Attenuation of Dysfunction in the Ischemia-Reperfused Liver by Glycyrrhizin

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ABSTRACT — The present study evaluated the effect of glycyrrhizin (GR) on an injury of the liver caused by ischemia-reperfusion in rats. In the liver ischemia-reperfusion model, levels of serum GOT, GPT and LDH activities and lipid peroxides in the liver tissue were significantly increased. On the contrary, total glutathione content in the liver tissue and NADPH cytochrome P-450 reductase activity of liver microsomes were decreased. Pretreatment with GR 20 mg/kg, i.v. 10 min before induction of ischemia resulted in significant decreases in serum GOT, GPT, LDH activities and the lipid peroxide level and a higher tissue glutathione content during the period of reperfusion. Electron microscopic studies revealed various hepatocellular damages with an almost intact sinusoidal endothelium in ischemia-reperfused livers. However, the degree of damage was less severe in the livers from the rats pretreated with 20 mg/kg GR. The results indicate that GR is able to provide partial protection against ischemia-reperfused damage.

It is now clear that oxygen-derived free radicals play an important role in several models of experimentally induced reperfusion injury (1). Although there are certainly multiple components to clinical ischemic and reperfusion injury, it appears likely that free-radical production may make a major contribution at certain stages in the progression of the injury (2). Moreover, there are many reports that reperfused tissues are protected in a variety of laboratory models by scavengers of superoxide radicals or hydroxyl radicals or by allopurinol or other inhibitors of xanthine oxidase (3–5).

Glycyrrhizin (GR), the main ingredient of licorice, consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid as shown in Fig. 1. GR has been used for the treatment of chronic liver diseases in Japan, and a distinct improvement of liver function tests has been reported (6). The effects of GR as an inducer of interferon (7), competitive inhibitor of ATP binding to the Na-K mem
brane pump (8), binding to protein kinases (9) and effects in altering corticosteroid receptor function (10) have been also reported. However, relatively little information is available concerning the antihapatotoxic mechanism of GR involved in the hepatocellular injury.

Recently, we have reported that administration of GR at a daily dose of 100 mg/kg for 10 days to rats suppressed the elevation of the lipid peroxide level, serum GOT, GPT, LDH activity levels and the decrease in glutathione content caused by ischemia-reperfusion in rat liver, and the mechanism to suppress the liver injury is due to the trapping action of hydroxyl radical induced by ischemia-reperfusion (11). It is known that the plasma concentration of GR injected intravenously reaches its peak in the 10 min following injection and declines in the following 10 hr. If GR possesses a hydroxyl radical-trapping action, a single injection of GR might be a useful administration route for the prevention of liver ischemia-reperfusion injury. In the present study, GR at a single dose of 20 mg/kg administered intravenously produced a significant improvement in posts ischemic hepatic function. Histologic evaluation of the GR-pretreated livers was carried out using electron microscopy, and this has given further insight into the mechanism of warm ischemic liver injury.

MATERIALS AND METHODS

Male Wistar rats weighing 200–250 g obtained from Seiwa Laboratory Animals, Inc. were kept in an environmentally controlled room (20–23°C, 50–60% humidity, illuminated from 7:00 to 19:00 hr) with food and water available ad libitum. Glycyrrhizin (20, 10 mg/kg) or physiological saline solution (0.1 ml/200 g body wt.) was injected via the tail vein 10 min before the induction of ischemia. Sham-operated animals were used as controls. The rats were sacrificed by decapitation.

Preparation of warm ischemic rats

To prepare the warm ischemia-reperfusion rats, the abdomen was opened through a mid-line incision under light ether anesthesia, and the left portal vein and hepatic artery were occluded with a microvessel clip. Later, the abdomen was closed and the animals were allowed to awaken. After 15 min of liver ischemia, the vascular clip was released, and the right lateral and caudate lobes were resected, leaving only the ischemic left lateral and median lobes behind (12). After 60 min of liver reperfusion, the rats were sacrificed.

Assay of lipid peroxides

The liver was perfused with 0.9% NaCl via the hepatic vein before homogenation. The lipid peroxides formed in the liver were determined according to Ohkawa et al. (13). Tetramethoxypropane was used as a standard for the assay, and lipid peroxide content was expressed as nmol of malonic dialdehyde (MDA) formed per mg protein.

Determination of glutathione

Total glutathione, both reduced and oxidized glutathiones, was measured by the method of Griffith (14).

