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European Journal of Pharmacology 505 (2004) 205–211

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Preventive effect of Y-27632, a selective Rho-kinase inhibitor, on ischemia/reperfusion-induced acute renal failure in rats

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Received 6 May 2004; received in revised form 13 October 2004; accepted 14 October 2004

Abstract

We evaluated the effects of Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate], a selective Rho-kinase inhibitor, on ischemic acute renal failure. Ischemic acute renal failure in rats was induced by clamping the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after the contralateral nephrectomy. Y-27632 administration (1, 10, and 100 μg/kg, i.p.) before ischemia dose-dependently attenuated the ischemia/reperfusion-induced renal dysfunction and histological damage, such as tubular necrosis. The ischemia/reperfusion-induced renal dysfunction was also overcome by postischemic treatment with Y-27632 at 100 μg/kg, i.p. Myeloperoxidase activity in the kidney after ischemia/reperfusion was significantly increased, being the maximal level at 6 h after the reperfusion, and this increase was also suppressed by Y-27632 (100 μg/kg, i.p.). These results indicate that Y-27632 prevents the development of ischemia/reperfusion-induced acute renal failure, and the effect is related to the suppression of the enhanced myeloperoxidase activity in an early phase after reperfusion, thereby suggesting that the Rho/Rho-kinase pathway plays a key role in the pathogenesis of ischemic acute renal failure.

Keywords: Ischemia; Reperfusion; Acute renal failure; Rho-kinase

1. Introduction

A small, monomeric G-protein Rho is known to function as a molecular switch in various cellular functions including regulation of cytoskeleton, cell adhesion, cell motility, G_1 to S cell cycle progression (Hall, 1998; Kaibuchi et al., 1999). Rho is activated by a variety of stimulants, such as lysophosphatidic acid, platelet-derived growth factor, norepinephrine, angiotensin II, and endothelin-1 (Amano et al., 1996; Aoki et al., 1998; Kim et al., 1997), and activated Rho stimulates target proteins, such as Rho-kinase, a serine/threonine kinase (Ishizaki et al., 1996). Rho-kinase regulates vascular contractility by increasing the level of phosphorylated myosin light chain and thereby elevating the Ca^{2+} sensitivity of vascular smooth muscle cells (Kimura et al., 1996; Somlyo and Somlyo, 1998; Feng et al., 1999). Several studies have demonstrated that the inhibition of Rho/Rho-kinase pathway induces the relaxation of vascular smooth muscle (Somlyo and Somlyo, 1998; Amano et al., 2000; Uehata et al., 1997).

Uehata et al. (1997) developed a potent and selective inhibitor of Rho-kinase, Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate], and demonstrated that the administration of Y-27632 to hypertensive animal models markedly reduced blood pressure, thereby suggesting that the Rho/Rho-kinase pathway plays a key role in the development of hypertension. In addition, a multicenter phase II clinical study (Shimokawa et al., 2002) indicated that another Rho-kinase inhibitor, fasudil (Asano et al., 1987), can exert antianginal effects. Pharmacological studies using these Rho-kinase inhibitors have demonstrated a close relationships between Rho/Rho-kinase pathway and the pathogenesis of vascular proliferative disorders and

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doi:10.1016/j.ejphar.2004.10.040

hypertensive diseases (Shimokawa, 2002). Most recently, Y-27632 has been reported to alleviate ischemia/reperfusion-induced injury in the heart and liver (Bao et al., 2004; Ikeda et al., 2003; Takeda et al., 2003). However, there is little available information on the pathological role of the Rho/Rho-kinase pathway in the postischemic renal injury.

The postischemic renal injury occurs frequently in patients after major surgery, trauma, and transplantation. In general, ischemic acute renal failure is induced not only by the ischemia itself but also by the following reperfusion. One of the major causal factors in the postischemic renal injury is infiltration/migration of neutrophil in renal tissues (Linas et al., 1992; Rabb et al., 1994), although several factors, such as ATP depletion, reactive oxygen species, phospholipase activation, and vasoactive peptides, are complicatedly involved in the pathogenesis of this renal damage (Edelstein et al., 1997). The Rho/Rho-kinase signalling system is known to regulate the neutrophil migration via myosin light chain phosphorylation (Niggli, 1999; Saito et al., 2002). Thus, we evaluated whether the ischemia/reperfusion-induced renal injury in rats would be improved by the pre- or postischemic treatment with Y-27632.

