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ORIGINAL ARTICLE

Ischemic Preconditioning and Intermittent Clamping Confer Protection Against Ischemic Injury in the Cirrhotic Mouse Liver

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Surgery on cirrhotic livers is fraught with complications, and many surgeons refrain from operating on patients with cirrhosis. Surgical procedures include temporal occlusion of blood flow resulting in ischemia. The mechanisms of protective strategies to prevent ischemic injury in patients with cirrhosis are not fully understood. The aim of this study was to evaluate how the cirrhotic liver tolerates an ischemic insult, whether mechanisms other than those observed in the normal liver are active, and whether intermittent clamping and preconditioning, which are known as safe surgical strategies in normal and steatotic livers, confer protection to the cirrhotic liver. We applied partial hepatic inflow occlusion to cirrhotic mice fed carbon tetrachloride according to different vascular occlusion protocols: intermittent clamping with 15 or 30 minute cycles of ischemia or ischemic preconditioning prior to 60 or 75 minutes of ischemia. Continuous ischemia (60 or 75 minutes) served as controls. The results showed that the cirrhotic liver was significantly more susceptible to 60 minutes of ischemia than the normal liver. Apoptosis was higher in the normal liver, whereas necrosis was a predominant feature in the cirrhotic liver. Both protocols of intermittent vascular occlusion and ischemic preconditioning dramatically prevented injury compared to continuous occlusion for 60 minutes. This protection was associated with reduced necrosis and apoptosis, and particularly reduced activation of the apoptotic pathway through mitochondria. In conclusion, this study extends the protective effects of ischemic preconditioning and intermittent clamping to the cirrhotic liver, highlighting a diminished apoptotic pathway with dramatic improvement in the development of necrosis. ***Liver Transpl 14:980-988, 2008.*** © **2008 AASLD.**

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Hepatocellular carcinoma, the most frequent liver tumor worldwide, is typically associated with liver cirrhosis. Because most patients with hepatocellular carcinoma are not candidates for liver transplantation, the only chance for a cure is resection.[1] Unfortunately, patients with a cirrhotic liver poorly tolerate surgery, particularly when inflow occlusion (that is, ischemia) is used to prevent bleeding during transection of the parenchyma.[2] In a large study including 747 patients who underwent hepatic resection, mortality was dramatically higher in patients with cirrhosis (8.7%) than in those exhibiting normal liver parenchyma (1%).[3] Additionally, the duration of vascular inflow occlusion correlates with the incidence of postoperative complica-

Abbreviations: AST, aspartate aminotransferase; C60, continuous clamping with 60 minutes of ischemia; C75, continuous clamping with 75 minutes of ischemia; CCl_4, carbon tetrachloride; H/E, hematoxylin-eosin; IC15, intermittent clamping with 15 minutes of ischemia; IC30, intermittent clamping with 30 minutes of ischemia; I/R, ischemia/reperfusion; PC60, ischemic preconditioning prior to 60 minutes of ischemia; PC75, ischemic preconditioning prior to 75 minutes of ischemia; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling.

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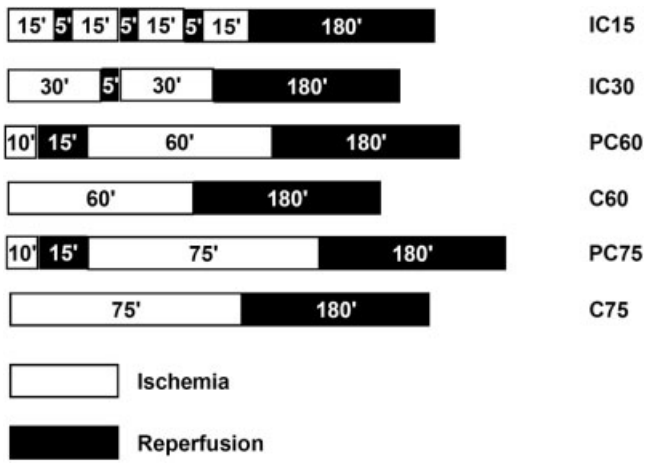

Figure 1. Overall scheme of the animal experiments. There were 2 cycles of intermittent clamping consisting of a 15- or 30-minute ischemic period and 5 minutes of short reperfusion. Ischemic preconditioning and continuous clamping were tested with 2 ischemic periods (60 and 75 minutes). Abbreviations: C60, continuous clamping with 60 minutes of ischemia; C75, continuous clamping with 75 minutes of ischemia; IC15, intermittent clamping with 15 minutes of ischemia; IC30, intermittent clamping with 30 minutes of ischemia; PC60, ischemic preconditioning prior to 60 minutes of ischemia; PC75, ischemic preconditioning prior to 75 minutes of ischemia.

tions, including hepatic failure and mortality.[4] The only strategy allowing extensive surgery under inflow occlusion in this population is intermittent clamping consisting of periods of 15 minutes of ischemia interrupted by 5 minutes of reperfusion. The mechanisms responsible for increased injury in the cirrhotic liver remain largely unexplored.

