Am J Physiol Gastrointest Liver Physiol 311: G305–G312, 2016.
First published June 30, 2016; doi:10.1152/ajpgi.00439.2015.

GGsTop, a novel and specific γ-glutamyl transpeptidase inhibitor, protects hepatic ischemia-reperfusion injury in rats

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Submitted 11 December 2015; accepted in final form 27 June 2016

Tamura K, Hayashi N, George J, Toshikuni N, Arisawa T, Hiratake J, Tsuchishima M, Tsutsumi M. GGsTop, a novel and specific γ-glutamyl transpeptidase inhibitor, protects hepatic ischemia-reperfusion injury in rats. *Am J Physiol Gastrointest Liver Physiol* 311: G305–G312, 2016. First published June 30, 2016; doi:10.1152/ajpgi.00439.2015.—Ischemia-reperfusion (IR) injury is a major clinical problem and is associated with numerous adverse effects. GGsTop [2-amino-4{[3-(carboxymethyl)phenyl]-(methyl)phosphono}butanoic acid] is a highly specific and irreversible γ-glutamyl transpeptidase (γ-GT) inhibitor. We studied the protective effects of GGsTop on IR-induced hepatic injury in rats. Ischemia was induced by clamping the portal vein and hepatic artery of left lateral and median lobes of the liver. Before clamping, saline (IR group) or saline containing 1 mg/kg body wt of GGsTop (IR-GGsTop group) was injected into the liver through the inferior vena cava. At 90 min of ischemia, blood flow was restored. Blood was collected before induction of ischemia and prior to restoration of blood flow and at 12, 24, and 48 h after reperfusion. All the animals were euthanized at 48 h after reperfusion and the livers were harvested. Serum levels of alanine transaminase, aspartate transaminase, and γ-GT were significantly lower after reperfusion in the IR-GGsTop group compared with the IR group. Massive hepatic necrosis was present in the IR group, while only few necroses were present in the IR-GGsTop group. Treatment with GGsTop increased hepatic GSH content, which was significantly reduced in the IR group. Furthermore, GGsTop prevented increase of hepatic γ-GT, malondialdehyde, 4-hydroxynonenal, and TNF-α while all these molecules significantly increased in the IR group. In conclusion, treatment with GGsTop increased glutathione levels and prevented formation of free radicals in the hepatic tissue that led to decreased IR-induced liver injury. GGsTop could be used as a pharmacological agent to prevent IR-induced liver injury and the related adverse events.

GGsTop; γ-glutamyl transpeptidase; γ-GT; ischemia; ischemia-reperfusion; 4-hydroxynonenal; TNF-α

HEPATIC ISCHEMIA-REPERFUSION (IR) injury is a major clinical problem and is associated with numerous adverse events such as hypovolemic shock (9), disseminated intravascular coagulation (34), liver transplant surgery (2), cardiac failure and arrest, increased toxicity of alcohol, and several other pathological conditions. Since liver requires higher amount of oxygen for the innumerable biochemical reactions, the impairment of blood flow rapidly causes hepatic hypoxia, which could progress to absolute anoxia, particularly in the pericentral regions of the hepatic lobe. Subsequent reperfusion leads to activation of Kupffer cells, which are resident macrophages of the liver, which in turn contribute to the production of reactive oxygen species (ROS) such as superoxides, hydrogen peroxide, and hydroxyl radicals that trigger cellular impairment leading to liver inflammation (19). Oxidative stress can also leads to increased lipid peroxidation and induces structural and functional derangements leading to cell death (22, 31). In addition, proinflammatory cytokines, chemokines, and other mediators produced by the impaired cells contribute to postischemic tissue injury, systemic inflammatory syndrome, and multiorgan failure. In conjunction with the activated complement factors, these inflammatory mediators activate and recruit neutrophils into the postischemic liver, which generates increased ROS and synthesize excessive proteases such as matrix metalloproteases (MMPs) and other degradative enzymes (16–18, 21). In addition to inflammatory response, ROS induces expression of endothelin-1 leading to vasoconstriction of sinusoids (5). This results in heterogeneous closure of many hepatic microvessels and prolongs ischemia in certain areas of the liver even after reperfusion (3, 31). Therefore, it is important to find appropriate modalities to arrest hepatic IR injury and subsequent tissue damage.

γ-Glutamyl transpeptidase (γ-GT), also named γ-glutamyl transferase or γ-glutamyl peptidyltransferase, is a heterodimeric enzyme found widely in organisms ranging from bacteria to mammals (14). The mammalian enzymes are anchored to the outside surface of plasma glutathione (GSH) and its S-conjugates via cleavage of the γ-glutamyl amide bound by hydrolysis and/or transpeptidation (13). Serum γ-GT has been widely used as an index of liver dysfunction and a marker of alcohol abuse (34). γ-GT catalyzes the first step in GSH degradation and transfers the γ-glutamyl moiety of GSH to water (hydrolysis) and amino acids or peptides (transpeptidation) into glutamate and γ-glutamyl-amino acids or peptides, respectively, with a by-product cysteinyl-glycine (26). This cysteinyl-glycine is one of the most reactive thiol compounds that possess very high physiological activity, and it has been reported that this particular thiol can reduce oxygen under normal physiological conditions by reducing ferric iron Fe^{3+} into Fe^{2+} (27). This process is known as iron redox cycling and produces ROS that subsequently facilitates an oxidative reaction (10, 11). The activity of γ-GT is highest in tissues with a transport function, such as kidney and biliary system (32).