2. Materials and methods

2.1. Animals and experimental design

Male Sprague–Dawley rats (10 weeks old, Japan SLC, Shizuoka) weighing 280–320 g were used. The animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed ad libitum access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period, uninephrectmized rats were divided into four groups: (1) sham-operated control; (2) untreated ischemic acute renal failure; (3) preischemic treatment with Y-27632 (1, 10, or 100 μg/kg, i.p.) in acute renal failure; and (4) postischemic treatment with Y-27632 (100 μg/kg, i.p.) in acute renal failure. To induce ischemic acute renal failure, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded for 45 min with a nontraumatic clamp. After the end of the ischemic period, the clamp was released for blood reperfusion. Y-27632 or vehicle (0.9% saline) was administered (preischemic treatment, 5 min before the ischemia; postischemic treatment, 5 min after the reperfusion) as an i.p. bolus injection at a volume of 1 ml/kg. In sham-operated control animals, the left kidney was treated identically, except for clamping. Animals exposed 45-min ischemia were housed in metabolic cages 24 h after the ischemia; 5-h urine samples were taken, and blood samples were drawn from the thoracic aorta at the end of urine collection period. The plasma was separated by centrifugation. These samples were used for measurements of renal functional parameters. The kidneys were excised and examined using a light microscope. In separate experiments, left kidneys were obtained at 2, 6, and 24 h after reperfusion to determine myeloperoxidase activities.

2.2. Renal functional parameters

Blood urea nitrogen and creatinine levels in plasma and urine were determined using a commercial assay kit, BUN-test-Wako and Creatinine-test-Wako (Wako, Osaka, Japan), respectively. Urinary osmolality was measured by freezing-point depression (Fiske Associates, Norwood, MA, USA). Urine and plasma sodium concentrations were determined using flame photometer (205D; Hitachi, Ibaraki, Japan). Fractional excretion of sodium (FE_{Na}, %) was calculated from the formula $FE_{Na}=U_{Na}V/(\text{plasma sodium concentration}\times\text{creatinine clearance})\times 100$, where $U_{Na}V$ is urinary excretion of sodium.

2.3. Renal myeloperoxidase assay

Myeloperoxidase activity in the kidney, which is recognized as an indicator of neutrophil infiltration into renal tissues, was determined using a method described by Chatterjee et al. (2000), with a small modification. Briefly, renal tissue samples obtained at 2, 6, and 24 h after reperfusion were weighed and homogenized for 30 s in 10 volumes of 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0). After freezing/thawing, homogenates were sonicated for 120 s and centrifuged for 10 min at 23,000×*g* at 4 °C. The supernatant was incubated at 60 °C for 2 h in a water bath and centrifuged again. The mixture of 50 μl of supernatant, 50 μl of 16 mM tetramethylbenzidine dissolved in dimethyl sulfoxide, and 400 μl of 80 mM phosphate buffer (pH 7.4) containing 75 μM H_2O_2 was incubated for 3 min at 37 °C. The rate of changes in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was expressed as absorbance/mg protein of the sample.

2.4. Histological studies

Excised left kidneys were processed for light microscopic observation, according to standard procedures. The kidneys were then preserved in phosphate-buffered 10% formalin, after which the kidneys were chopped into small pieces, embedded in paraffin wax, cut at 4 μm, and stained with hematoxylin and eosin. Histopathological changes were analyzed for tubular necrosis, proteinaceous casts, and medullary congestion, as suggested by Solez et al. (1974). Tubular necrosis and proteinaceous casts were graded as follows: no damage (0), mild (1, unicellular,

patchy isolated damage), moderate (2, damage less than 25%), severe (3, damage between 25% and 50%), and very severe (4, more than 50% damage). Degree of medullary congestion was defined by no congestion (0), mild (1, vascular congestion with identification of erythrocytes by ×400 magnification), moderate (2, vascular congestion with identification of erythrocytes by ×200 magnification), severe (3, vascular congestion with identification of erythrocytes by ×100 magnification), and very severe (4, vascular congestion with identification of erythrocytes by ×40 magnification). The scoring of the histological data was done by independent observers in a double-blind manner.