It is also unknown whether the same pathways of injury are activated in normal and cirrhotic livers. Activation of the apoptotic pathway followed by patchy necrosis is a central feature of reperfusion injury in the normal liver.[5-7] The degree of endothelial and hepatocellular apoptosis correlates with the elevation of several markers of hepatic injury, such as aspartate aminotransferase (AST) release and animal survival,[8,9] and importantly, antiapoptotic strategies have been shown to be highly protective in a variety of models.[10-13] The degree of necrosis, typically documented after 24 hours of reperfusion, also correlates with the incidence of liver failure and animal survival.[14]

Clinical and experimental studies have shown that the diseased liver, such as the fatty liver, tolerates ischemic insults poorly and that the mechanisms of injury leading to organ failure differ from those observed in livers exhibiting normal parenchyma.[15-17] For example, the fatty liver developed a rapid form of necrotic change rather than apoptosis.[8,18-20] Interestingly, despite the activation of different pathways of injury, the fatty liver is protected by strategies effective in the normal liver, such as intermittent clamping and ischemic preconditioning.[21]

The aim of this study was to evaluate in a murine model of carbon tetrachloride (CCl_4)–induced liver injury (1) how the cirrhotic liver tolerates an ischemic insult, (2) whether mechanisms other than those observed in the normal liver are active, and (3) whether intermittent clamping and ischemic preconditioning confer protection.

MATERIALS AND METHODS

Animal and Experimental Model of Cirrhosis

Male C57BL/6 mice were used in all experiments. Mice were fed a standard laboratory chow with free access to water. They were kept under constant environmental conditions with a 12-hour light-dark cycle. The animals were maintained in accordance with the Keimyung University Medical Science Institutional Animal Care Committee guidelines.

To induce hepatic fibrosis, C57BL/6 mice were fed 50% CCl_4 in a soybean oil (Junsei Chemical, Tokyo, Japan) solution (1 mL/kg) twice a week for 12 weeks. Hematoxylin-eosin (H/E) and trichrome staining [Fig. 2D (shown later)] was performed to score the histologic activity index for the establishment of an experimental cirrhosis model, and 2 pathologists scored 3 times in a blinded fashion.[22] Samples with a histologic activity index score over 14 were selected for this study.

Partial Hepatic Inflow Occlusion

All of the surgical steps were performed under inhalation anesthesia with isoflurane (Forane, Choongwae-Abbott, Korea). After a midline laparotomy, all structures in the portal triad to the left and median hepatic lobes were occluded by a microvascular clamp (Aesculap, San Francisco, CA) according to each time protocol of ischemia. A model of segmental (70%) hepatic ischemia was used. This method of partial hepatic ischemia prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. For the first set of experiments, we compared the normal liver to the cirrhotic liver after 60 minutes of ischemia and 3 hours of reperfusion. For the second set of experiments, we grouped 3 experimental arms: 2 intermittent ischemia cycles and ischemic preconditioning. Cycles of intermittent ischemia for 15 or 30 minutes followed by subsequent reperfusion for 5 minutes was repeated for a total duration of ischemia of 60 minutes. Ischemic preconditioning was introduced with 10 minutes of ischemia and 15 minutes of reperfusion prior to continuous clamping for 60 minutes. We tested an extended period (75 minutes) of ischemia to confirm the effect of ischemic preconditioning. Five mice per group were used for all experiments in this study (Fig. 1). During the ischemic period, the abdomen was closed. Reperfusion was initiated by removal of the clamp. The animal was allowed to recover from anesthesia during the 3 hours of reperfusion.

Histological Examination

To examine the histological change, H/E and Masson's trichrome staining was performed on 4-μm paraffin-