Acivicin [L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125; produced by *Streptomyces sviceus*)] has been widely used as an inhibitor of γ-GT (6). Accatino et al. (1) reported that inhibition of γ-GT with acivicin resulted in decreased secretion of the enzyme and its biliary levels and a

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marked increase of biliary GSH secretion and its levels in rat model of hepatic IR injury. However, acivicin irreversibly inhibits various glutamine amidotransferases, including imidazole glycerol phosphate synthase and guanine monophosphate synthetase, and inactivates a number of biosynthetic enzymes for purine and pyrimidine, amino acids, and amino sugars, which results in a potent cytotoxicity (7, 12). These findings indicate that γ-GT is not a natural target of acivicin but is inhibited fortuitously by acivicin.

GGsTop [2-amino-4{[3-(carboxymethyl)phenyl](methyl)-phosphono}butanoic acid], a novel phosphonate and mechanism-based irreversible inhibitor of γ-GT, exhibits its activity toward human γ-GT over 100-fold higher compared with acivicin and specifically inhibits γ-GT but does not affect glutamine amidotransferase (13). GGsTop is an electrophilic phosphonate phenyl ester, chemically stable, nontoxic, that can be used for in vivo purposes. GGsTop covalently binds between the side chain oxygen of Thr-381 of human GGT1 (hGGT1) and the phosphate of GGsTop, resulting in an enzyme-inhibitor complex (28). Yamamoto et al. (33) reported that no abnormalities were observed in general symptoms, body weight, and amount of food intake for 2 wk after intravenous administration of GGsTop at a single dose of 30 or 100 mg/kg body wt. Only less than 4% of the compound is hydrolyzed in neutral water over a month (33). Therefore, we hypothesized that in vivo administration of GGsTop could protect the liver against IR injury and subsequent adverse effects. The aim of the present investigation was to evaluate the protective effects of GGsTop on IR-induced hepatic injury in rats.

MATERIALS AND METHODS

Animals and experimental design. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 86-23, revised 1996). The protocol was also approved by the Animal Care and Research Committee of Kanazawa Medical University on the Ethics of Animal Experiments. Wistar male rats were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). They were housed in a temperature- and humidity-regulated room with 12-h light/dark cycles and allowed ad libitum access to food and water.

At about 9 wk old, male rats were randomly divided into four groups of six rats each as sham group (body weight 307.6 ± 6.8 g), IR group (body weight 293.1 ± 4.2 g), sham treated with GGsTop (body weight 298.6 ± 3.7 g), and IR treated with GGsTop (body weight 306.0 ± 7.1 g). The animals were anesthetized with intraperitoneal injections of pentobarbital (4.8 mg/100 g body wt), and the abdomen was shaved and disinfected with 70% ethanol. An upper abdominal ventral incision was made without harming the internal organs, and 1 ml of blood was obtained from inferior vena cava of all rats in the four groups.

Ischemia was induced by clamping the portal vein and hepatic artery with a vascular microclip (30–60 g/mm^2, catalog no. AM-1, Bear Medic, Kuji-gun, Ibaraki-ken, Japan) to the left lateral and median lobes of the liver. This procedure yields ~70% partial ischemia (8, 22). The right and caudate lobes (30% of liver mass) retain intact portal and arterial inflow and venous outflow, preventing intestinal congestion (8). In both the ischemic groups of animals, 1 ml of normal saline (IR group) or 1 ml of normal saline containing 1 mg/kg body wt of GGsTop (Institute for Chemical Research, Kyoto University, Japan) (IR-GGsTop group) was injected into the inferior vena cava, and the portal vein and hepatic artery were clamped instantly (less than 5 s). The laparotomy was closed by suturing immediately after the start of ischemia and the animals were observed for 80 min maintained at 37°C on a warm pad. Abdominal incision was made again and 1 ml of blood was obtained from inferior vena cava at 90 min after ischemia, and blood flow was restored by unclamping the vessels (23). The color of ischemic lobes was restored gradually within 1 to 1.5 min. Then laparotomy was closed again using suture. Two groups of rats that are not subjected to IR were considered as sham groups. One sham group was treated with GGsTop and counted as sham-GGsTop group.

At 12 and 24 h after reperfusion, 1 ml of blood was obtained from orbital venous plexus of each rat via a capillary tube. At 48 h after reperfusion, all the animals were euthanized and the blood was collected immediately. The livers were quickly removed and the median lobe was cut into 3-mm pieces and fixed in 10% phosphate-buffered formalin for histopathology, and the remaining liver tissue was flash frozen in liquid nitrogen and stored at −80°C until assayed. A schematic representation of the study protocol is presented in Fig. 1.

Measurement of ALT, AST, and γ-GT levels in serum. Blood was allowed to clot for 3–5 h at 37°C and the serum was separated by the conventional method. Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured via an autoanalyzer. Serum γ-GT activity was determined by using L-γ-glutamyl-3-carboxy-4-nitroanilide (no. FG11354, Funakoshi, Bunkyo-ku, Tokyo, Japan) according the method of Theodorsen and Stromme (29).

Measurement of γ-GT, GSH, IL-1β, and MDA in the liver. Tissue hepatic levels of γ-GT, GSH, interleukin-1β (IL-1β), and malondialdehyde (MDA) were measured in the liver tissues as follows. About 100 mg of frozen liver tissue was homogenized in 1 ml of ice-cold 50 mM Tris·HCl buffer (pH 8) containing 150 mmol/l NaCl, 1 mmol/l EDTA, and 1% Triton X-100. Then 50 μl of the homogenate was treated with 150 μl of 5% 5-sulfosalicylic acid solution and vortexed well. It was allowed to stand on ice for 10 min and centrifuged at 12,000 *g* for 10 min at 4°C. Then 50 μl of the supernatant was used to measure hepatic γ-GT activity as per the protocol used for serum (29). The activity of γ-GT in the liver homogenate was presented as units per milligram protein. One unit of γ-GT catalyzes the transfer of one micromole of the glutamyl moiety from γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine per minute at 37°C.

