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Partial protective effect of Y-27632, a Rho kinase inhibitor, against hepatic ischemia–reperfusion injury in rats

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Abstract

(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)-cyclohexanecarboxamide dihydrochloride (Y-27632), a Rho kinase inhibitor, has a suppressive effect on the functions of polymorphonuclear leukocytes. In this study, the influence of Y-27632 on ischemia–reperfusion injury of the liver was examined in rats. Y-27632 (3 mg/kg) or vehicle alone was intravenously injected into rats 60 min before occlusion. Blood samples were obtained for 48 h after reperfusion. At the end of the experiment, the hepatic content of myeloperoxidase, which reflects the number of polymorphonuclear leukocytes in liver tissues, was determined. The increases in serum hepatic aminotransferases and inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-6] after reperfusion were partially, but significantly, inhibited by Y-27632. The increased hepatic myeloperoxidase content was significantly lowered by Y-27632. These results suggest that Y-27632 has a partial protective effect against hepatic ischemia–reperfusion injury through the suppression of polymorphonuclear leukocytes and inflammatory cytokines.

Keywords: Hepatic ischemia–reperfusion; Polymorphonuclear leukocyte; Rho kinase inhibitor; Tumor necrosis factor-α; Interleukin-6

1. Introduction

Stimulated by many endogenous and exogenous substances, polymorphonuclear leukocytes adhere to endothelial cells and subsequently generate cytotoxic O_2^- and release enzymes and cytokines (Altstaedt et al., 1996; Weiss, 1989). Although activated polymorphonuclear leukocytes and the O_2^- produced by polymorphonuclear leukocytes play a crucial role in host defense, they are also involved in various conditions such as systemic inflammatory response syndrome and organ damage in chronic illness (Fridovich, 1978; Fujishima and Aikawa, 1995; Yao et al., 1998).

Recent advances in technology and immunosuppressive drugs have remarkably improved the outcome of human liver allografts, including living related liver transplantation. However, donors sometimes suffer from hepatic ischemia–reperfusion injury after a Pringle Maneuver during liver resection. Therefore, to protect against hepatic ischemia–reperfusion injury, effective treatment, including drug therapy, is needed during the perioperative period.

(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)-cyclohexanecarboxamide dihydrochloride (Y-27632) is an inhibitor of a 160-kDa serine/threonine kinase called Rho-associated coiled coil-forming protein kinase (p160 ROCK), which regulates actin stress fiber, focal cell adhesion, and vascular smooth muscle cell contraction (Amano et al., 1997; Hirata et al., 1992; Uehata et al., 1997). It has been reported that p160 ROCK is expressed in polymorphonuclear leukocytes and that Y-27632 suppresses the motile functions of these cells (Niggli, 1999). We recently demonstrated that the drug inhibits the O_2^- production by and aggregation of activated polymorphonuclear leukocytes (Kawaguchi et al., 2000). We also observed an inhibitory effect of Y-27632 on the chemotaxis of polymorphonuclear leukocytes (unpublished). Because animal

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studies showed that hepatic ischemia–reperfusion injury is mainly caused by activated polymorphonuclear leukocytes (Jaeschke et al., 1990, 1993; Langdale et al., 1993), we speculated that Y-27632 has a protective effect against hepatic injury. To address the issue, Y-27632 or vehicle was intravenously injected before occlusion of the left portal vein and hepatic artery in rats. Serum concentrations of liver aminotransferases and inflammatory cytokines after reperfusion were determined in animals treated with or without Y-27632.

2. Methods

2.1. Ischemia–reperfusion injury

Thirteen-week-old adult male Wistar rats (SLC, Shizuoka, Japan) were maintained in a specific pathogen-free room in the animal center of Jichi Medical School and were allowed free access to standard rat chow and water.

We had observed that 3 mg/kg was an adequate dose of Y-27632 under the present conditions (data are shown in Results). Sixty minutes before occlusion, Y-27632 (3 mg/kg) or vehicle alone was intravenously injected. Transient normothermic ischemia of the liver was induced as previously described (Mizuta et al., 1999). A midline incision was made on the abdominal wall under ether anesthesia. A left portal vein and a hepatic artery were occluded with a micro-clip for 60 min ($n=7$ in each group). Sham-operation was also performed ($n=6$ in each group). Peripheral blood (1 ml in each) was obtained from the inguinal vein just before occlusion (-1 h) and at 3, 6, 9, 12, 24 and 48 h after reperfusion. Serum concentrations of alanine aminotransferase and asparate aminotransferase were measured by an ultraviolet method using an automatic analyzer (Hitachi 7170, Tokyo, Japan).