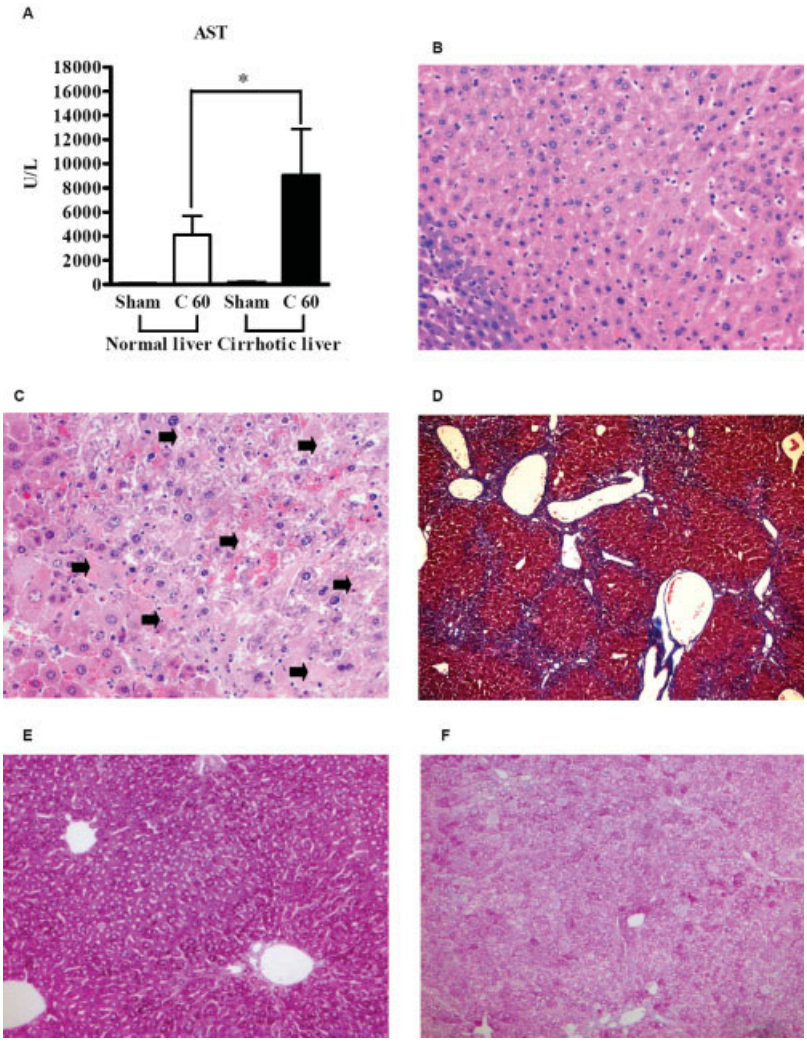

Figure 2. Demonstration of injury caused by 60 minutes of ischemia in normal and cirrhotic livers and pathologic observations. Mice were fed CCl_4 for 12 weeks (cirrhotic) or were given oil as a control. Liver injury was quantified by serum AST levels and H/E staining late after 60 minutes of inflow occlusion and 3 hours of reperfusion. (A) Normal livers show reduced serum AST levels after 3 hours of reperfusion. Much more prevalent necrotic hepatocytes (arrow) can be observed in (C) a cirrhotic liver than (B) a normal liver after 3 hours of reperfusion. (D) Trichrome staining shows the circular nodulation and accumulated fibrous matrix of a cirrhotic liver. Periodic acid Schiff staining shows the hepatic glycogen content in (E) normal and (F) cirrhotic livers. Glycogen storage, presented in purple, was reduced in cirrhotic livers compared to normal livers. The asterisk indicates a significant difference (*t* test) of $P < 0.05$. Abbreviation: C60, continuous clamping with 60 minutes of ischemia.

embedded sections of the liver. For H/E staining, xylene and a series of graded alcohols were used. Bouin's fixative, Weigert's iron hematoxylin solution, Biebrich scarlet–acid fuchsin solution, and phosphomolybdic–phosphotungstic acid solution (all from Sigma, St. Louis, MO) and aniline blue solution (BDH, United Kingdom) were used for Masson's trichrome stain.

Determination of the Area Injured by Ischemia/Reperfusion

Hepatocellular necrosis was determined in H/E-stained tissue with a semiquantitative scale by a point counting method in a blinded fashion according to the previous description.[23,24] The entire fields of ischemic liver sections per slide were investigated to determine the percentage of the injured area.

Serum AST Level

Blood samples were obtained from the inferior vena cava after 3 hours of reperfusion. Blood cells were precipitated by immediate centrifugation at 10,000*g* for 8 minutes. Enzyme levels were measured with a serum multiple biochemical analyzer (COBAS Integra 800, Roche Diagnostic GmbH, Manheim, Germany) in the Department of Clinical Pathology at Keimyung University Dongsan Medical Center.

Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick End Labeling (TUNEL) Stain

After ischemia/reperfusion, the livers were fixed *in vivo* by perfusion with 4% paraformaldehyde (Sigma) in phosphate-buffered saline administered through the portal vein. The liver was cut into 3- to 5-mm sections and was stored in 70% alcohol after additional overnight fixation in 4% paraformaldehyde. Tissues were then incubated in 30% sucrose in phosphate-buffered saline, embedded in 7.5% gelatin, and finally frozen in isopentane submerged in dry ice and 95% alcohol slush. Frozen sections (5 μm) of the fixed tissue were placed on coated slides and were treated with terminal deoxynucleotidyl transferase from calf thymus in the presence of fluorescein deoxyuridine triphosphate and