Hepatic GSH content was measured by using 50 μl of the supernatant diluted with 50 μl of distilled water employing glutathione assay kit (catalog no. CS0260, Sigma-Aldrich, St. Louis, MO). Hepatic IL-1β level was measured by using 50 μl of the supernatant diluted with 50 μl of distilled water using rat IL-1β assay kit (no. 27193, Immuno-Biological Laboratories, Fujioka, Gunma, Japan). Hepatic MDA content was determined with 30 μl of the supernatant diluted 1:1 with distilled water by using MDA assay kit (no. NWK-MDA01, Northwest Life Science, Vancouver, WA).

Histopathological evaluation of the liver tissue. The formalin-fixed liver tissues were processed in an automatic tissue processor optimized for liver tissue, embedded in paraffin blocks, and cut into sections of 5-μm thickness. The sections were stained with hematoxylin and eosin (H&E) as per the standard protocol. The stained sections were examined under an Olympus BX50 microscope attached with a DP 71 digital camera (Olympus, Tokyo, Japan) and photographed. The degree of hepatic necrosis was quantified as per

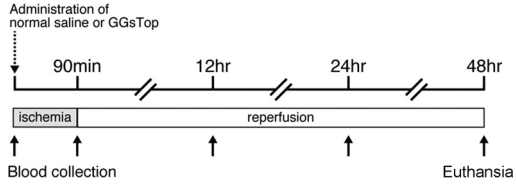

Fig. 1. Schematic illustration of the experimental design.

the protocol of Ishak et al. (15) and presented as scores of 0–4, where 0 is absence of necrosis, 1 is mild (focal necrosis, few lobules), 2 is mild/moderate (focal necrosis, most lobules), 3 is moderate continuous necrosis (<50% of lobules), and 4 is severe continuous necrosis (>50% of lobules). The score was calculated after examination of ten lobules in each liver.

Immunohistochemical staining for 4-HNE and TNF-α. Immunohistochemical staining of tumor necrosis factor-α (TNF-α) and 4-hydroxynonenal (4-HNE) was carried out on paraffin liver sections to examine the increased production of TNF-α and ROS caused by IR, respectively. The liver sections were deparaffinized with xylene and alcohol and hydrated to water. For antigen retrieval and exposure, the slides for TNF-α staining were autoclaved at 95°C for 20 min in citric acid buffer (catalog no. S2031, DAKO, Bunkyo-ku, Tokyo, Japan) and cooled to room temperature. Immunohistochemistry was performed using a broad-spectrum histostain kit (Invitrogen, Carlsbad, CA). After blocking, the liver sections were treated with TNF-α rabbit polyclonal antibody (catalog no. ab6671, Abcam, Chuo-ku, Tokyo, Japan) and 4-HNE mouse monoclonal antibody (no. HNE-J2, Nikken Seil, Shizuoka, Japan) and incubated in a moisturized chamber (Evergreen Scientific, Los Angeles, CA) at 4°C overnight. The sections were then washed thrice in cold phosphate-buffered saline and incubated with broad-spectrum biotinylated secondary antibody for 2 h at room temperature. The slides were washed again and treated with streptavidin-peroxidase conjugate and incubated for another 1 h. The final stain was developed by using 3% 3-amino-9-ethylcarbazole in *N*,*N*-dimethylformamide. The stained sections were washed and counterstained with Mayer's hematoxylin for 2 min and mounted with aqueous-based mounting medium. The slides were examined under a microscope (Olympus BX50) attached with a digital camera (Olympus DP71) and photographed. The staining intensity in 10 randomly selected microscopic fields was quantified by using WinRoof image analyzing software (Mitani, Fukui, Japan). Data are presented as percentage square micrometers, where the sample with maximum staining intensity (μm^2) was considered as 100%.

Statistical analysis. Arithmetic mean and standard deviation (SD) were calculated for all the data and presented as means ± SD. All data were analyzed and compared by one-way analysis of variance or Student's *t*-test as per the situation. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Serum levels of ALT, AST, and γ-GT before and after ischemia-reperfusion and effect of treatment with GGsTop. As depicted in Fig. 2, there was no difference in the serum levels of ALT, AST, and γ-GT before ischemia among sham, IR, sham-GGsTop, and IR-GGsTop groups. In sham and sham-GGsTop groups, the mean serum levels of ALT, AST, and γ-GT at 90 min after ischemia and at 12, 24, and 48 h after reperfusion were not altered compared with the respective levels before ischemia. Serum ALT level at 90 min after ischemia in both IR and IR-GGsTop group was significantly increased (224.3 ± 163.1 and 163.7 ± 45.4 IU/l, respectively) compared with the respective levels before ischemia. The increased level was not significantly different between the two groups. At 12 h after reperfusion, serum ALT level was dramatically increased to 2,719 ± 1,325 IU/l in the IR group, which was 10-fold higher compared with the level at 90 min after ischemia. In the IR-GGsTop group also, serum ALT level was increased to 775.7 ± 337.2 IU/l at 12 h after reperfusion, but the level was significantly ($P < 0.05$) lower compared with the IR group. At 24 h after reperfusion, serum ALT level in the IR group was still high (2,507 ± 1,664 IU/l), but at 48 h the