Microsomal cytochrome P-450 and b5 contents and NADPH cytochrome P-450 reductase activity

Livers were removed, weighed and homogenized in 10 vol. 1.15% KCl containing 1 mM EDTA (pH 7.4). The homogenates were centrifuged at 10,000 × g for 20 min. The supernatants were again centrifuged at 105,000 × g for 60 min. Pellets were resuspended in 10 mM phosphate buffer containing 1.15% KCl and 1 mM EDTA, pH 7.4. Cytochrome P-450 and b5 contents were determined by the method of Omura and Sato (15). NADPH cytochrome P-450 reductase activity was measured by the method of Phillips and Langdon (16).

Electron microscopy

For the morphological study, the samples were fixed with modified Dalton's fixative (17) and then dehydrated in a graded series of ethanol solutions and embedded in epoxy resin. Ultrathin sections were prepared on a
Reihert ultramicrotome, picked up on copper grids and stained with methanolic uranyl acetate and lead citrate. The specimens were observed in a JEM-100CX electron microscope.

**Biochemical assay**

Glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and lactate dehydrogenase (LDH) activities in serum were determined by the Shimadzu CL20 Auto Analyzer.

**Protein determination**

The protein concentration was determined by the method of Lowry et al. (18) using bovine serum albumin as a standard.

**Chemicals**

Glycyrrhizin was a gift from Minophagen Co. (Tokyo, Japan). 2-Thiobarbituric acid, tetramethoxypropane, sodium dodecyl sulfate and glutathione reductase were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NADPH and glutathione reductase were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Other reagents were of analytical grade.

**Statistical analysis**

Data are expressed as means ± S.E. The mean values of the SEM index were analyzed for significant differences by Student’s t-test.

**RESULTS**

Changes in serum, liver tissue and microsomal parameters after 60 min reperfusion following 15 min ischemia

Figure 2 shows the changes in serum GOT, GPT and LDH on an injury of the liver caused by ischemia-reperfusion in rats. These serum parameters increased relatively during the periods of ischemia, except for GPT. When reflow of hepatic blood was allowed, the levels of LDH, GOT and GPT in serum were increased about 8-fold, 12-fold and 30-fold after 60 min of reperfusion, respectively.

As shown in Fig. 3, the levels of MDA, which is derived from lipid peroxides in the liver tissue, and the total glutathione contents in these tissues remained unchanged during the ischemia period. When reflow of hepatic blood was allowed, a 2-fold increase of MDA level was observed after 60 min of reperfusion. On the contrary, total glutathione content in the liver tissue decreased about 33% during the reflow periods.

As shown in Fig. 4, liver microsomal cytochrome P-450 and b5 contents and NADPH cytochrome P-450 reductase activity remained unchanged during the ischemic period, while NADPH cytochrome P-450 reductase activity significantly decreased by approximately 10% during the reflow period.

**Treatment with glycyrrhizin**

The levels of serum GOT, GPT, LDH and
Fig. 3. Changes in lipid peroxides and total glutathione content in liver tissue after ischemia and reperfusion. Rats were subjected to hepatic ischemia for 15 min and subsequent reperfusion for 60 min. (○) malondialdehyde formation, (●) total glutathione content. Each point represents a mean ± S.E. of 6 rats. *P < 0.05, **P < 0.01, compared with the untreated rats (Student's t-test).

Fig. 4. Changes in liver microsomal contents of cytochrome P-450 and b5 and NADPH cytochrome P-450 reductase activity. Rats were subjected to hepatic ischemia for 15 min and subsequent reperfusion for 60 min. (■) cytochrome P-450, (△) cytochrome b5 content, (○) NADPH cytochrome P-450 reductase activity. Each point represents a mean ± S.E. of 6 rats. *P < 0.05, compared with the untreated rats (Student's t-test).

Table 1. Effects of glycyrrhizin administration on serum GOT, GPT and LDH levels after 60 min reperfusion following 15 min ischemia