2.5. Drugs

Y-27632 (Mitsubishi Pharma, Osaka, Japan) was dissolved in saline solution. Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and Wako.

2.6. Statistical analysis

Values were expressed as mean±S.E.M. Statistical analysis was performed using one-way analysis of variance followed by a Dunnett-type multiple-comparison test. Histological data were analyzed using Kruskal–Wallis nonparametric test combined with a Steel-type multiple-comparison test. For all comparisons, differences were considered significant at $P<0.05$.

3. Results

3.1. Renal function after the ischemia/reperfusion and effect of preischemic treatment with Y-27632

As shown in Fig. 1, renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured 24 h after the reperfusion. As compared with sham-operated rats, untreated acute renal failure rats showed significant increases in blood urea nitrogen (110.6±5.7 versus 27.4±1.8 mg/dl), plasma creatinine concentration (2.80±0.28 versus 0.67±0.02 mg/dl), urine flow (92.6±5.5 versus 30.6±3.6 μl/min/kg), and FENa (2.52±0.22 versus 0.26±0.09%) and significant decreases in creatinine clearance (1.04±0.21 versus 5.12±0.57 ml/min/kg) and urinary osmolality (420±33 versus 1545±72 mOsm/kg). Preischemic treatment with Y-27632 (1, 10, or 100 μg/kg, i.p.) dose-dependently attenuated the acute renal failure-induced renal dysfunction. The administration of Y-27632 (100 μg/kg) to