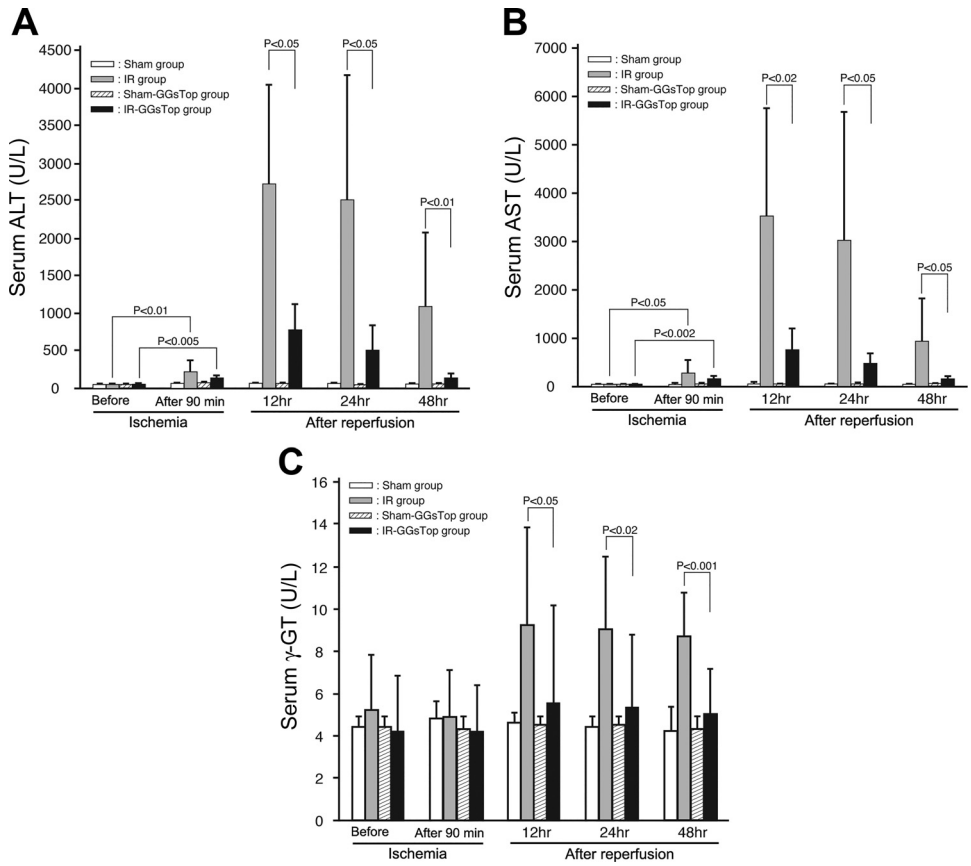

Fig. 2. Serum levels of ALT, AST, and γ-GT before and after ischemia-reperfusion and effects of GGsTop treatment. *A*: serum ALT levels. Serum ALT levels at 12, 24, and 48 h after reperfusion in the IR-GGsTop group were significantly lower than the respective levels in the IR group. *B*: serum AST levels. Serum AST levels at 12, 24, and 48 h after reperfusion in IR-GGsTop group were significantly lower compared with the respective levels in the IR group. *C*: serum γ-GT activities. Treatment with GGsTop prevented increase of serum γ-GT levels after reperfusion. Values are means ± SD ($n = 6$).

level decreased to 1,080 ± 994.3 IU/l, which was significantly ($P < 0.01$) lower compared with the level at 12 h after reperfusion. On the other hand, in the IR-GGsTop group, serum ALT level decreased to 506.0 ± 342.8 IU/l at 24 h and to 135.5 ± 70.19 IU/l at 48 h after reperfusion, which was significantly lower ($P < 0.05$ at 24 h and $P < 0.01$ at 48 h) compared with the respective levels in the IR group (Fig. 2*A*). Serum AST levels after ischemia and reperfusion in IR and IR-GGsTop groups depicted an almost similar pattern as in the case of serum ALT (Fig. 2*B*).

Serum γ-GT levels in ischemia and after reperfusion are demonstrated in Fig. 2*C*. There was no difference in serum γ-GT activities between IR and IR-GGsTop groups after ischemia. In the IR group, serum γ-GT activities at 12, 24, and 48 h after reperfusion were significantly higher ($P < 0.05$) than at 90 min after ischemia. On the other hand, in the IR-GGsTop group, there was no difference between serum γ-GT activities before and after reperfusion at any time point. Serum γ-GT activities at 12, 24, and 48 h were significantly lower ($P < 0.05$, $P < 0.02$, and $P < 0.001$, respectively) after reperfusion in the IR-GGsTop group compared with the IR group.

Alteration of γ-GT, GSH, IL-1β, and MDA in the liver after ischemia-reperfusion and the effects of GGsTop treatment. As depicted in Fig. 3*A*, there was no difference in hepatic γ-GT activity between sham and sham-GGsTop groups. Hepatic γ-GT activity in the IR group was 8.72 ± 1.30 units/mg protein, which was significantly higher compared with sham group (4.38 ± 1.47 units/mg protein). On the other hand, hepatic γ-GT activity in the IR-GGsTop group was 4.66 ± 1.09 units/mg protein, which was significantly lower compared with the IR group.

Figure 3*B* represents the hepatic GSH content in the sham, IR, sham-GGsTop, and IR-GGsTop groups. GSH content in the IR group was lower (4.18 ± 3.07 μmol/mg protein) compared with sham group (5.77 ± 1.27 μmol/mg protein), although the difference was not significant. There was no difference in hepatic GSH content between sham-GGsTop and IR-GGsTop groups.

As demonstrated in Fig. 3*C*, there was no difference in hepatic IL-1β levels among sham, IR, sham-GGsTop, and IR-GGsTop groups.

Hepatic MDA levels in sham, IR, sham-GGsTop, and IR-GGsTop groups are presented in Fig. 3*D*. MDA level in the IR group significantly ($P < 0.01$) increased to 3.47 ± 0.45 μmol/mg protein compared with sham group (1.86 ± 0.15 μmol/mg protein). In IR-GGsTop group, the mean hepatic MDA level (1.94 ± 0.31) was not different from the level in sham-GGsTop group (1.87 ± 0.25 μmol/mg protein). Hepatic MDA level in the IR group was significantly higher ($P < 0.01$) compared with the IR-GGsTop group (Fig. 3*D*).

