasudil; Ach = acetylcholine; P = papaverine; Occl = coronary occlusion.
NMMA, Sigma). All drugs were diluted in a physiologic saline immediately before use.

Statistical analysis. Results are expressed as means ± SEM. Vascular responses (Figs. 2a to 2c, 3c, 4c, 6c, 7a to 7c, 8a) were analyzed by one-way analysis of variance followed by Scheffe’s post-hoc test for multiple comparisons. Difference in the effects of serotonin, acetylcholine, and papaverine on subepicardial microvessels before and after I/R (Figs. 3a, 3b, 4a, 4b, 5at o5 d , 6a, and 6b), and difference between infarct size/risk area and transmural collateral flow with or without hydroxyfasudil (Fig. 8b) were examined by a multiple regression analysis using a model in which the change in coronary diameter was set as a dependent variable (y) and vascular size as an explanatory variable (x) while the statuses of hydroxyfasudil and hydroxyfasudil plus L-NMMA were set as dummy variables (D1, D2) in the following equation:

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y = a_0 + a_1x + a_2D_1 + a_3D_2,
\]

where \(a_0\) through \(a_3\) are partial regression coefficients. The criterion for statistical significance was at p < 0.05.

RESULTS

Coronary vasodilator effects of hydroxyfasudil. Intra-coronary administration of hydroxyfasudil caused a significant coronary vasodilation of both small arteries and arterioles (Figs. 2a and 2b, both p < 0.05, 10 \(\mu\)g/kg vs. 30 and 100 \(\mu\)g/kg) in a dose-dependent manner under control conditions with a resultant increase in CBF (Fig. 2c, p < 0.05, C vs. 10, 30 and 100 \(\mu\)g/kg). Intracoronary hydroxyfasudil did not significantly alter mean aortic pressure or heart rate (Table 1).

Hemodynamics and blood gases during I/R injury. In each experimental condition, mean aortic pressure and heart rate at baseline were constant and comparable (Table 1), and oxygen partial pressure (PO2), carbon dioxide partial pressure (PCO2), and pH were maintained within the physiologic ranges (pH 7.35 to 7.45, PCO2 25 to 40 mm Hg, PO2 >70 mm Hg) throughout the experiments. Hemodynamic...
Coronary I/R significantly impaired the coronary vasodilation to acetylcholine in both-sized arteries (both p < 0.01) and L-NMMA further reduced the vasodilation (Figs. 4a, 4b, and 5b both p < 0.01), whereas hydroxyfasudil completely preserved (small artery p < 0.05, arteriole p < 0.01) the acetylcholine-induced coronary vasodilator response after I/R (Figs. 4a and 4b). The vasoconstriction by L-NMMA was significantly attenuated by hydroxyfasudil in both-sized arteries (both p < 0.05) with decrement of CBF (Figs. 4a to 4c). When the coronary vasodilator response to acetylcholine was expressed as a function of basal coronary diameter, hydroxyfasudil preserved the response after I/R injury at all-sized coronary arteries either in the absence of or presence of L-NMMA alone (c) compared with that in the presence of L-NMMA alone (b). Number of vessels per animals used was 19/7 under control conditions (before I/R; y = -0.3x + 35.9, r = 0.85; after I/R; y = -0.2x + 18.1, r = 0.80), 13/4 for L-NMMA alone (before I/R; y = -0.2x + 35.1, r = 0.76; after I/R; y = -0.2x + 12.2, r = 0.88), 14/7 for hydroxyfasudil (before I/R; y = -0.2x + 27.9, r = 0.73; after I/R; y = -0.2x + 27.4, r = 0.80), and 16/7 for hydroxyfasudil plus L-NMMA (before I/R; y = -0.2x + 31.8, r = 0.83; after I/R; y = -0.2x + 19.2, r = 0.86). *p < 0.01. Open circles = before I/R; solid circles = after I/R.

