Rho-kinase inhibitor prevents hepatocyte damage in acute liver injury induced by carbon tetrachloride in rats

Hitoshi Ikeda,1,2 Yukio Kume,1 Kazuaki Tejima,1,2 Tomoaki Tomiya,2 Takako Nishikawa,2 Naoko Watanabe,2 Natsuko Ohtomo,2 Masahiro Arai,3 Chihiro Arai,1 Masao Omata,2 Kenji Fujiwara,4 and Yutaka Yatomi4

1Department of Laboratory Medicine and 2Department of Gastroenterology, The University of Tokyo, Bunkyo-ku; 3Toshiba Hospital, Shinagawa-ku, Tokyo; and 4Yokohama Rosai Hospital, Kohoku-ku, Yokohama-shi, Kanagawa, Japan

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Rho-kinase inhibition is now known to be useful for the treatment of ischemic disease, including cerebral vasospasm after subarachnoid hemorrhage, angina pectoris, or pulmonary hypertension, based on the inhibitory action on cell contractility (1, 33, 34). Furthermore, recent accumulating evidence reveals that Rho-kinase inhibition improves various kinds of diseases besides ischemic disease; Rho-kinase inhibition attenuates angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-deficient mice by inhibiting apoptosis and proteolysis (43), aldosterone-induced renal injury in rats (38), or development of diabetes and nephropathy in insulin-resistant diabetic rats (15).

Regarding liver damage, the administration of Rho-kinase inhibitor leads to improvement of liver fibrosis in rats induced by carbon tetrachloride (CCL4) (21, 22) or dimethylnitrosamine (39). This improvement of liver fibrosis by Rho-kinase inhibitor has been explained by its effects on hepatic stellate cells: inhibition of cell contraction (13, 45) and migration (41, 45), and stimulation of apoptosis (10). Interestingly, however, in the experiment to determine the effect of Rho-kinase inhibitor on CCL4-induced liver fibrosis, not only the improvement of liver fibrosis but also the reduction of serum alanine aminotransferase (ALT) level was reported, where the authors speculated a direct action of Rho-kinase inhibitor to prevent the hepatocyte damage caused by CCL4 (21). To examine this possibility, we investigated whether Rho-kinase inhibitor could prevent the hepatocyte damage in acutely CCL4-intoxicated rats.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were given a standard pellet diet and water ad libitum and were used in all experiments. All animals received humane care, and the experimental protocol was approved by the Animal Research Committee of The University of Tokyo.

Materials. HA-1077 was purchased from Biomol Research Laboratories. Anti-Bcl-2, anti-Bcl-x, anti-Akt, and anti-phospho-Akt (Ser472/473/474) were obtained from BD Biosciences; anti-Bax (N-20) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (V-18) were obtained from Santa Cruz Biotechnology.

Acute liver injury in rats. Rats, weighing 140–180 g, were randomized into the following treatment groups: vehicle, HA-1077 (10 mg/kg body wt) (44), CCL4 (3.0 ml/kg body wt) as a 20% solution in olive oil) (7), CCL4 + HA-1077, CCL4 + wortmannin (15 μg/kg body wt) (44), and CCL4 + HA-1077 + wortmannin. CCL4 was adminis-

Address for reprint requests and other correspondence: H. Ikeda, Dept. of Laboratory Medicine, The Univ. of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan (e-mail: ikeda-lim@h.u-tokyo.ac.jp).

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tered subcutaneously, and HA-1077 and wortmannin were adminis-
tered intraperitoneally (44) at the same time as CCl4 injection.

**Determination of liver function.** Blood samples were collected from rats through the inferior vena cava at 24 h after administrations of CCl4, HA-1077, or wortmannin. The serum level of ALT was determined using an automated analyzer (Hitachi 7170; Hitachi Instruments Service, Tokyo, Japan).

**Histological examination.** Liver histology in formalin-fixed and paraffin-embedded specimens of the liver stained with hematoxylin and eosin was studied with a light microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan) at magnifications ×100, ×200, and ×400 and with a digital camera (Nikon DXM1200F).

**Cells.** Hepatocytes were isolated from rats weighing 140–180 g by in situ perfusion of the liver with type I collagenase (29). The isolated cells were seeded at a density of 5 × 10^4 cells/cm^2 and cultured in Williams’ medium E (ICN Biomedicals, Costa Mesa, CA) with 10% fetal calf serum (GIBCO, Grand Island, NY), 0.9 μM dexamethasone (Takeda Pharmaceutical Industries, Osaka, Japan), and 10 μM Actrapid MC insulin (Novo Industri, Copenhagen, Denmark). At 2 h after seeding, the medium was replaced with serum- and hormone-free Williams’ medium E containing 0.2 μg/ml aprotonin (Sigma Chemical) (24).