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<tbody>
<tr>
<td>Sham operation</td>
<td>1475 ± 341</td>
<td>192 ± 23</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>15162 ± 1594**</td>
<td>1178 ± 132##</td>
<td>629 ± 123##</td>
</tr>
<tr>
<td>GR-10</td>
<td>14597 ± 3027</td>
<td>1091 ± 180</td>
<td>498 ± 90</td>
</tr>
<tr>
<td>GR-20</td>
<td>9086 ± 1071*</td>
<td>578 ± 87**</td>
<td>234 ± 53*</td>
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LDH: lactate dehydrogenase; GOT: glutamic oxaloacetic transaminase; GPT: glutamic pyruvic transaminase; W.U.: Wroblewski units; K.U.: Karmen units. Sham operation: Sham operated rats were treated in the same manner, except for clamping. GR-10, GR-20: Rats were given glycyrrhizin at 10 mg/kg or 20 mg/kg, i.v., 10 min before induction of hepatic ischemia. ##P < 0.01, compared with the sham operation group. *P < 0.05, **P < 0.01, compared with the ischemia-reperfusion group (Student's t-test). Each value represents a mean ± S.E. of 6–8 rats.
lipid peroxide levels in liver tissue increased with the ischemia-reperfusion of livers, significantly decreased by the pretreatment with 20 mg/kg GR (Table 1). The treatment with GR also suppressed the decrease in total glutathione content in liver tissue (Table 2). The pretreatment with GR at 10 mg/kg had no significant effect (Tables 1 and 2). There was no significant difference in liver microsomal parameters between GR-treated and saline-treated livers from animals that had undergone ischemia-reperfused injury (data not shown).

**Electron microscopic findings**

Electron microscopy of the nonischemic control livers revealed intact, healthy-appearing hepatocytes as well as intact, well-

<table>
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<tr>
<th>Group</th>
<th>Lipid peroxide [nmol MDA/mg]</th>
<th>Glutathione [μmol/g]</th>
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<tbody>
<tr>
<td>Sham operation</td>
<td>3.25 ± 0.54</td>
<td>5.62 ± 0.47</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>5.62 ± 0.24*</td>
<td>4.17 ± 0.25*</td>
</tr>
<tr>
<td>GR-10</td>
<td>4.68 ± 0.61</td>
<td>4.67 ± 0.24</td>
</tr>
<tr>
<td>GR-20</td>
<td>4.14 ± 0.29*</td>
<td>5.10 ± 0.19*</td>
</tr>
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Sham operation: Sham operated rats were treated in the same manner, except for clamping. GR-10, GR-20: Rats were given glycyrrhizin at 10 mg/kg or 20 mg/kg, i.v. 10 min before induction of hepatic ischemia. *P < 0.05, compared with the sham operation group. *P < 0.05, compared with the ischemia-reperfusion group (Student's t-test). Each value represents a mean ± S.E. of 6–8 rats.

**Fig. 5.** Electron micrograph of nonischemic liver. × 5,200. Note intact endothelium (arrows) and normal appearing hepatocytes (H). The mitochondria are oval or rod-like in shape and possess lamellar cristae and a relatively dense matrix.
Fig. 6. Electron micrograph of an ischemia-reperfused liver. × 5,200. A. Note that the sinusoid (S) is lined by the endothelial cells with fenestrae (arrows). The mitochondria (M) become slightly swollen. B. Note the nonviable hepatocyte with disrupted mitochondria (M) and destruction of the space of Disse. The sinusoidal lumen is filled with numerous erythrocytes (Er).
preserved sinusoidal endothelial cells. The hepatocytes possessed mitochondria with lamellar cristae and a somewhat dense matrix, rough endoplasmic reticulum, smooth endoplasmic reticulum and lysosomes. The sinusoidal lining was composed of the endothelium with fenestrations, forming a smooth covering over the microvilli protruding from the parenchymal cells (Fig. 5). The ischemia-reperfused livers resulted in various morphological changes. When the sinusoidal endothelium remained intact, hepatocytes were relatively viable (Fig. 6A). Most of the hepatocytes, however, possessed a great number of somewhat swollen mitochondria which had irregular forms and a clear matrix. Chromatin clumping, loss of the plasma membrane, flocculent densities in mitochondria and vacuolization of the cytoplasm were also observed, as was a marked loss of the granulations of the endoplasmic reticulum. In contrast, the sinusoidal endothelium was disrupted in areas adjacent to nonviable hepatocytes (Fig. 6B). The nonviable hepatocytes had disrupted mitochondria and destruction of the space of Disse was also seen.

Thin sections from ischemia-reperfused livers pretreated with GR at 20 mg/kg showed relatively normal-appearing hepatocytes. Most of the hepatocytes were well-preserved and were similar to those of nonischemic hepatocytes in ultrastructure, except that the mitochondria looked a little swollen. The cristae showed a lamellar configuration, and the matrix had almost normal density. Most of the sinusoidal endothelial lining was relatively pre-

Fig. 7. Electron micrograph of an ischemia-reperfused liver pretreated with glycyrrhizin at 20 mg/kg. × 5,200. Note the relatively normal-appearing hepatocytes and intact endothelium (arrows).
served, although it was found to be destroyed in part (Fig. 7).