Effects of Rho-kinase inhibition on serotonin-induced coronary responses. Intracoronary administration of serotonin caused coronary vasoconstriction of small arteries and arterioles. CBF was comparable under all conditions in both-sized arteries and arterioles. Number of vessels per animals used was 7/6 for control (mean diameter 120 ± 7 µm), 5/4 for L-NMMA (123 ± 8 µm), 6/4 for hydroxyfasudil (118 ± 8 µm), and 5/4 for hydroxyfasudil plus L-NMMA (125 ± 9 µm) in small arteries; and 12/6 for control (70 ± 6 µm), 8/4 for L-NMMA (69 ± 7 µm), 8/5 for hydroxyfasudil (68 ± 7 µm), and 11/6 for hydroxyfasudil plus L-NMMA (71 ± 5 µm) in arterioles. C = control; L = L-NMMA; HF = hydroxyfasudil. I/R = ischemia/reperfusion. B = before papaverine; A = after papaverine.

Figure 5. Coronary microvascular responses to acetylcholine before and after coronary I/R injury in dogs in vivo. Under control conditions, I/R significantly impaired coronary vasodilator response to acetylcholine (a), whereas hydroxyfasudil completely preserved the responses in the absence (c) or presence of L-NMMA (d) compared with those before I/R (Table 1).

Figure 6. Endothelium-independent coronary vasodilation before and after I/R. Under control conditions (before I/R), intracoronary administration of acetylcholine caused a significant coronary vasodilation to a greater extent in arterioles than in small arteries (Figs. 4a, 4b, and 5a, p < 0.01). Coronary I/R significantly impaired the coronary vasodilation to acetylcholine in both-sized arteries (both p < 0.01) and L-NMMA further reduced the vasodilation (Figs. 4a, 4b, and 5b both p < 0.01), whereas hydroxyfasudil completely preserved (small artery p < 0.05, arteriole p < 0.01) the acetylcholine-induced coronary vasodilator response after I/R (Figs. 4a and 4b). The vasoconstriction by L-NMMA was significantly attenuated by hydroxyfasudil in both-sized arteries (both p < 0.05) with decrement of CBF (Figs. 4a to 4c). When the coronary vasodilator response to acetylcholine was expressed as a function of basal coronary diameter, hydroxyfasudil preserved the response after I/R injury at all-sized coronary arteries either in the absence of or presence of L-NMMA alone (c) compared with that in the presence of L-NMMA alone (b). Number of vessels per animals used was 19/7 under control conditions (before I/R; y = -0.3x + 35.9, r = 0.85; after I/R; y = -0.2x + 18.1, r = 0.80), 13/4 for L-NMMA alone (before I/R; y = -0.2x + 35.1, r = 0.76; after I/R; y = -0.2x + 12.2, r = 0.88), 14/7 for hydroxyfasudil (before I/R; y = -0.2x + 27.9, r = 0.73; after I/R; y = -0.2x + 27.4, r = 0.80), and 16/7 for hydroxyfasudil plus L-NMMA (before I/R; y = -0.2x + 31.8, r = 0.83; after I/R; y = -0.2x + 19.2, r = 0.86). *p < 0.01. Open circles = before I/R; solid circles = after I/R.
(Figs. 4a, 4b, and 5c, df 2, 25, p < 0.01) or presence (Figs. 4a, 4b, and 5d, df 2, 24, p < 0.01) of L-NMMA compared with that in the presence of L-NMMA alone (Figs. 4a, 4b, and 5b).

**Endothelium-independent coronary vasodilation.** Coronary vasodilator response to papaverine was comparable under all conditions in both small arteries and arterioles (Figs. 6a and 6b). Similarly, the increase in CBF to papaverine (Fig. 6c) was also comparable under all conditions in both-sized arteries. Those coronary vasodilator responses were resistant to the blockade of NO synthesis with L-NMMA (Figs. 6a and 6b).