**TdT-mediated dUTP nick-end labeling staining.** For the liver specimen, sections 3 μm thick were collected on glass slides coated with poly-l-lysine, and apoptotic cells were detected using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon). The number of TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells was determined as the mean in five different areas at 400-fold magnification in each section. For the cultured hepatocytes, the cells at day 1 on Lab Tek slides (4 wells) were incubated with HA-1077 for 24 h. Apoptotic cells were detected with an in situ cell death detection kit (Roche Applied Science) using the manufacturer’s instructions.

**Cytoplasmic histone-associated DNA fragments assay.** Cultured hepatocytes at day 1 were incubated with various concentrations of HA-1077 for 24 h. Cell death detection ELISA (Roche Applied Science) was used to quantitatively determine cytoplasmic histone-associated DNA fragments associated with apoptotic cell death.

**Caspase-3 activity assay.** Caspase-3 activity was measured using a colorimetric CaspACE kit (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, after homogenization of liver tissue in cell lysis buffer (100 mg/ml), homogenates were centrifuged for 20 min at 10,000 g, and the supernatant was used for the measurement of caspase-3 activity. Caspase-3 activity also was measured at day 1 in the cultured hepatocytes, which were incubated with HA-1077 for 24 h.

**Immunoblot analysis.** After experimental treatment, the medium was discarded and hepatocytes were incubated in 50 mM Tris, pH 7.5, 250 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 0.1 mM Na2VO4, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml aprotonin. Samples containing the same amount of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and were transferred to a sheet of polyvinylidene difluoride membrane (Amersham). To block nonspecific binding, the membrane was soaked in blocking agent derived from skim milk (Blockace; Snow Brand Milk Product, Sapporo, Japan) for 1 h at room temperature. The membrane was then incubated with primary antibody (dilution 1:1000; Bcl-2, Bax, Akt, and phospho-Akt; dilution 1:2000; GAPDH) overnight at 4°C. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:1000) for 1 h at room temperature. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham) and recorded by a chemiluminescence recording system (LAS 1000; Fuji-film, Tokyo, Japan).

**Statistical analysis.** When indicated, statistical analysis was performed by Student’s t-test, and P < 0.05 was considered significant.

**RESULTS**

**Rho-kinase inhibitor prevents hepatocyte damage in acute liver injury induced by CCl4.** To determine whether Rho-kinase inhibition could prevent hepatocyte damage in CCl4-intoxicated rats, we employed the model of acute liver injury induced by CCl4: 3.0 ml/kg CCl4 were administered in rats with or without Rho-kinase inhibitor, HA-1077, at 10 mg/kg body wt, and serum ALT level and liver histology were examined at 24 h after the administration. Because HA-1077 at 10 mg/kg body wt was used to show that inhibition of Rho-kinase leads to cardiovascular protection in rats in vivo (44), the equivalent dose of HA-1077 was examined in rats in this study. As demonstrated in Fig. 1, the serum ALT level was increased to 593 ± 174 IU/l (mean ± SE, n = 8) by CCl4 administration, whereas it was significantly reduced to 82 ± 14 IU/l by cotreatment with HA-1077 (P < 0.05, mean ± SE, n = 8). On the other hand, the serum ALT level of untreated rats was 46 ± 5 IU/l (mean ± SE, n = 3), and administration of HA-1077 alone did not alter the serum ALT level (38 ± 2 IU/l; mean ± SE, n = 3). The ballooning of hepatocytes is one of the earliest, most frequent, and most conspicuous changes seen in the liver injured by CCl4 (31). Histological analysis showed that the cotreatment with HA-1077 reduced this change of ballooning of hepatocytes (Fig. 2, A and B).

Because hepatocyte damage has been attributed to apoptosis in addition to necrosis in acute liver injury induced by CCl4 (31), we examined apoptotic cells in the liver of CCl4-intoxicated rats with or without treatment of HA-1077. Figure 2, C–E, depicts TUNEL-positive cells in the livers of CCl4-intoxicated rats with or without treatment of HA-1077. TUNEL-
positive cells appeared less apparent in the livers of CCl4-intoxicated rats without HA-1077 treatment (Fig. 2D) compared with those with HA-1077 treatment (Fig. 2C); TUNEL staining was noted in the ballooned hepatocytes in the CCl4-intoxicated livers without HA-1077 treatment (Fig. 2E). As shown in Fig. 2F, HA-1077 significantly reduced the number of TUNEL-positive cells in the livers of CCl4-treated rats ($P < 0.01; n = 3$).
Because caspase-3 plays a key role in various forms of apoptosis, we investigated whether this enzyme activity could be altered in the CCl4-intoxicated livers with HA-1077 treatment. As demonstrated in Fig. 2G, HA-1077 treatment significantly reduced caspase-3 activity in the CCl4-intoxicated livers ($P < 0.01; n = 3$).