Treatment with GGsTop prevented hepatic necrosis. The histological alterations of liver after IR and the effects of GGsTop treatment are presented in Fig. 4*A*. There were no histological alterations in the liver tissue obtained from sham

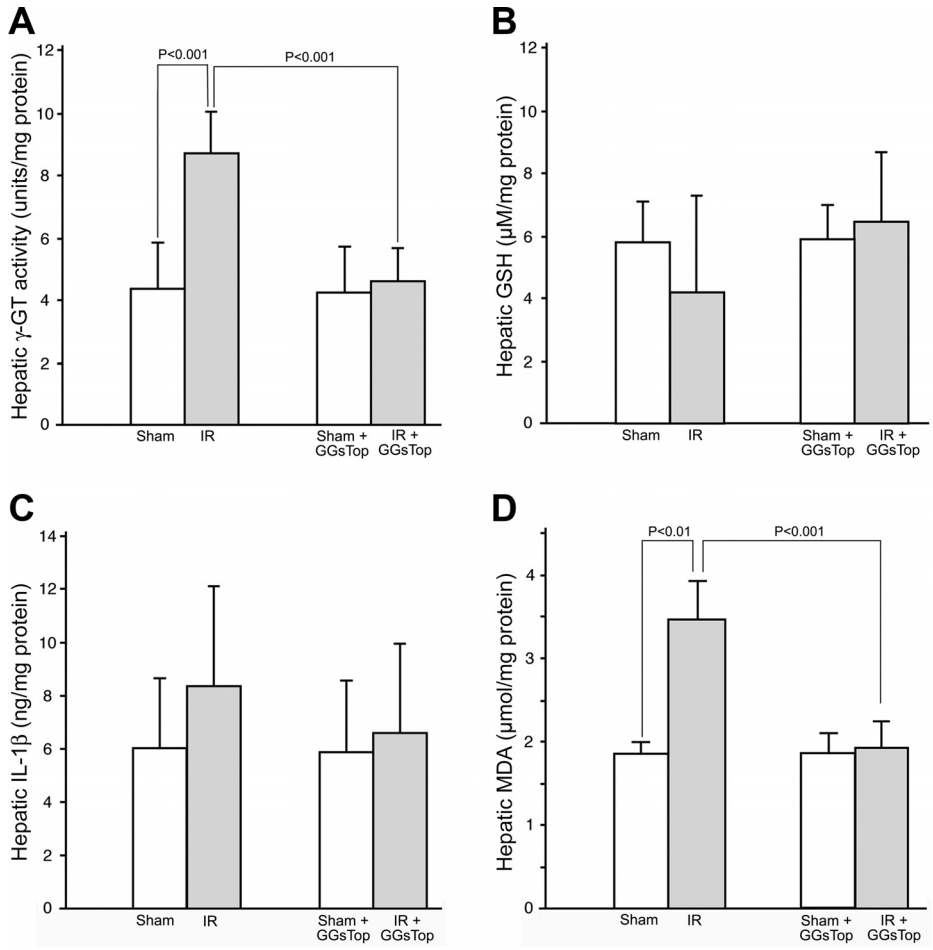

Fig. 3. Levels of γ-GT, GSH, IL-1, and MDA in the liver after ischemia-reperfusion and effects of GGsTop treatment. *A*: hepatic γ-GT activities. Treatment with GGsTop completely prevented the increase of hepatic γ-GT activity. *B*: hepatic GSH contents. There was no significant difference in hepatic GSH content between the groups. *C*: hepatic IL-1β. Hepatic IL-1β did not show any significant alteration between the groups. *D*: hepatic MDA levels. There was significant increase of MDA in the IR group. Treatment with GGsTop maintained normal levels of hepatic MDA. Values are means ± SD ($n = 6$).

and sham-GGsTop groups. Massive necrosis with infiltration of mononuclear cells was observed in many hepatic lobules in the IR group. Massive necrosis was more prominent in pericentral areas (arrow). On the other hand, only few necroses were present in the rat livers obtained from IR-GGsTop group. Quantitative analysis of the degree of hepatic necrosis presented in Fig. 4*C* clearly indicates a significant decrease ($P < 0.001$) after treatment with GGsTop.

GGsTop decreased production of 4-HNE and TNF-α. The results of the immunohistochemical staining of 4-HNE are presented in Fig. 4*B*. The staining for 4-HNE was completely absent in the livers obtained from sham and sham-GGsTop groups. On the other hand, marked and strong staining of 4-HNE was present in the hepatocytes around necrosis in the IR group, while little and weak staining was observed, especially in pericentral areas in the IR-GGsTop group (Fig. 4*C*).