The experiment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Jichi Medical School Guide for Laboratory Animals.

2.2. Myeloperoxidase assay

The hepatic content of myeloperoxidase, which reflects the number of polymorphonuclear leukocytes in the liver, was determined by the method of Shindler et al. (1976). In brief, about 1 cm^3 of liver tissue obtained 48 h after reperfusion was frozen in liquid nitrogen and stored at -80 °C until measurements were performed. Samples were homogenized in phosphate-buffered saline (10 ml) and centrifuged at 100,000 × g at 4 °C for 30 min. Reaction mixture containing the supernatant (10 μl) and 2, 2′ -azino-di-(3-ethyl benzthiazoline-6-sulphonic acid (ABTS) buffer (100 μM, 200 μl) with H_2O_2 was incubated for 60 min at 25 °C in a 96-well plate. The optical density at 414 nm was determined with a plate reader (SPECTRAmax 340, Molecular Devices, Sunnyvale, CA, USA). Myeloperoxidase activity was calculated based on an extinction coefficient of 3.6×10^4 ml^{-1} cm^{-1} for ABTS and normalized to protein levels (μ/mg protein).

2.3. Hepatic histology

Hepatic tissues were fixed in 20% neutral formalin solution, embedded in paraffin, and cut into thin sections using conventional techniques. The sections were stained with hematoxylin–eosin.

2.4. TNF-α and IL-6 assay

To determine the effects of Y-27632 (3 mg/kg) on tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, blood (1 ml in each) was obtained from the inguinal vein just before

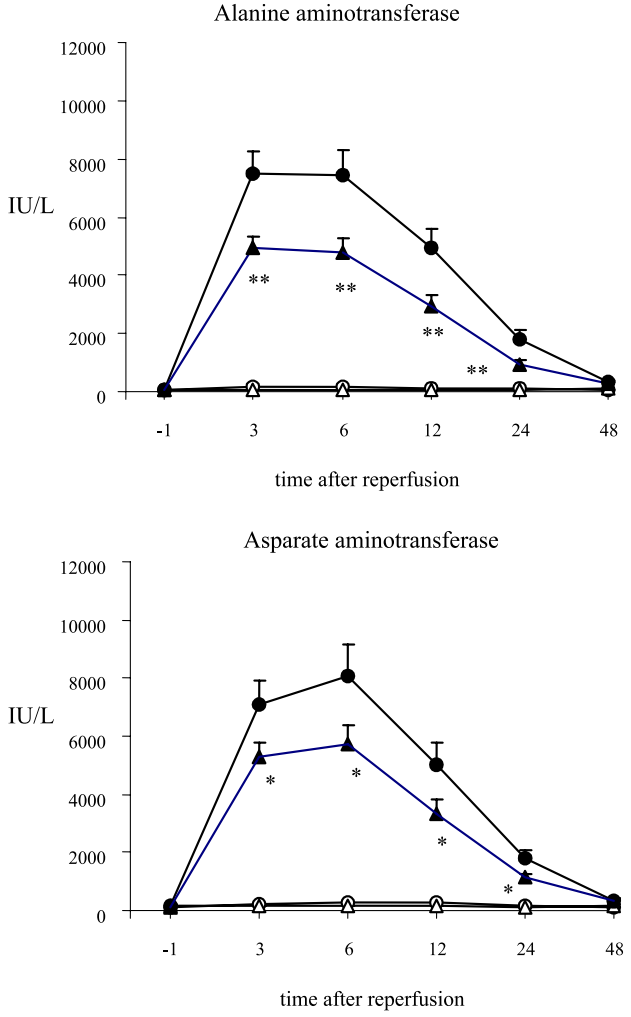

Fig. 1. The time course of serum alanine aminotransferase and asparate aminotransferase concentrations during 48 h after hepatic reperfusion in rats. *$p<0.05$, **$p<0.01$ vs. —●—. Mean ± S.E.; —○—, sham-operated ($n=6$); —△—, sham-operated + Y-27632 ($n=6$); —●—, ischemia–reperfusion ($n=7$); —▲—, ischemia–reperfusion + Y-27632 ($n=7$).