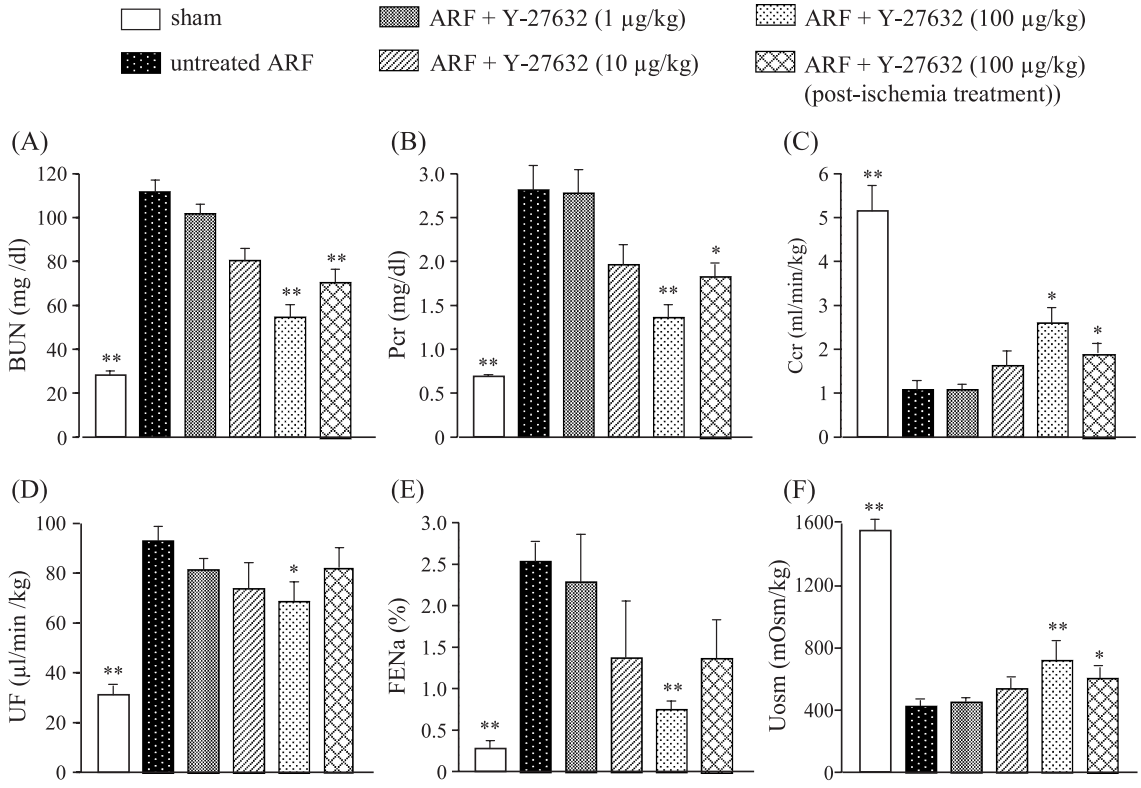

Fig. 1. Effects of Y-27632 administered before ischemia (1, 10, 100 μg/kg, i.p.) or after reperfusion (100 μg/kg, i.p.) on blood urea nitrogen (BUN, A), plasma creatinine concentration (Pcr, B), creatinine clearance (Ccr, C), urine flow (UF, D), fractional excretion of sodium (FENa, E), and urinary osmolality (Uosm, F) at 24 h after ischemia/reperfusion. Y-27632 was given 5 min before ischemia or 5 min after reperfusion. Each column and bar represents the mean±S.E.M. (n=6). *P<0.05, **P<0.01, compared with untreated ARF rats; ARF: acute renal failure.

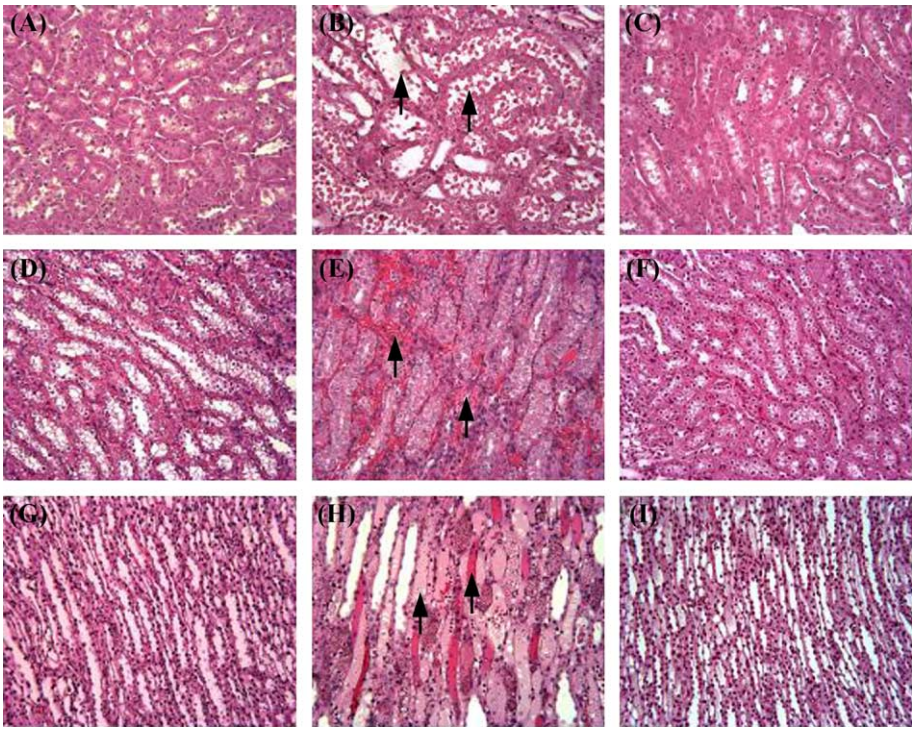

Fig. 2. Light microscopy of outer zone outer stripe (A–C), outer zone inner stripe (D–F), and inner zone (G–I) of medulla of the kidney of ARF rats untreated (B, E, H) and treated with Y-27632 (100 μg/kg, i.p., C, F, I) at 24 h after ischemia/reperfusion, and sham-operated rats (A, D, G). Arrows indicate severe tubular necrosis (B), congestion and hemorrhage (E), and proteinaceous casts in tubuli (H) in untreated ARF rats. These lesions were markedly attenuated in the kidney of Y-27632-treated rats (C, F, I). No histopathological lesion was observed in the kidney of sham-operated rat (A, D, G). Y-27632 was given 5 min before the ischemia; ARF: acute renal failure (hematoxylin–eosin staining, magnification ×200).

sham-operated animals produced no effects in their renal functional parameters (data not shown).