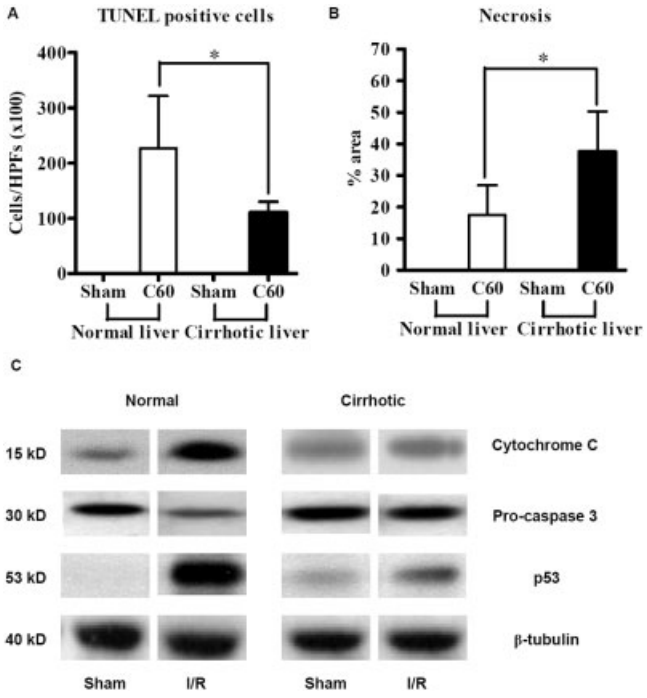

Figure 3. Demonstration of the mode of injury in livers after 60 minutes of ischemia and 3 hours of reperfusion. Markers of apoptosis and necrosis were quantified. (A) TUNEL-positive hepatocytes. (B) Area of necrosis as a percentage of the whole area. (C) Western blots detecting cytochrome c, procaspase 3, and p53. Beta-tubulin served as a loading control. Representative pictures of TUNEL staining in (D) the sham-operated liver and (E) the liver subjected to 1-hour ischemia/3-hour reperfusion show clear TUNEL-positive nuclei in the injured liver. Another feature of apoptosis observed by H/E staining demonstrates condensed and eosinophilic nuclei (arrows) shown in (G) normal and (I) cirrhotic injured livers compared to (F) normal and (H) cirrhotic sham controls. The asterisk indicates a significant difference (*t* test) of $P < 0.05$. Abbreviations: C60, continuous clamping with 60 minutes of ischemia; I/R, ischemia/reperfusion; HPF, high power field.

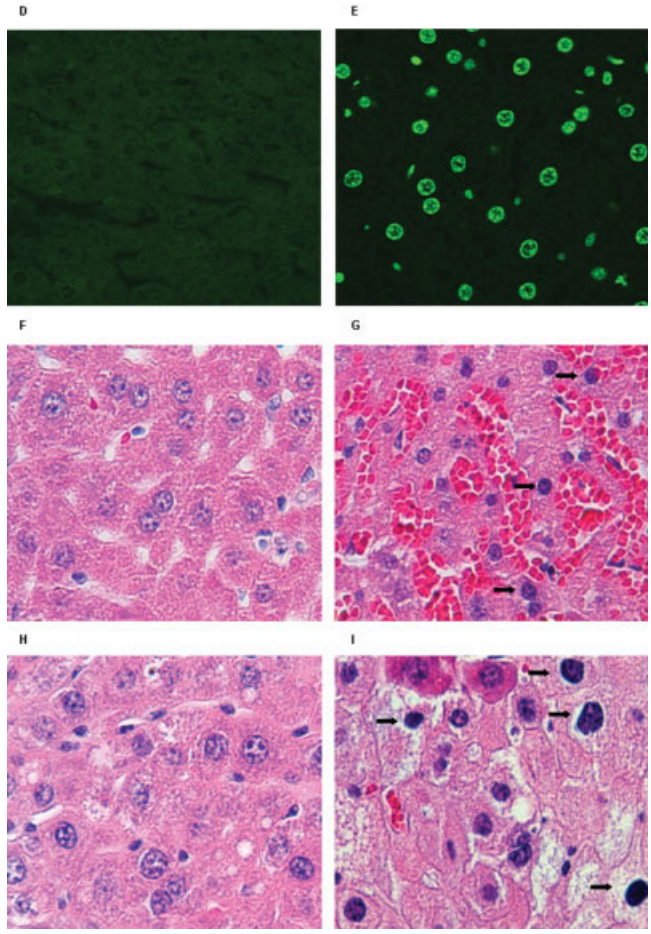

Figure 3. (continued)

deoxyribonucleotide triphosphate according to the supplier's recommended protocol (Boehringer Mannheim Co., Indianapolis, IN). This was followed by poststaining using horseradish peroxidase–conjugated anti-fluorescein antibody and development using test sections pretreated with DNase I and staining without deoxynucleotide substrate, respectively. Morphometric analysis of the fluorescent cells was performed for each slide to determine the percentage of TUNEL-positive cells under high-power magnification (×400) in 30 random fields.