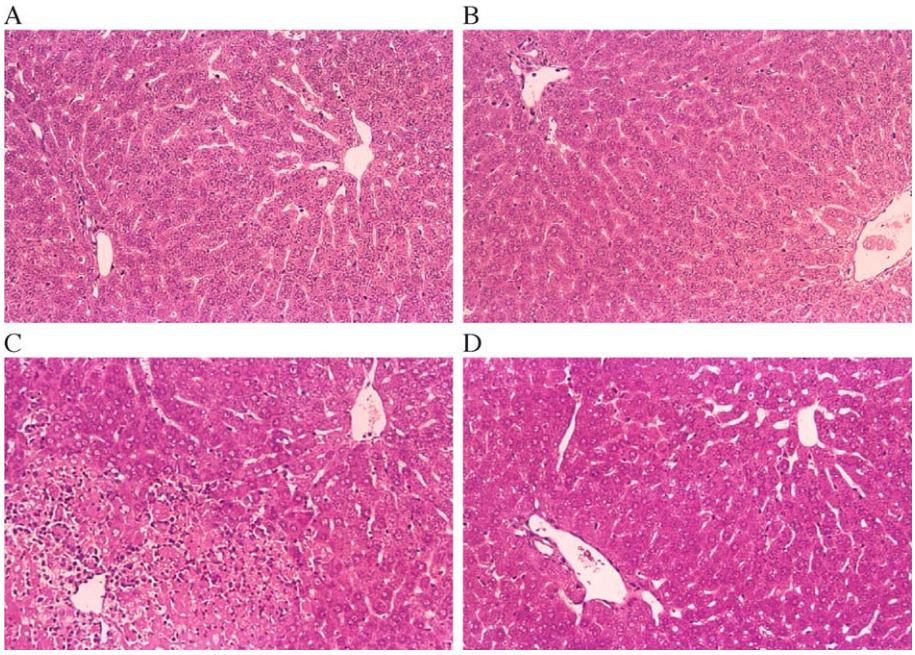

Fig. 2. Histological findings (A) sham-operated; (B) sham-operated + Y-27632; (C) ischemia–reperfusion; (D) ischemia–reperfusion + Y-27632.

occlusion and at 3 h after reperfusion. Serum concentrations of TNF-α and IL-6 were measured with immunoassay kits (Biosource International, CA, USA).

2.5. Statistical analysis

Data are expressed as the means ± S.E. Statistical analysis was done by one-way analysis of variance. The results for alanine aminotransferase and asparate aminotransferase were analyzed by unpaired *t*-test (ischemia–reperfusion group vs. ischemia–reperfusion + Y-27632 group) followed by two-way analysis of variance.

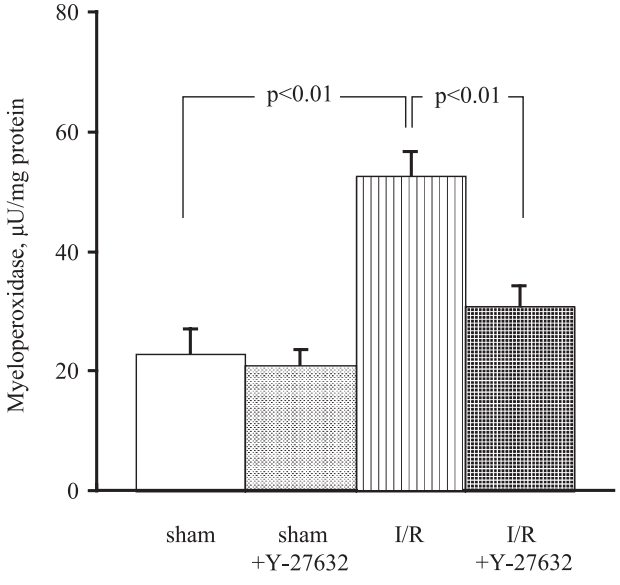

Fig. 3. The hepatic myeloperoxidase content 48 h after hepatic reperfusion in rats. Means ± S.E.; sham: sham-operated ($n = 6$ in each); I/R: ischemia–reperfusion ($n = 7$ in each).

3. Results

A preliminary study showed that systolic blood pressure was significantly decreased at 60 min after a bolus injection of more than 3 mg/kg of Y-27632. In addition, 1 mg/kg of Y-27632 did not inhibit the increase in alanine aminotransferase and asparate aminotransferase at 6 h after hepatic ischemia–reperfusion [mean ± S.E. (IU/l), $n = 3$: alanine aminotransferase, 7596 ± 538 (vehicle) vs. 8159 ± 468 (Y-27632), asparate aminotransferase, 7096 ± 621 (vehicle) vs. 7728 ± 982 (Y-27632)]. Therefore, we used 3 mg/kg of Y-27632 in the following experiment.