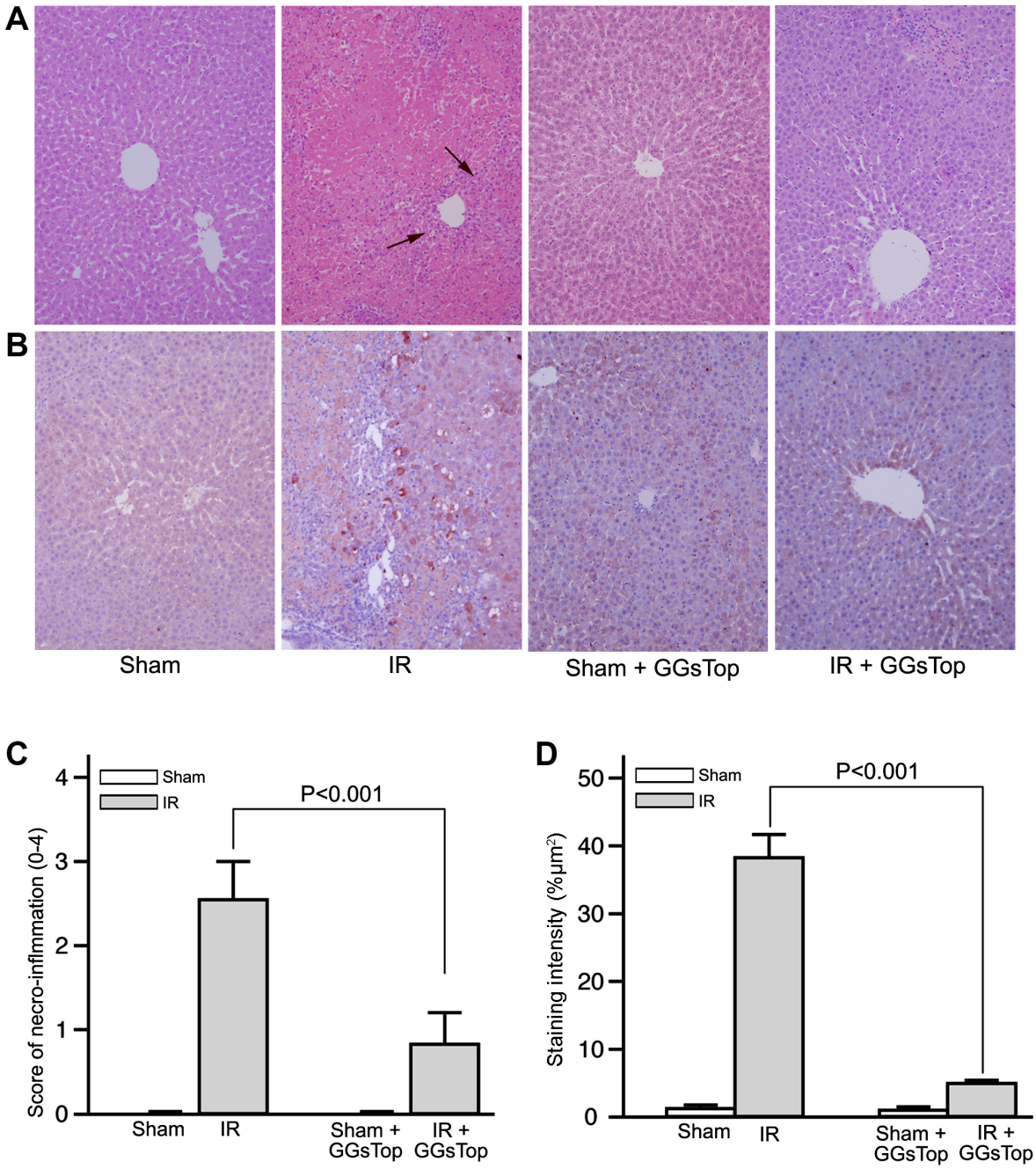

Fig. 4. Histopathological alterations and staining of 4-HNE after ischemia-reperfusion and effects of GGsTop treatment. *A*: H&E staining of liver sections from sham, IR, sham-GGsTop, and IR-GGsTop groups. Massive hepatic necrosis was present in the IR group, which was prominent in pericentral areas (arrow). Only mild necrosis was present in IR-GGsTop group. GGsTop treatment prevented massive hepatic necrosis and retained intact cellular architecture (×100). *B*: immunohistochemical staining for 4-HNE in the liver sections from sham, IR, sham-GGsTop, and IR-GGsTop groups. Marked staining of 4-HNE was present in hepatocytes around necrosis in the IR group, while only weak staining was present in pericentral area in IR-GGsTop group. Treatment with GGsTop prevented formation of 4-HNE during reperfusion (×100). *C*: quantification of the scoring of hepatic necrosis. *D*: quantitative analysis of the staining intensity of 4-HNE. Data are means ± SD of 6 rats per group.

Quantitative analysis of the staining intensity of 4-HNE is presented in Fig. 4*D*. The intensity of 4-HNE staining was significantly high ($P < 0.001$) in the IR group compared with IR-GGsTop group.

The staining of TNF-α in sham, IR, sham-GGsTop, and IR-GGsTop groups are depicted in Fig. 5. Staining for TNF-α was completely absent in sham and sham-GGsTop groups. Intense and strong staining of TNF-α was observed in macrophages that infiltrated into the necrotic zone in the IR group (arrow). In contrast, staining for TNF-α was very weak in the livers obtained from IR-GGsTop group (Fig. 5*A*). The number of TNF-α-positive cells was also much less compared with the IR group. Quantification of the staining intensity of TNF-α is presented in Fig. 5*B*. The staining intensity was significantly high ($P < 0.001$) in the IR group compared with the IR-GGsTop group.

DISCUSSION

Ischemic tissue injury is a major problem in healthcare and occurs when the blood supply to an area of tissue is cut off. The incidence of ischemic injury could lead to serious problems including myocardial infarction, stroke, and other thrombotic process and also affects transplant surgery. In addition, as an "insult to injury," the restoration of blood supply or reperfusion of ischemic tissue led to increased injury mainly due to enhanced production of free radicals. So it is important to prevent generation of free radicals and ROS during reperfusion. In the present study, we demonstrated that GGsTop, a specific γ-GT inhibitor, could prevent reperfusion-induced hepatic injury in rats.