Cytochrome c Extraction

The cryopreserved tissue was homogenized gently in cytochrome c extraction buffer [20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM ethylene diamine tetraacetic acid, and protease inhibitors] with a Tenbroeck glass grinder (Heaton, Millville, NJ). The nuclei and mitochondria were precipitated by centrifugation (10,000*g* for 10 minutes). The protein content of supernatants was determined by the Bradford protein assay (Bio-Rad, Hercules, CA).

Western Blotting

The tissue was homogenized in a cell lysis buffer (50 mM trishydroxymethylaminomethane, 150 mM NaCl, 5 mM ethylene diamine tetraacetic acid, 0.5% Nonidet P40, 100 mM phenylmethylsulfonyl fluoride, 1 M dithiothreitol, 10 mg/mL leupeptin, and 10 mg/mL aprotinin; all from Sigma) and centrifuged at 12,000*g* for 30 minutes. The protein content of supernatants was determined by the Bradford protein assay (Bio-Rad). Samples were boiled with a 4× sodium dodecyl sulfate sample buffer for 10 minutes. Fifty micrograms of protein was loaded onto each well of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then electrophoresis was performed at 100 V for 3 hours. The proteins were transferred onto a nitrocellulose filter and probed with cytochrome c (BD Biosciences, San Diego, CA), caspase 3, Bid, Bax, p53, and beta-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary antibody conjugated to horseradish peroxidase and detected with enhanced chemiluminescence detection reagents (Amersham, Piscataway, NJ). Five samples were used for each group

shown by representative pictures. For clarity the sham group is not shown because the levels of proteins were similar to that of the 15-minute intermittent clamping group [Fig. 4D (shown later)].

Statistical Analysis

The results are expressed as mean ± standard deviation. Data were analyzed with SPSS software, version 10.0.0 (SPSS, Inc., Chicago, IL). The differences between the groups were evaluated with the Student *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Is the Cirrhotic Liver More Susceptible to Ischemic Insult than the Normal Liver?

In the first set of experiments, we tested whether animals with CCl_4-induced cirrhotic livers display enhanced injury following an ischemic insult in comparison with lean animals. Serum AST levels and histology served as markers of injury.

We compared normal livers to cirrhotic livers following 60 minutes of ischemia and 3 hours of reperfusion. AST levels were significantly higher in cirrhotic animals (Fig. 2A). Liver biopsies were taken after 3 hours of reperfusion and were stained with H/E. Ischemic livers from CCl_4-treated animals disclosed more necrotic areas than normal livers (Fig. 2B) and were characterized by clusters of hepatocytes having no nucleus, ballooning, and eosinophilic cytoplasm, mostly in zone II areas (Fig. 2C).

Are the Mechanisms of Ischemic Injury Different Between the Cirrhotic and Normal Livers?

Next, we evaluated whether the type of ischemic injury of the normal and diseased livers disclosed significant differences. We focused on the evidence of the apoptotic markers after 60 minutes of ischemia and 3 hours of reperfusion of the ischemic livers, including TUNEL staining and western blotting of cytochrome c, procaspase 3, and p53.

Previous studies showed that apoptosis of endothelial cells and hepatocytes are prominent features of cell death after ischemia and reperfusion injury in the normal liver.[6,25,26] TUNEL staining is a well-established method for detecting apoptotic nuclei (Fig. 3E). A number of hepatocytes were TUNEL-positive in the continuous clamping groups, and there were significantly more in the normal group than in the cirrhosis group after ischemia/reperfusion (Fig. 3A). At a higher magnification, H/E staining could show apoptotic cells having condensed and eosinophilic nuclei, a sign of apoptosis (Fig. 3G, I), in comparison with sham-operated normal and cirrhotic controls (Fig. 3F,H).

We investigated the degree of necrosis by morphometry of the liver biopsy specimens taken after 60 minutes of ischemia and 3 hours of reperfusion in normal and cirrhotic animals. Ischemia and reperfusion in cirrhotic livers were associated with a higher degree of necrosis (37.5%) in comparison with the normal liver (19.8%; Fig. 3B).

The changes of apoptotic molecular markers were investigated by western blotting. We observed increased cytochrome c release into cytoplasm, whereas in the cirrhotic liver, cytochrome c release into the cytoplasm was limited. Another indication of apoptosis, caspase 3 activation, also demonstrated that in the normal liver, ischemia/reperfusion caused activation of caspase 3. This is shown by the loss of procaspase 3 in the normal liver but not the cirrhotic liver by western blotting (Fig. 3C) and activity assay of the enzyme (Supplementary Fig. 1C). To further substantiate this observation, we tested the expression of p53, which is known to induce apoptosis or cell cycle arrest as a response to DNA damage.[27-30] The ischemic insult resulted in massive production of p53 in the normal liver but not in the cirrhotic liver.