**Rho-kinase inhibition reduces hepatocyte apoptosis in vitro with activation of phosphatidylinositol 3-kinase/Akt pathway.** We next examined whether Rho-kinase inhibitor could directly prevent hepatocyte damage using rat hepatocytes in vitro. Rat hepatocytes in primary culture do not survive for long periods of time in the absence of serum unless extracellular matrices, growth factors, and/or coculture is employed (8, 30, 36). It has been shown that aprotinin, a protease inhibitor, is capable of maintaining viability of rat primary hepatocytes in culture for over 2 days (2, 8). We used this relatively simple serum-free culture system with aprotinin alone to check a possible direct action of Rho-kinase inhibitor on hepatocytes. First, we checked the status of the cells cultured with serum-free medium with 0.2 µg/ml aprotinin. Although aprotinin did maintain hepatocytes for over 2 days, the significant amount of the cells cultured in the medium with aprotinin alone underwent apoptosis compared with those cultured in the medium with fetal calf serum; histone-associated DNA fragmentation was detected 1.6 times more in the cells cultured with aprotinin alone than in those with fetal calf serum (data not shown). We then examined the effect of HA-1077 on hepatocyte apoptosis by using this serum-starved culture system with aprotinin; rat hepatocytes in culture (day 1) were treated with HA-1077 for 24 h, and the fragmentation of DNA into oligonucleosomal lengths was examined using the TUNEL technique. The treatment with 30 µM HA-1077 appeared to decrease the number of cells containing fragmented DNA (Fig. 3B) compared with the untreated cells (Fig. 3A), and we quantitatively determined the number of cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death. The treatment with HA-1077 reduced histone-associated DNA fragmentation in a concentration-dependent manner, as shown in Fig. 3C. At a concentration of 10 µM, HA-1077 significantly reduced histone-associated DNA fragmentation by 25%, and at 30 µM, by 35%. HA-1077 at 30 µM was used to examine the effect of Rho-kinase inhibition on endothelial cells (44), and 10 µM HA-1077 was specific to inhibit Rho-kinase in smooth muscle cells (23). We then investigated whether caspase-3 activity could be altered in the reduction of DNA fragmentation by HA-1077. When the cells were treated with 30 µM HA-1077 for 24 h, caspase-3 activity was significantly reduced, as demonstrated in Fig. 4A. These results suggest that Rho-kinase inhibition may increase hepatocyte survival cultured.

Next, a possible mechanism by which Rho-kinase inhibition reduces hepatocyte apoptosis was examined by quantifying any...
associated changes in cellular content of proteins that enhance or inhibit apoptosis of the Bcl-2 family. As shown in Fig. 4B, immunoblot analysis of Bcl-2, Bax, or Bcl-x expression in the cells was determined by immunoblotting. GAPDH expression was also analyzed as a control for protein loading. A representative immunoblot of 3 experiments is shown.

Because phosphatidylinositol 3-kinase (PI3-kinase)/Akt is considered a key factor for cell apoptosis (6), we examined a possible involvement of activation of PI3-kinase/Akt pathway in the HA-1077-induced survival in cultured hepatocytes. As shown in Fig. 5, a sharp increase in the level of phosphorylation of Akt was determined at 15 min after the addition of 30 μM HA-1077. We then examined the effect of wortmannin, a PI3-kinase/Akt inhibitor, on the reduction of apoptosis by Rho-kinase inhibition. As depicted in Fig. 6, in the presence of 30 nM wortmannin, the reduction of apoptosis by 30 μM HA-1077 was abrogated. These results suggest that the activation of PI3-kinase/Akt pathway is involved in the mechanism of the reduction of apoptosis by Rho-kinase inhibition.

PI3-kinase/Akt inhibitor abrogates the preventive effect of Rho-kinase inhibitor on hepatocyte damage in acute liver injury induced by CCl₄. Because the activation of PI3-kinase/Akt pathway was found to be involved in the antiapoptotic effect of Rho-kinase inhibition on hepatocytes in vitro, we next examined the significance of activation of PI3-kinase/Akt pathway in the hepatocyte protection by Rho-kinase inhibitor in CCl₄-induced acute liver injury in vivo. The effect of wortmannin, 15 μg/kg body wt, on this acute liver injury model by CCl₄ was determined in the presence or absence of HA-1077. Wortmannin at 15 μg/kg body wt was previously used to inhibit PI3-kinase/Akt pathway in rats in vivo (44). As shown in Fig. 1, wortmannin abrogated the reduction of serum ALT level caused by cotreatment with HA-1077 (322 ± 114 IU/l; n = 8). It also is notable that wortmannin alone did not alter serum ALT level in acute liver injury model by CCl₄ (545 ± 333 IU/l; n = 8). This
result indicates that the activation of PI3-kinase/Akt pathway is necessary for the hepatocyte protection by Rho-kinase inhibitor in CCl4-induced acute liver injury.