3.2. Histological renal damage after ischemia/reperfusion and effects of preischemic treatment with Y-27632

Histopathological examination revealed severe lesions in the kidney of untreated acute renal failure rats (24 h after the ischemia/reperfusion). These changes were characterized by tubular necrosis in the outer zone outer stripe of medulla (Fig. 2B; scores, 3.33±0.21; Fig. 3), medullary congestion and hemorrhage in the outer zone inner stripe of medulla (Fig. 2E; scores, 3.67±0.21; Fig. 3), and proteinaceous casts in tubuli in the inner zone of medulla (Fig. 2H; scores, 3.50±0.22; Fig. 3). Preischemic treatment with Y-27632 attenuated the development of all these lesions in a dose-

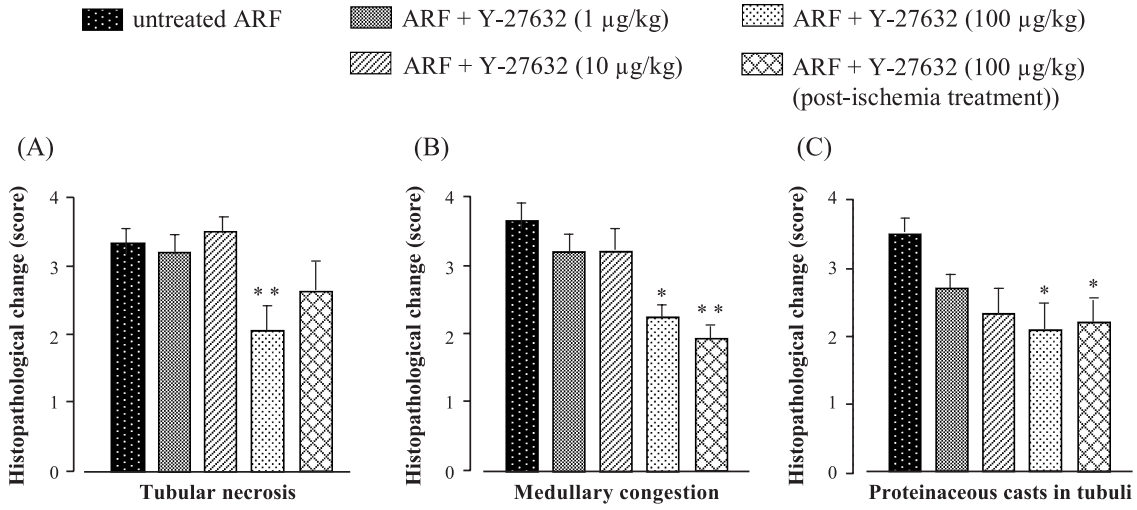

Fig. 3. Effects of Y-27632 administered before ischemia (1, 10, 100 μg/kg, i.p.) or after reperfusion (100 μg/kg, i.p.) on histopathological changes in kidneys of ARF rats. Each column and bar represents the mean±S.E.M. (n=6) of histopathological score. Grades of score: no change (0), mild (1), moderate (2), severe (3), very severe (4). *P<0.05, **P<0.01, compared with untreated ARF rats; ARF: acute renal failure.