These experiments indicate that the apoptotic pathway is more activated by an ischemic insult in the normal liver than in the cirrhotic liver.

Do Intermittent Clamping and Preconditioning Protect the Cirrhotic Liver Against Ischemic Injury?

Ischemic preconditioning and intermittent clamping are well-established protective strategies against prolonged periods of ischemia in the normal liver in human[31-33] and animal models.[8,34,35] Intermittent clamping is commonly used in patients to perform surgery under prolonged periods of ischemia with a protocol of 15 minutes of ischemia interrupted by 5 minutes of reperfusion. We previously showed in an animal model of intermittent clamping of the normal liver using longer periods of ischemia (30 minutes) a similar degree of protection.[36] To our knowledge, no data are convincing regarding a protective strategy in either the clinical or experimental setting of cirrhosis. Therefore, we tested 3 protective strategies in our models of cirrhosis in mice. Two strategies of intermittent clamping were applied, that is, successive periods of either 15 or 30 minutes of ischemia interrupted by 5 minutes of reperfusion for a total ischemic time of 60 minutes (Fig. 1). The third strategy was ischemic preconditioning using a preconditioning ischemic time of 10 minutes followed by 15 minutes of reperfusion and a 60-minute period of continuous ischemia. Finally, a group that was exposed to 75 minutes of continuous ischemia was added to further evaluate the effects of ischemic preconditioning in the cirrhotic model.

We used the same markers described previously. As shown in Fig. 4, all protocols demonstrated a high degree of protection. The hepatic architecture of the liver examined in H/E biopsies taken after 3 hours of reperfusion revealed minimal derangement in both intermittent occlusion groups; that is, 30 minutes of intermittent clamping was as protective as shorter periods of 15 minutes. In contrast, in the absence of protective strat-