**Activation of Rho-kinase by ischemia-reperfusion causes down-regulation of eNOS protein expression.** Rho-kinase activity after a 90-min period of ischemia was significantly greater in the ischemic LAD than in the nonischemic LCX in the control group (Fig. 7a, p < 0.01). This Rho-kinase activation was significantly suppressed by hydroxyfasudil in the ischemic LAD (Fig. 7a, p < 0.01). Expression of eNOS protein in the ischemic endocardium of LAD area was significantly decreased compared with the non-ischemic endocardium of LCX area, which was again improved by hydroxyfasudil (p < 0.05; **p < 0.01).

**DISCUSSION**

The major findings of the present in vivo study in the canine coronary microcirculation were that: 1) a specific Rho-kinase inhibitor hydroxyfasudil preserved the endothelium-dependent coronary vasodilator responses after coronary I/R injury, 2) hydroxyfasudil also reduced myocardial infarct size, and 3) NO may be involved in those cardiovascular protective effects of hydroxyfasudil. To the best of our knowledge, this is the first report that demonstrates the usefulness of a Rho-kinase inhibitor to prevent coronary I/R injury in vivo.

**Validations of experimental model and methodology.** On the basis of the previous reports (4,12,20), we chose the adequate dose of hydroxyfasudil, acetylcholine, papaverine, and L-NMMA to examine the effects of the Rho-kinase inhibition, endothelium-dependent and -independent vasodilator responses, and inhibition of NO synthesis on coronary vascular responses before and after coronary I/R, respectively. The methodologic validity of the present study has been confirmed previously (15). After 60 to 90 min of ischemia, ultrastructural damage of coronary endothelium was observed particularly in the subendocardium in the present study, a consistent finding to the previous study (21).

**Hydroxyfasudil as a specific Rho-kinase inhibitor in the coronary microcirculation in vivo.** Shimokawa et al. (11) have recently demonstrated that hydroxyfasudil is a specific Rho-kinase inhibitor that markedly inhibits coronary vasospastic responses in a porcine model; its inhibitory effect on Rho-kinase is 100 times greater than on protein kinase C and...
L-NMMA significantly attenuated serotonin-induced coronary smooth muscle contraction (12). In the present study, intracoronary injection of L-NMMA significantly reduced the serotonin-induced coronary constriction to serotonin in both-sized arteries. Our present results are in agreement with those of Lamping et al. The beneficial vasodilator effect of hydroxyfasudil on coronary vascular response to serotonin is mediated by its action on both vascular smooth muscle and the endothelium as shown in Figure 3. Thus, it is possible that the beneficial effect of Rho-kinase blockade with hydroxyfasudil is mediated by its action on both vascular smooth muscle and the endothelium (22). Serotonin released by aggregating platelets has been implicated for coronary vasospasm in the presence of damaged vascular endothelium (5,23).

**Beneficial effects of a Rho-kinase inhibitor on coronary I/R injury.** In the present study, hydroxyfasudil exerted beneficial effects on I/R-induced endothelial injury in the canine coronary microcirculation in vivo through the NO-dependent mechanism (Figs. 4 and 5). This dose of hydroxyfasudil (100 μg/kg) selectively inhibits Rho-kinase activity and effectively prevents serotonin-induced coronary hyperconstriction. Recent studies have demonstrated that cGMP-dependent protein kinase inhibits RhoA phosphorylation by inhibiting the membrane binding of RhoA, in which the NO-mediated mechanism may inhibit the RhoA/Rho-kinase pathway (24–26). It was previously demonstrated that statins attenuate I/R injury of the heart and the brain in rats and mice, demonstrating the Rho-mediated and NO-dependent protective effect of statins (27,28). Hydroxyfasudil also inhibits the production of superoxide anions in neutrophils (29) and various chemoattractant-induced migration of those cells (14) in a canine model of cerebral ischemia. Furthermore, treatment with hydroxyfasudil in human saphenous vein endothelial cells reversed the hypoxia-induced decrease in eNOS activity as examined by the citrulline conversion assay and 4,5-diaminofluorescein diacetate fluorescence method (13). In the present study, I/R increased Rho-kinase activity, and hydroxyfasudil significantly inhibited the Rho-kinase activation. These findings suggest that NO is involved in the protective effect of hydroxyfasudil with an increase in eNOS activity and a decrease in Rho-kinase activity during reperfusion injury.