Serum concentrations of alanine aminotransferase and asparate aminotransferase increased strongly and reached peak levels around 6 h after liver reperfusion and then gradually decreased (Fig. 1). Y-27632 partially, but significantly ($p < 0.05$), prevented the hepatic ischemia–reperfu-

Table 1
The effect of Y-27632 on serum TNF-α and IL-6 concentrations

	1 h before occlusion	3 h after ischemia–reperfusion
TNF-α (pg/ml)		
Without Y-27632	2.2 ± 0.3	17.3 ± 2.5
With Y-27632	3.6 ± 0.4	7.4 ± 1.4**
IL-6 (pg/ml)		
Without Y-27632	28.6 ± 8.7	177.7 ± 20.8
With Y-27632	17.2 ± 3.4	69.7 ± 24.8**

Means ± S.E. $n = 7$ in each.
** $p < 0.01$ vs. without Y-27632.

sion-related elevation of these aminotransferases. The ischemia–reperfusion-induced damage in hepatic parenchymal cells was ameliorated by the drug (Fig. 2C,D). In addition, the ischemia–reperfusion-induced increase in hepatic myeloperoxidase content was significantly ($p < 0.01$) lowered by the drug (Fig. 3). Y-27632 did not significantly ($p < 0.01$) inhibit the increase in TNF-α and IL-6 induced by ischemia–reperfusion (Table 1).

4. Discussion

When the function of polymorphonuclear leukocytes is evaluated, there is always concern about unexpected effects on this physiological parameter, such as infection. In this study, the animals were kept and blood sampling was performed in a specific pathogen-free room. Therefore, we think that all findings were due to hepatic ischemia–reperfusion alone.

Activated polymorphonuclear leukocytes play a major role in hepatic ischemia–reperfusion injury through a CD11b-dependent mechanism in rats (Jaeschke et al., 1990, 1993). Several drugs are reported to have protective effects by inhibiting the functions of polymorphonuclear leukocytes in this model. For example, tacrolimus, a potent immunosuppressant, protects against such liver damage by inhibiting the migration of polymorphonuclear leukocytes (Garcia-Criado et al., 1997) while the ameliorating effects of prostaglandin E_1 and adenosine are mainly mediated by the suppression of polymorphonuclear leukocyte adhesion and degranulation, respectively (Harada et al., 2000; Natori et al., 1997). In this study, the elevated hepatic myeloperoxidase content in rats with ischemia–reperfusion injury was lowered by Y-27632. This finding is partly explained by the inhibitory effect of the drug on the motile functions of activated polymorphonuclear leukocytes (Niggli, 1999). Thus, we think that Y-27632 inhibited the infiltration of polymorphonuclear leukocytes after reperfusion and consequently protected against hepatic injury. Recently, we showed that the drug inhibits the O_2^- production by and the aggregation of activated polymorphonuclear leukocytes (Kawaguchi et al., 2000), which might be additionally involved in the protective mechanism of Y-27632 against hepatic ischemia–reperfusion injury.

Several factors are also reported to be involved in ischemia–reperfusion injury. Endothelin-1 elicits stellate cell contraction and disrupts blood flow in an early stage of reperfusion and causes hepatic damage (Pannen et al., 1997). Pretreatment with bosentan (an endothelin receptor antagonist) attenuates liver injury in the hepatic ischemia–reperfusion model in rats (Fong et al., 1990). Cytokines such as TNF-α and IL-6 also initiate and maintain the inflammatory response, resulting in reperfusion injury (Arii et al., 1994). In this study, serum concentrations of TNF-α and IL-6 were elevated after hepatic reperfusion, a response which was blunted by pretreatment with Y-27632. Thus, we think that the suppression of inflammatory cytokines is also involved in the protective mechanism of Y-27632.

In this study, Y-27632 had a protective effect against ischemia–reperfusion-induced hepatic injury. Therefore, combination therapy with other agents with different inhibitory mechanisms should be evaluated to achieve a greater improvement of hepatic function in this animal model, which could lead to effective drug therapy for the prevention of hepatic damage in the donors of living related liver transplantation.

In summary, we demonstrated that Y-27632 partially protected against hepatic ischemia–reperfusion injury, probably through suppression of activated polymorphonuclear leukocyte functions and elevated inflammatory cytokines in rats.

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