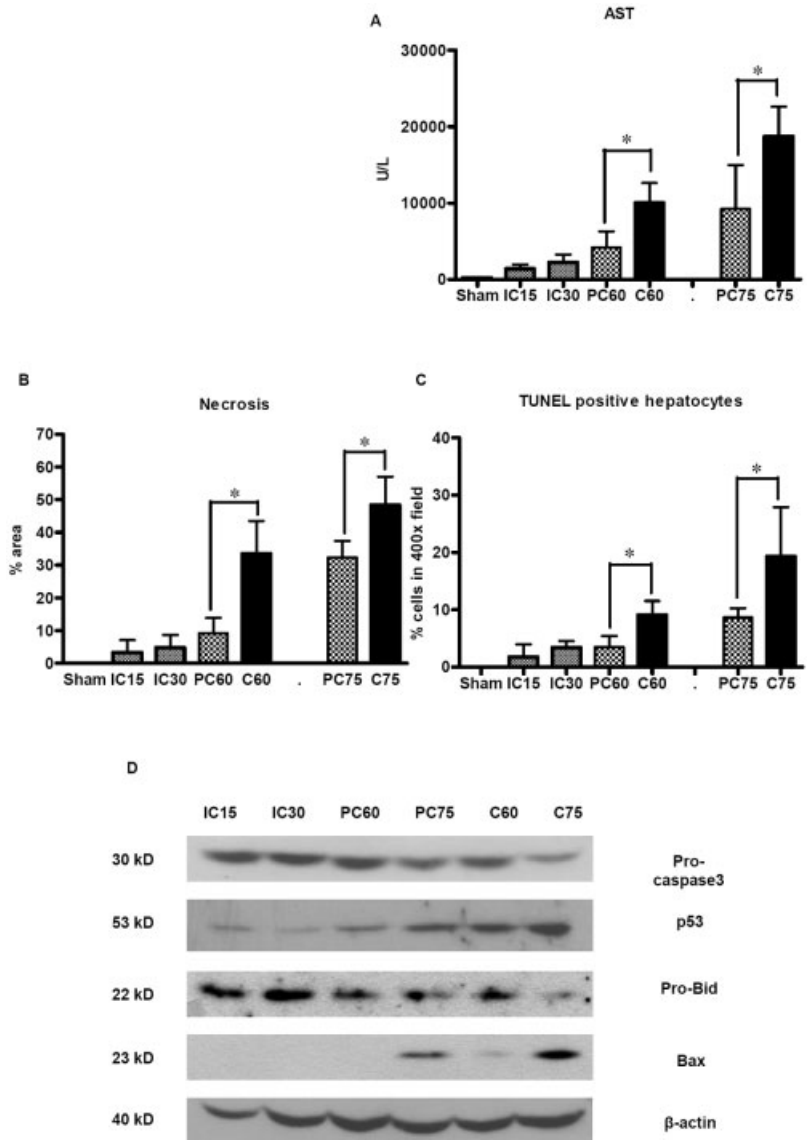

Figure 4. Determination of injury and markers of apoptosis in the liver protected by intermittent clamping or preconditioning. (A) Hepatic injury quantified by serum AST levels 3 hours after reperfusion. (B) Area of necrosis as a percentage of the whole area. (C) TUNEL-positive hepatocytes. (D) Western blot demonstrating proapoptotic markers: procaspase 3, p53, pro-Bid, and Bax. Beta actin served as a loading control. The asterisk indicates a significant difference (*t* test) of $P < 0.05$. Abbreviations: C60, continuous clamping with 60 minutes of ischemia; C75, continuous clamping with 75 minutes of ischemia; IC15, intermittent clamping with 15 minutes of ischemia; IC30, intermittent clamping with 30 minutes of ischemia; PC60, ischemic preconditioning with 60 minutes of ischemia; PC75, ischemic preconditioning with 75 minutes of ischemia.

egies, livers subjected to continuous occlusion of 60 minutes and 3 hours of reperfusion showed dramatic morphological changes characterized by disruption of zone II, ballooning, pyknotic nucleus, eosinophilic cytoplasm, and anuclear cells. In extending the ischemic time to 75 minutes, we observed more diffusely injured areas of necrosis and more TUNEL-positive hepatocytes than in the 60-minute ischemia group (Fig. 4B,C).

Ischemic preconditioning was also associated with significantly decreased injury compared to continuous clamping (Fig. 4A-C), although the degree of protection was less than that observed with intermittent clamping. The number of TUNEL-positive hepatocytes and area of necrosis were significantly reduced in the preconditioning group. The same effects were observed after 75 minutes of ischemia. To investigate the mechanism of ischemic preconditioning in the cirrhotic liver, we performed western blotting of procaspase 3, p53, pro-Bid, and Bax (Fig. 4D). Conversion of procaspase 3 was prominent after continuous ischemia, whereas the protective strategies exhibited higher levels of the inactive procaspase 3. The level of p53, which induces apoptosis, was high in the control group with 75 minutes of ischemia, and this suggested that besides necrosis, ischemic injury resulted in substantial apoptosis. The protective strategies demonstrated reduced p53 levels indicating reduced apoptosis, and this was consistent with TUNEL staining. Bid and Bax are known to influence mitochondrial integrity, leading to apoptosis. The presence of Bid was indirectly determined by probing for pro-Bid. As shown for procaspase 3, pro-Bid was converted to Bid after 75 minutes of ischemia, whereas protective strategies prevented Bid formation. Bax, another proapoptotic protein, was found in the 75-minute control but was less prominent in the ischemic preconditioning groups.

From this set of experiments, we conclude that ischemic preconditioning prevents not only necrosis but also apoptosis caused by ischemia/reperfusion in the cirrhotic liver.

Considering all this together, we conclude that intermittent clamping using periods of either 15 or 30 minutes of ischemia and ischemic preconditioning confer a high degree of protection, in terms of apoptosis and necrosis, in the cirrhotic liver subjected to long periods of continuous ischemia.

DISCUSSION

We established a model of cirrhosis by 12 weeks of CCl_4 feeding to mice. We compared normal and cirrhotic livers subjected to the same injury to establish how the cirrhotic liver tolerates the injury. Next, we tested well-known protective surgical techniques, intermittent clamping and ischemic preconditioning, on cirrhotic livers. We found increased necrotic changes in cirrhotic liver parenchyma but less apoptosis than in normal livers subjected to the same ischemic insult. Overall, the degree of hepatic injury was significantly higher in cirrhotic livers. Intermittent clamping cycles of 15 and 30 minutes conferred a high and comparable degree of protection against ischemic injury in the cirrhotic liver. Ischemic preconditioning also significantly ameliorated ischemic insult by reducing necrosis and apoptosis in cirrhotic livers.

CCl_4 is well known as a hepatotoxic chemical inducing postnecrotic cirrhosis in animals.[37-39] Continuous intoxication of CCl_4 induces a cirrhotic phenotype in mouse and rat livers.[40] Cirrhosis is a chronic and severe hepatic architectural change in human beings. Because it is difficult to make an identical human "cirrhosis" model in an experimental animal, we tried to establish the closest model to human cirrhosis. After a long period (12 weeks) of CCl_4 feeding, the liver showed circular nodulation and accumulation of a fibrous matrix surrounding nodules. The mice did not show any complications of cirrhosis, that is, portal hypertension, ascites, variceal bleeding, or hepatic coma. This state of cirrhosis is similar to Child A cirrhosis in humans, which is the only population to undergo major hepatic surgery.[1] To evaluate the cirrhotic state in the experimental groups, we used Knodell scoring concerning piecemeal necrosis, lobular necrosis, portal inflammation, and fibrosis.[22,41,42]

Intermittent clamping has been considered a safe surgical intervention for surgery on patients with cirrhosis,[43-45] although some groups failed to identify a beneficial effect in patients with cirrhosis who underwent hepatic surgery.[46] Previously, we tested 15 and 30 minutes of intermittent cycles in the normal mouse liver, showing a similar protective effect in both cycles against ischemia/reperfusion injury.[36] Intermittent clamping was protective in cirrhotic mouse livers challenged with an ischemic insult, and this protection was similar to that in the normal liver. Both intermittent clamping protocols were protective, although hepatic injury was slightly increased in the group subjected to a 30-minute intermittent cycle following ischemic injury. Nevertheless, we suggest that 30 minutes of intermittent ischemia might be better for cirrhotic liver surgery to reduce intraoperative bleeding and operation time and to diminish postoperative mortality and morbidity.