One of the aims of the present study was to investigate whether ischemia alone or reperfusion after ischemia causes more severe liver damage. Therefore, we carried out 90 min of warm ischemia although it is longer than the period of time with vascular occlusion habitual at the clinical practice. Besides, thrombosis of portal vein and/or hepatic artery results in longer than 90 min of ischemia. This model was successfully used by Peralta et al. (24); they subjected the animals to 90 min of ischemia, followed by 90 min of reperfusion. We obtained blood from the rats at 90 min after ischemia and at 12, 24, and 48 h after reperfusion. The prominent biomarkers of liver damage, serum levels of ALT and AST, were around 10-fold higher at 12 h after reperfusion compared with their levels at 90 min after ischemia. The results indicate that reperfusion could exacerbate the liver damage induced by ischemia. The elevated

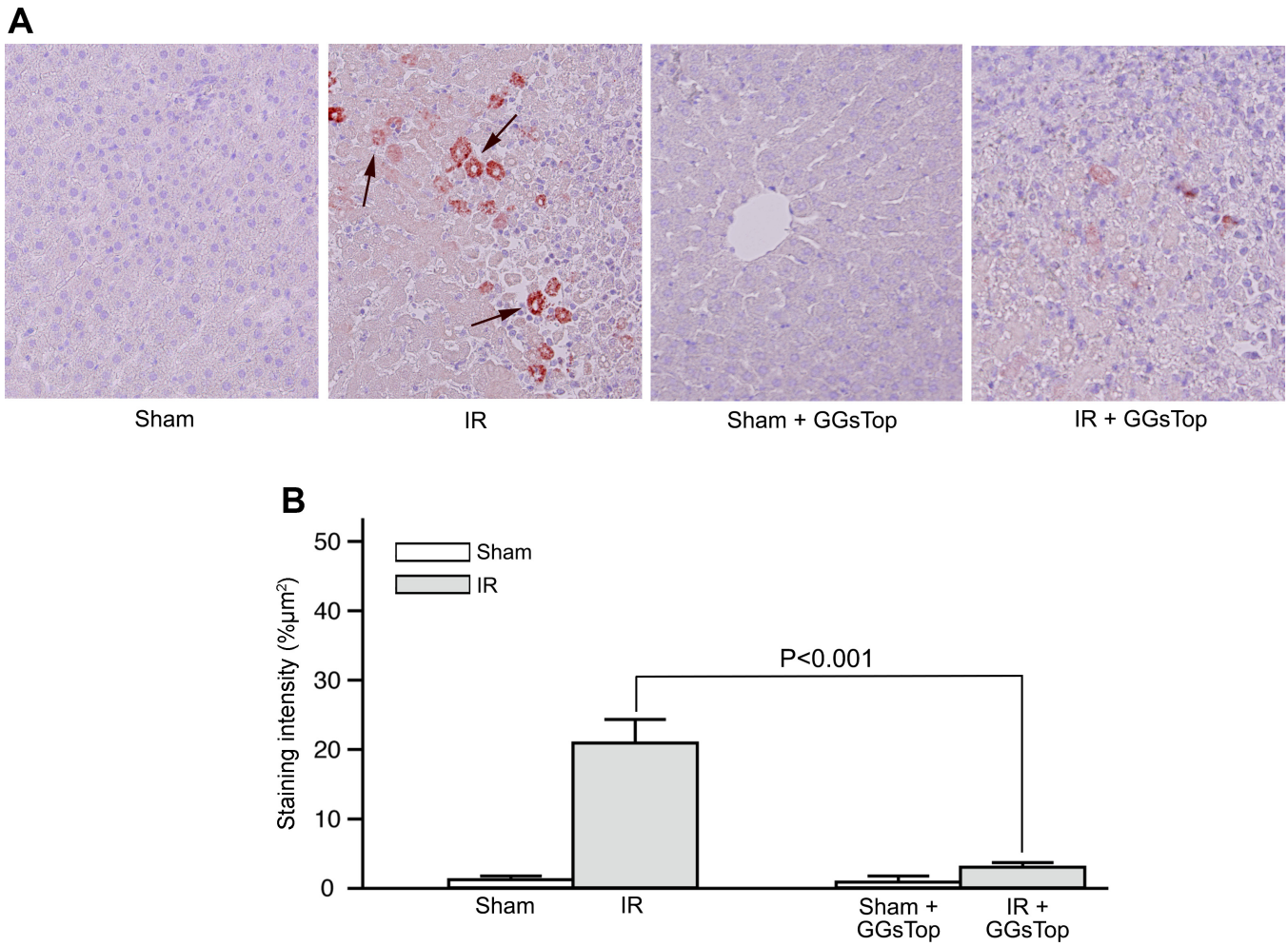

Fig. 5. Immunohistochemical staining of TNF-α after ischemia-reperfusion and effects of GGsTop treatment. *A*: staining of TNF-α in the liver sections from sham, IR, sham-GGsTop, and IR-GGsTop groups. Staining for TNF-α was completely absent in sham and sham-GGsTop groups. Marked staining of TNF-α was present in hepatic macrophages that infiltrated into necrotic zone in the IR group, while only feeble staining was present in IR-GGsTop group (×200). *B*: quantitative analysis of the staining intensity of TNF-α. Data are means ± SD of 6 rats per group.

levels of serum ALT and AST at 12 h were retained almost the same level at 24 h also after reperfusion, suggesting that hepatic IR injury may be persistent for at least 24 h. At 48 h after reperfusion, the elevated serum AST and ALT levels decreased to almost 50% of its level at 24 h after reperfusion. However, the levels were still significantly high compared with their level at 90 min after ischemia, suggesting that it may take over 48 h to return the elevated AST and ALT levels to normal after IR-induced hepatic injury.