DISCUSSION

Liver cells are protected from oxygen-derived radical injury by naturally occurring free-radical scavengers and antioxidant pathways. When these protective mechanisms are overwhelmed, however, liver tissues become susceptible to damage by oxygen radicals that peroxidate lipids and disturb cell membrane function (19). Therefore, many compounds have been demonstrated to protect against oxidative damage by inhibiting free radicals and reactive oxygen species. Catalase, superoxide dismutase (SOD) and allopurinol have been shown to protect against reperfusion injury mediated by oxygen free radicals (4, 5, 20). Yoshikawa et al. (21) have reported that the combination of SOD and catalase is more effective than the treatment of SOD alone. However, it appears that the presence of SOD and catalase is not always sufficient to protect against hydroxyl radical-induced damage by enzymes that generate superoxide anion and H₂O₂.

Das et al. (22) indicated the presence of hydroxyl radical during early reperfusion in the ischemia-reperfused tissue, which decreased in intensity as the reperfusion progressed. Zweier et al. (23) demonstrated that endothelial cells subjected to anoxia and reoxygenation, conditions observed in ischemic and reperfused tissues, generate a burst of superoxide-derived hydroxyl radicals that in turn cause cell injury and cell death. Hydroxyl radical is extremely reactive and readily reacts with a number of compounds, including lipids and proteins (1), while it has been demonstrated that the antioxidants, dimethylsulfoxide and dimethylthiourea, beneficially possess hydroxyl radical-trapping ability which contributes to their suppressive effect on myocardial ischemic injury (24, 25). These reports support the important role of hydroxyl radicals in ischemic organ injury and moreover, introduce a potential therapeutic approach to organ ischemia-reperfusion and preservation through the direct inhibition of hydroxyl radicals. Previously, we found that GR is one of the most potent hydroxyl radical scavengers developed to date, judging from the result of the 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)-OH radical trapping ability of GR as observed by electron spin resonance (11).

In this study, a single dose of GR (10 mg or 20 mg/kg) was injected intravenously 10 min before the induction of ischemia. Dose-dependent protective effects against warm ischemia-reperfusion injury were clearly demonstrated after rats pretreated with even a single dose of GR. Serum GOT, GPT, LDH and lipid peroxides and glutathione content in liver tissue exhibited significant improvement when compared with ischemia-reperfused controls (saline-treated) in the livers from rats pretreated with 20 mg/kg of GR. When a lower dose of 10 mg/kg of GR was administered, no significant improvement in liver function was demonstrated for any parameter measured when compared with ischemia-reperfused controls. From these results, it is suggested that the hydroxyl radical trapping action of GR is partially concerned with its suppressive effect on an injury of liver caused by ischemia-reperfusion, but another action may also play an important role in protecting the fragile membrane.

The morphologic appearance of the non-ischemic control livers was clearly identical to those of normal liver without any evidence of hepatocellular or endothelial injury. The general pattern of injury found in the ischemia-reperfused livers and the ischemia-reperfused livers pretreated with GR (20 mg/kg) both had almost the same characteristics, showing mitochondria with irregular, swollen configurations. Nonviable hepatocytes were scattered among the viable cells. However, the degree of injury appeared less severe in the group pretreated with GR; the foci of damage were smaller and less abundant than in the ischemia-reperfusion (saline-treated) group. The sinusoidal endothelium was relatively spared even in the most severely damaged
warm ischemia-reperfused livers, while hepatic injury involving parenchymal cells occurred. This is of singular importance because the primary abnormality seen in livers after cold ischemic or preservation injury is endothelial disruption (26). Although sinusoidal endothelial injury was apparent in livers injured by cold ischemia-reperfusion, the hepatocytes showed manifest normal hepatocellular architectures (27, 28). This difference in morphology between after warm and cold ischemia-reperfusion has only recently been appreciated and may lend further credence to the belief that warm and cold ischemia represent two different and distinct types of injury (29).

In the present study, the morphologic findings reveal a pattern of hepatic injury involving primarily parenchymal cells after warm ischemia-reperfusion that differs significantly from the primarily endothelial injury seen subsequent to cold ischemia. Therefore, pharmacologic amelioration of ischemic injury will involve the administration of GR, which will not only protect against parenchymal cellular injury and disruption, but will also maintain the endothelial integrity of the organ.

REFERENCES