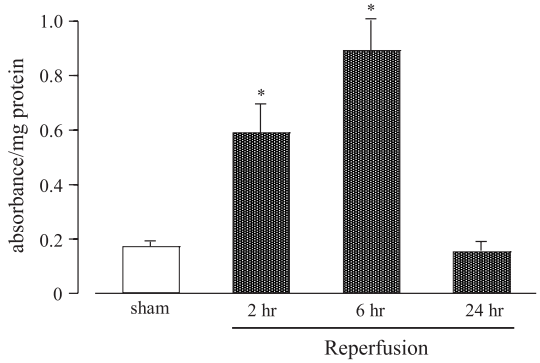

Fig. 4. Myeloperoxidase activities in the kidney of sham and ARF rats at 2, 6, and 24 h after ischemia/reperfusion. Each column and bar represents the mean±S.E.M. (n=6). *P<0.01, compared with sham-operated rats; ARF: acute renal failure.

dependent manner (Fig. 3). Typical photographs of marked improvements observed by Y-27632 (100 μg/kg) treatment are shown in Fig. 2C, F, and I (scores, 2.00±0.45, 2.17±0.17, and 2.00±.52, respectively; Fig. 3).

3.3. Effects of postischemic treatment with Y-27632 on ischemia/reperfusion-induced renal dysfunction and histological renal damage

The effect of postischemic treatment with Y-27632 (100 μg/kg) on the renal dysfunction induced by the 45-min ischemia and reperfusion was shown in Fig. 1. The agent significantly (except for urine flow and FENa) and efficiently improved the renal dysfunction. The histological deterioration induced by the ischemia/reperfusion was also attenuated by Y-27632 administered after the reperfusion (Fig. 3). These improvements by Y-27632 were somewhat less potent compared with the cases of preischemic treatment.

3.4. Effects of Y-27632 on renal myeloperoxidase activities after the ischemia/reperfusion

To evaluate contribution of neutrophil infiltration to ischemic acute renal failure, renal myeloperoxidase activities were determined at 2, 6, and 24 h after the reperfusion. As shown in Fig. 4, there were significant increases in renal myeloperoxidase activities, reaching its maximum level at 6 h after the reperfusion. Preischemic treatment with Y-27632 (10 or 100 μg/kg) dose-relatedly suppressed the increased myeloperoxidase activities (Fig. 5).

4. Discussion

We found that Y-27632 overcame the ischemia/reperfusion-induced renal dysfunction with postischemic, as well as preischemic treatments. In addition, both treatments showed a protective effect against ischemic acute renal failure-induced histological injuries in medullary regions. This is the first report indicating that a selective Rho-kinase inhibitor attenuates the postischemic renal injury in vivo. Thus, Rho-kinase inhibition may be useful in the treatment of ischemia/reperfusion-induced acute renal failure. It is especially noteworthy that the postischemic treatment can efficiently improve this injury, because many clinical cases of ischemic acute renal failure cannot be predicted.

The main functional change in the ischemia/reperfusion-induced acute renal failure is a decrease in glomerular filtration rate and its consequent effect on uremic toxin accumulation, as indicated in this study (Brady et al., 2000). In addition, the medullary thick ascending limb of the loop of Henle and the proximal tubule (pars recta), both situated in the outer medulla of the kidney, are the nephron segments that are most susceptible to ischemic injury, probably because of their high ATP requirements for active solute transport and the regional differences in renal blood flow that render the outer medulla more hypoxic than other regions of the kidney (Brady et al., 2000). These tubular injuries seem to account for marked increase in urine volume and altered urinary osmolality, as seen in this study.

The molecular mechanisms underlying the ischemia/reperfusion-induced renal injury are poorly understood, but several causal factors (ATP depletion, reactive oxygen species, phospholipase activation, vasoactive peptides, etc.) are known to be contributive to the pathogenesis of this renal disease (Edelstein et al., 1997). Another causal factor is infiltration/migration of inflammatory cells, such as neutrophils (Linas et al., 1988; 1992; Rabb et al., 1994). Linas et al. (1988, 1992) noted that mild renal ischemia and primed neutrophils synergistically enhanced renal ischemic injury. In addition, monoclonal antibodies to neutrophil adhesion molecules are known to decrease the