In the present study, the vasodilator effects of hydroxyfasudil were significantly attenuated by L-NMMA (Figs. 3 and 4). The eNOS expression was decreased in the ischemic area of the endocardium compared with that of the epicardium under control conditions, which was improved by hydroxyfasudil (Fig. 7). We have previously demonstrated that endocardial arteriolar dilation during reactive hyperemia is more sensitive to L-NMMA than epicardial arte-
riolar dilation (30). These findings indicate that the perfusion of the endocardium is more dependent on NO than that of the epicardium and that endothelial damage after I/R in arterioles may be greater in the endocardium than in the epicardium.

In the present study, hydroxyfasudil exerted cardiovascular protective effects on coronary I/R injury, as did preconditioning (31,32). However, the mechanism by which hydroxyfasudil and preconditioning protect coronary I/R injury appears to be different. Endogenous NO does not alter the infarct size after I/R and is not involved in the protective mechanism of preconditioning in pigs or rabbits (33,34). It has been suggested that preconditioning preserves myocardial creatine phosphate and intracellular pH (35). Furthermore, ischemic preconditioning increases adenosine production and activates protein kinase C, which also enhances adenosine production during I/R injury.

In the present study, hydroxyfasudil significantly reduced myocardial infarct size with increment of coronary collateral blood flow, at least in part, thorough the NO-mediated mechanism (Fig. 8). Shimokawa et al. (11) demonstrated that hydroxyfasudil inhibits both MLC mono- and diphosphorylations. Satoh et al. (14) showed that hydroxyfasudil also protects the brain from ischemic injury through inhibition of superoxide production and neutrophil infiltration. Mohri et al. (36) demonstrated that fasudil suppresses coronary microvascular spasm in patients with microvascular angina. Wolfrum et al. (37) recently demonstrated that inhibiting Rho-kinase has cardioprotective effects to reduce infarct size by activating phosphatidylinositol 3-kinase/protein kinase Akt/eNOS pathways. All these mechanisms may be involved in the beneficial effects of hydroxyfasudil on the I/R-induced myocardial injury.

Hydroxyfasudil increases blood supply to the ischemic region of the myocardium and prevents I/R-induced myocardial injury. Furthermore, it has been recently demonstrated that an estrogen receptor modulator, raloxifene, also reduces I/R-induced myocardial infarct size, whereas an inhibitor of NO synthesis (L-NAME) or a blocker of calcium-activated K$^+$ channels (charybdotoxin) partly attenuates the effect of raloxifene (19). These results suggest that cardioprotective effects of those inhibitors may be mediated in part by the compensatory effects of NO and endothelium-derived hyperpolarizing factor (20). Several studies using NO synthase inhibitors (38,39) or eNOS-deficient mice (40) demonstrated an increase in infarct size after I/R. The effect of NO synthesis inhibition on the infarct size might be species- and dose-dependent.

Clinical implications and conclusions. The present study has demonstrated for the first time that hydroxyfasudil, a specific Rho-kinase inhibitor, has NO-dependent cardiovascular protective effects on coronary I/R injury in vivo. Rho-kinase inhibitor has also an antianginal effect in a canine model of angina (41), patients with effort angina (42), and those with vasospastic angina (43). Moreover, it has been recently reported that hydroxyfasudil may be effective for the treatment of pulmonary hypertension (44). Indeed, Rho-kinase inhibitors may be useful for the treatment of a wide range of cardiovascular diseases (10). The present study suggests that Rho-kinase inhibitors may also be useful for the treatment of coronary I/R injury in humans.

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