**DISCUSSION**

The current study indicates that Rho-kinase inhibitor prevents hepatocyte damage in acute liver injury induced by CCl4 administration in rats, where histological analysis revealed the reduction of the number of apoptotic cells with reduced caspase-3 activity in the liver. Thus, as speculated previously (21), the reduction of serum ALT in CCl4-induced liver fibrosis in rats by Rho-kinase inhibitor may be mediated at least in part by its direct protective effect on hepatocytes. In the course of clarifying the mechanism underlying this preventive effect of Rho-kinase inhibitor on hepatocyte damage, we have found that Rho-kinase inhibitor directly inhibits apoptosis of hepatocytes in rats cultured in serum-free condition.

Reports showing the significance of Rho/Rho-kinase pathway in hepatocytes have been scarce. Unlike fibroblasts or smooth muscle cells, no apparent changes in cell shape or cell adhesion were detected in cultured rat hepatocytes with the addition of the stimulator or inhibitor of Rho or Rho-kinase (not shown), which might suggest that the role of Rho/Rho-kinase would be minor in hepatocytes. However, GTP-bound RhoA, the active form of Rho, is expressed in cultured rat hepatocytes, although in relatively small amounts, and furthermore, Rho activation is involved in the regulation of growth factor-induced DNA synthesis of those cells (11), suggesting that Rho/Rho-kinase pathway may play some role in hepatocytes. In line with this speculation, we have found that Rho-kinase inhibition leads to reduction of apoptosis in cultured rat hepatocytes in serum-free condition. This was found to quantitatively determine cytoplasmic histone-associated DNA fragments associated with apoptotic cell death and was confirmed by reduction of caspase-3 activity and enhanced expression of Bel-2. Activation of Akt by Rho-kinase inhibition seems important to this finding, because a specific inhibitor of PI3-kinase/Akt pathway, wortmannin, abrogated the protective effect of Rho-kinase inhibition on apoptosis of cultured hepatocytes.

As to the contribution of Rho/Rho-kinase pathway to apoptosis, it was first discovered that Rho/Rho-kinase pathway is activated during the execution phase of apoptosis to stimulate apoptotic membrane blebbing (4, 19, 28). Subsequently, reports of participation of Rho/Rho-kinase pathway in not only the execution phase but also the initial phase of apoptosis have been accumulating (18, 25, 26, 32), in which there has been a distinct effect. Rho-kinase inhibition enhanced apoptosis in rat smooth muscle cells (32) or human endothelial cells (18), and in contrast, Rho-kinase inhibition reduced apoptosis in mouse myocytes (3) or mouse vascular wall (43). We demonstrated that Rho-kinase inhibition enhanced apoptosis in rat hepatic stellate cells in the previous study (10), and in the current study we have found that Rho-kinase inhibition reduced apoptosis in rat cultured hepatocytes induced by serum-free condition. The positive or negative regulation of cell survival by Rho-kinase inhibition seems to depend on cell types. The mechanism to explain this distinct effect should be further clarified.

In addition to the improvement of liver fibrosis (21, 22, 39), the administration of Rho-kinase inhibitor is reported to lead to reduction of hepatic ischemia-reperfusion injury in rats (7, 9, 14, 20, 35, 40) and to improvement of septic liver injury in mice (42). These effects are explained by the facts that Rho-kinase inhibitor suppresses infiltration of polymorphonuclear leukocytes, production of inflammatory cytokines (14, 35, 40, 42), or generation of reactive oxygen species (35) and improves microcirculatory disruption through inhibition of hepatic stellate cell contraction (20). Because the mechanism of CCl4-induced liver injury involves inflammatory cytokines (5) and reactive oxygen species (37), the prevention of CCl4-induced acute liver injury by Rho-kinase inhibitor in the current study may be explained by the same mechanism i.e., the suppression of inflammatory cytokines or reactive oxygen species. However, the finding that wortmannin canceled the hepatocyte-protective effect of Rho-kinase inhibitor in CCl4-induced acute liver injury (Fig. 1) suggests that an antiapoptotic effect of Rho-kinase inhibitor on hepatocytes might operate, at least in part, considering the in vitro finding that Akt activation is a key to Rho-kinase inhibitor-induced anti-apoptosis in hepatocyte. Partial cancellation of Rho-kinase inhibitor-induced hepatocyte protection by wortmannin may be in line with this speculation.

In conclusion, Rho-kinase inhibitor prevented hepatocyte damage in acute liver injury induced by CCl4 in rats and merits consideration as a hepatocyte-protective agent in liver injury due to not to just CCl4 but to other insults, considering its direct antiapoptotic effect on hepatocytes in vitro.

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