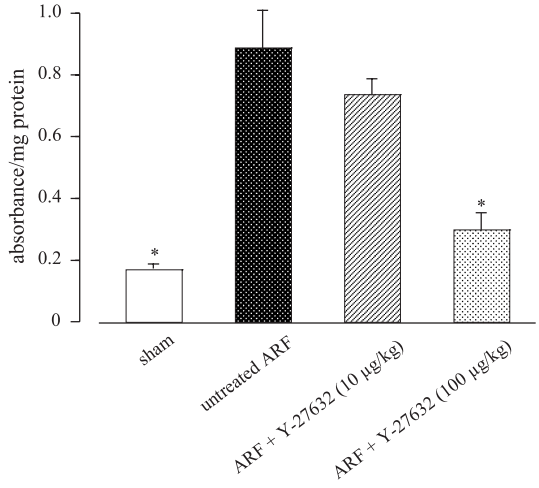

Fig. 5. Effect of Y-27632 (10, 100 μg/kg, i.p.) administered before ischemia on myeloperoxidase activities in the kidney of ARF rats at 6 h after ischemia/reperfusion. Y-27632 was given 5 min before the ischemia. Each column and bar represents the mean±S.E.M. (n=6). *P<0.01, compared with untreated ARF rats; ARF: acute renal failure.

postischemic renal injury (Rabb et al., 1994). Neutrophils release myeloperoxidase, which produces a powerful oxidant, hypochlorous acid from hydrogen peroxide and chloride ion (Eiserich et al., 1998). Therefore, renal myeloperoxidase activities have been used as an index of neutrophil infiltration into renal tissues (Chatterjee et al., 2000; Meldrum et al., 2002). In this study, we observed that renal myeloperoxidase activity in untreated acute renal failure rats increased significantly 2 to 6 h after the reperfusion. However, this increase was suppressed by Y-27632 treatment, suggesting that the infiltration/migration of neutrophils in the postischemic kidney was attenuated by the Rho-kinase inhibition. This attenuation seems to be closely related to the protective effect of Y-27632 against ischemia/reperfusion-induced renal injury. However, since we did not evaluate directly the neutrophil infiltration in the postischemic kidney, further studies are required to determine the mechanisms underlying the ameliorating effect of Y-27632 against the ischemia/reperfusion-induced renal injury.

Rho/Rho-kinase signalling system plays an important role in neutrophil migration and adhesion. Niggli (1999) found that Rho-kinase was activated in human neutrophils exposed to chemotactic peptide and was involved in their motile functions by regulating myosin light chain phosphorylation. A recent study using myocardial ischemia/reperfusion animal model clearly demonstrated that the ischemia/reperfusion up-regulated Rho expression and increased Rho-kinase activity, whereas Y-27632 could decrease ischemia/reperfusion-induced accumulation of neutrophil in the heart, improved cardiac function following ischemia/reperfusion, and attenuated myocardial infarction (Bao et al., 2004). Taken together, a selective and potent inhibitor of Rho-kinase seems to be an attractive agent for application in postischemic organ damage.

There is accumulating evidence that several vasoconstrictor substances, such as norepinephrine and endothelin-1, are at least partly responsible for the pathogenesis of the ischemia/reperfusion-induced renal injury (Brady et al., 2000). Rho-kinase regulates vascular contractility by increasing the level of phosphorylated myosin light chain and thereby elevating the Ca^{2+} sensitivity of vascular smooth muscle cells (Kimura et al., 1996; Somlyo and Somlyo, 1998; Feng et al., 1999). Several studies have demonstrated that the inhibition of Rho/Rho-kinase pathway induces the relaxation of vascular smooth muscle (Somlyo and Somlyo, 1998; Amano et al., 2000; Uehata et al., 1997). Thus, Y-27632-induced attenuation of ischemia/reperfusion-induced renal injury may be related to at least partly the agent-induced renal vasodilation.

In conclusion, Y-27632 prevents the development of ischemia/reperfusion-induced renal injury, thereby suggesting that the Rho/Rho-kinase pathway plays a key role in the pathogenesis of ischemic acute renal failure. A selective and potent inhibitor of Rho-kinase seems to be an attractive agent for application in the ischemic acute renal failure.

Acknowledgement

This work was supported by Grant-in-Aid for High Technology Research and a Grant-in-Aid for Scientific Research 14570092 (to Y.M.) from the Ministry of Education, Science Sports, and Culture of Japan. The authors thank Mitsubishi Pharma (Osaka, Japan) for providing Y-27632.

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