Ischemic preconditioning is not well studied in the cirrhotic liver. It is generally accepted that the cirrhotic liver is more susceptible to an ischemic insult. Surgeons should consider the functional remnant liver volume and ischemic times to decide which method will be used in the surgery of a patient with cirrhosis. Smyrniotis et al.[47] suggested that ischemic preconditioning in cirrhotic livers works only with short periods of ischemic insult. Li et al.[48] reported protective effects of ischemic preconditioning in patients with cirrhosis subjected to less than 20 minutes of ischemia. In our animal model, we found ischemic preconditioning protects cirrhotic livers subjected to 60 and 75 minutes of continuous ischemia following 3 hours of reperfusion. Although necrosis, which is a major ischemic injury of the cirrhotic liver, of the ischemic preconditioning group was still significantly different from the control group after 75 minutes of ischemia, the protective effect was decreased compared to that of the 60-minute ischemia group. The mice tolerated the long ischemic periods because the nonischemic part of the liver remained undisturbed and prevented mesenteric venous congestion. To confirm our conclusions, we performed a survival study (resecting nonischemic lobes after ischemia) for intermittent clamping groups. All cirrhotic animals in the 2 intermittent clamping groups survived until 10 days after the operation, whereas 6 of 10 cirrhotic animals of the control group died within a week. In the same vein, the protective effect of ischemic preconditioning reduced AST levels and areas of necrosis after 24 hours of reperfusion (Supplementary Fig. 1A,B).

The type of cell death in the liver caused by ischemic injury is still under debate. In the normal liver, the apoptotic pathway is activated after reperfusion, and this results in apoptosis, first of endothelial cells, followed by hepatocytes. In contrast, we showed that steatotic livers instead develop severe necrotic changes usually after 8 to 24 hours of reperfusion of an ischemic insult.[24] The cirrhotic liver, similar the steatotic liver, is associated with a defect in energy storage, as examplified by low glycogen and adenosine triphosphate contents.[49] Moreover, an ischemic stress results in glycogen consumption, which produces glucose and adenosine triphosphate,[50] and severe ischemic insults may induce a hypoglycemic state. Apoptosis is an active process of cell death, which is fully energy-dependent. When the energy storage is depleted in the liver, an otherwise apoptotic process may switch to necrosis.[51,52] Our mouse model of cirrhosis induced by long-term CCl_4 administration is associated with major depletion of glycogen (Fig. 2E,F). This finding is consistent with similar observations in patients with cirrhosis.[49] This feature may explain the development of significant necrosis already 3 hours after reperfusion, despite evidence of an early activation of the apoptotic pathway.

Protective mechanisms triggered by ischemic preconditioning have been well described in the normal[8,26,53,54] and steatotic[21] livers. To our knowledge, no data are currently available regarding the protection

of ischemic preconditioning in the cirrhotic liver. We observed reduced activation of the apoptotic pathway followed by a dramatic reduction in necrosis when the ischemic preconditioning protocol was applied. The putative mechanisms associated with this protective effect were not investigated in this study. Considering the main effect on the development of necrosis, we may speculate that ischemic preconditioning, besides an antiapoptotic effect, results in some maintenance of the cellular energy status. Further investigation will be needed to better understand the protective pathways. It will also be interesting to test whether the protective mechanism of ischemic preconditioning is also active with intermittent clamping, as we previously observed in the normal liver.[34]

In summary, the search for protective strategies against ischemic injury in the cirrhotic liver is clinically very relevant, as surgery is increasingly performed in this high-risk population. We showed that a cirrhotic liver, even disclosing no signs of liver failure or portal hypertension, is twice as susceptible to ischemic injury as a liver displaying normal parenchyma. We also demonstrated that the 2 standard surgical protective strategies, intermittent clamping and ischemic preconditioning, protective in normal and steatotic livers, also confer significant protection in the presence of cirrhosis, particularly in preventing the development of necrosis. Both intermittent clamping protocols disclosed similar protection. Further studies are now warranted in patients, and if similar findings are identified, then intermittent clamping with a cycle of 30 minutes should be preferred if prolonged periods of ischemia are used, whereas ischemic preconditioning might become a promising strategy for shorter periods, maybe up to 60 minutes.

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