It has been well demonstrated that GGsTop is a novel, highly specific, potent, and irreversible inhibitor of γ-GT, so we employed the compound in the present study. It was shown that GGsTop is a phosphonate-based and mechanism-based irreversible inhibitor of GGT, exhibits its activity toward human GGT (hGGT) more than 100 times higher than that of acivicin, inhibits only GGT, and does not inhibit glutamine amidotransferases (13). In addition, Yamamoto et al. (33) demonstrated that GGsTop prevents ischemia-reperfusion-induced acute renal injury and stated that the renoprotective effect of GGsTop seems to be attributed to the suppression of oxidative stress by inhibiting γ-GT activity, thereby preventing the degradation of glutathione. Furthermore, Tuzova et al. (30) reported that inhibition of γ-GT activity in lung lining fluid increased glutathione levels and protected lung airway epithelial cells against oxidant injury associated with inflammation in a mouse of model of asthma and recommended GGsTop as a novel pharmacological agent for the treatment of asthma. In the present study we observed that treatment with GGsTop significantly decreased the serum levels of ALT and AST at 12, 24, and 48 h after reperfusion. In addition, GGsTop treatment also prevented the increase of γ-GT after reperfusion at all time points and maintained the same level as in sham group. These data suggests that GGsTop could be used as a pharmacological agent to prevent IR-induced hepatic injury.

Transfer of γ-glutamyl moiety of GSH by γ-GT with the by-product cysteinyl-glycine results in production of ROS and subsequent oxidative reaction (10, 11). In the present study, there was no increase of serum γ-GT activity at 90 min after ischemia in either the IR or the IR-GGsTop group. However, after reperfusion, serum γ-GT activity increased significantly in the IR group. Hepatic γ-GT activity was also increased in the IR group. On the other hand, in the IR group, hepatic GSH content decreased and hepatic MDA, 4-HNE, and TNF-α increased. These results indicate that the increased γ-GT activity induced by IR may play a significant role in IR-induced hepatic injury.

Significantly elevated hepatic γ-GT activity was observed in rat livers treated with Lieber-DeCarli liquid diet containing 5% ethanol (20). Rats treated with ethanol after IR depicted increased hepatic injury compared with the rats with IR alone (unpublished data), suggesting that the increased hepatic γ-GT activity could be one of the causes to produce IR-induced hepatic injury. In the present study, hepatic γ-GT activity in the sham group treated with GGsTop was not altered compared with sham group without GGsTop treatment. One of the possibilities in which GGsTop did not reduce hepatic γ-GT activity in the sham-GGsTop group could be that GGsTop may circulate and affect extrahepatic organs. To test this, we measured γ-GT activity in the kidney after treatment with GGsTop, since kidney has the highest γ-GT activity compared with other organs. But there was no significant difference in γ-GT activity in the kidney between sham and sham-GGsTop groups (data not shown). In the present study, we euthanized the animals at 48 h after injection of GGsTop to remove liver and kidney. This long duration of time could also be contributed to the absence of difference in the hepatic γ-GT activity between sham and sham-GGsTop groups.

Reperfusion after ischemia leads to the activation of Kupffer cells, which serve as liver macrophages. Activated Kupffer cells release several inflammatory mediators and produce ROS that leads to cell impairment contributing to liver injury. The increased inflammatory mediators further activate and recruit neutrophils into the postischemic liver (16, 17). Activated Kupffer cells and infiltrated neutrophils contribute to increased production of IL-1β after reperfusion (4). Using a rat leg model of IR, Seekamp et al. (26) noticed that IL-1β increased dramatically after reperfusion and peaked at 2 h; they suggested IL-1β may contribute to the development of postischemic syndrome. However, in the present study we did not observe any significant difference in hepatic IL-1β levels between sham, IR, sham-GGsTop, and IR-GGsTop groups. This could be attributed to the time point of sample collection after reperfusion: Seekamp et al. collected at 2 h and the present study at 48 h.

In the present study, we employed GGsTop, a highly specific and potent inhibitor of γ-GT, to prevent ROS-mediated cellular impairment and hepatic injury during reperfusion after ischemia. Treatment with GGsTop completely prevented increase of γ-GT activity in serum after reperfusion. In addition, hepatic γ-GT activity was significantly lower in the IR-GGsTop group compared with the IR group, which suggests GGsTop treatment prevented the increase of both serum and hepatic γ-GT activity induced by IR. This led to an increase of hepatic GSH content in the IR-GGsTop group, which in turn resulted in significant decrease of hepatic necrosis. These data are substantiated by marked decrease in hepatic MDA, TNF-α, and 4-HNE in the IR-GGsTop group. The observation of increased infiltration of hepatic macrophages (Kupffer cells) and the strong staining of TNF-α proved that the activated macrophages secretes TNF-α in the IR group, which was very minimal in the IR-GGsTop group. These results strongly suggest that GGsTop could prevent ROS-mediated hepatic injury during experimentally induced IR in rats.

In conclusion, in the present study we observed a significant elevation of serum and hepatic γ-GT activity in experimentally induced IR. Treatment with GGsTop completely prevented the elevation of γ-GT, which led to an increase of GSH levels and subsequent reduction of oxidative stress and ROS, which altogether resulted in decreased IR-induced liver injury. Taken together, the results of the present study indicate that GGsTop could be used as a pharmacological agent to prevent IR-induced hepatic injury and related adverse events.

ACKNOWLEDGMENTS

The authors are thankful to Mitsuru Araya for excellent technical assistance to carry out the animal experiments.

GRANTS

This work was supported in part by Grant for Specially Promoted Research from Kanazawa Medical University (SR 2013–2015) to M. Tsutsumi. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.T., N.H., J.G., N.T., J.H., M. Tsuchishima, and M. Tsutsumi conception and design of research; K.T., N.H., and M. Tsutsumi performed experiments; K.T., N.H., and J.G. prepared figures; K.T. and M. Tsutsumi drafted manuscript; K.T., N.H., J.G., N.T., T.A., J.H., M. Tsuchishima, and M. Tsutsumi approved final version of manuscript; N.H., J.G., and M. Tsutsumi analyzed data; J.G., N.T., T.A., J.H., M. Tsuchishima, and M. Tsutsumi interpreted results of experiments; J.G., N.T., T.A., J.H., M. Tsuchishima, and M. Tsutsumi edited and revised manuscript.

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