Ischemic Preconditioning Increases the Tolerance of Fatty Liver to Hepatic Ischemia-Reperfusion Injury in the Rat

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Hepatic steatosis is a major risk factor in ischemia-reperfusion. The present study evaluates whether preconditioning, demonstrated to be effective in normal livers, could also confer protection in the presence of steatosis and investigates the potential underlying protective mechanisms. Fatty rats had increased hepatic injury and decreased survival after 60 minutes of ischemia compared with lean rats. Fatty livers showed a degree of neutrophil accumulation and microcirculatory alterations similar to that of normal livers. However, in presence of steatosis, an increased lipid peroxidation that could be reduced with glutathione-ester pretreatment was observed after hepatic reperfusion. Ischemic preconditioning reduced hepatic injury and increased animal survival. Both in normal and fatty livers, this endogenous protective mechanism was found to control lipid peroxidation, hepatic microcirculation failure, and neutrophil accumulation, reducing the subsequent hepatic injury. These beneficial effects could be mediated by nitric oxide, because the inhibition of nitric oxide synthesis and nitric oxide donor pretreatment abolished and simulated, respectively, the benefits of preconditioning. Thus, ischemic preconditioning could be an effective surgical strategy to reduce the hepatic ischemia-reperfusion injury in normal and fatty livers under normothermic conditions, including hepatic resections, and liver transplantation. (*Am J Pathol* 2002, 161:587–601)

The ischemia-reperfusion (I/R) injury is an important cause of liver damage occurring during surgical procedures that include hepatic resections and liver transplantation.^{1–3} Hepatic steatosis is a major risk factor after liver surgery because steatotic livers tolerate poorly I/R injury.

The occurrence of postoperative liver failure after hepatic resection in a steatotic liver exposed to normothermic ischemia has been reported.^{4–6} In addition, the use of steatotic livers for transplantation is associated with an increased risk for primary nonfunction or dysfunction after surgery.^{2,7–9}

Several hypotheses have been suggested to explain the decreased tolerance of steatotic liver to I/R injury compared with normal livers. These include increased 1) lipid peroxidation,^{10,11} 2) neutrophil infiltration,^{12,13} 3) microcirculatory alterations,^{14,15} and 4) release of proinflammatory mediators such as tumor necrosis factor (TNF)- α .¹³ Understanding the mechanisms of liver failure in steatotic livers will help to increase the tolerance of fatty livers to I/R injury and consequently decrease the inherent risk of liver surgery.

Currently, only a few pharmacological protective strategies, consisting of anti-TNF therapy, are clinically available in normothermic conditions and no protective strategy is clinically available for liver transplantation.¹⁶ Strategies focused on the improvement of hepatic microcirculation or the inhibition of oxygen-free radical-mediated injury have resulted in decreased injury after I/R in fatty rats livers but were insufficient to prevent hepatic injury.^{15,17–20} Multiple mechanisms are thus potentially involved in the impaired tolerance to ischemic injury of steatotic livers, and consequently, various pharmacological strategies may need to be combined to effectively protect the fatty liver. Moreover, the different mechanisms of cell death in fatty versus nonfatty livers,²¹ point to the potential differences in the mechanisms involved in hepatic I/R injury in both types of the livers. Accordingly, those strategies that are effective in the normal liver may not be useful in the presence of steatosis. These observations point to the difficulties for effectively preventing the steatotic liver from hepatic I/R injury.

Ischemic preconditioning, firstly described in the heart by Murry and colleagues²² nearly a decade ago, is an

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endogenous protective mechanism by which brief periods of vascular occlusion, confer protection against subsequent sustained I/R. Despite intensive investigations, the underlying mechanisms remain to be elucidated. However, it has been suggested that the benefits of ischemic preconditioning could be mediated by the synthesis of vasoactive mediators, such as nitric oxide (NO).²³

The effectiveness of ischemic preconditioning against the hepatic I/R injury in experimental models of normothermic and cold ischemia in normal livers,^{24–27} points to their potential clinical application in hepatic surgery, as recently reported in normothermic conditions.²⁸ We report here the results of an experimental study aimed to evaluate whether preconditioning could be an effective strategy to reduce hepatic I/R injury in the presence of steatosis. This may open the way to new therapeutic avenues for the ischemic preconditioning of fatty livers. Moreover, experiments were designed to elucidate the potential underlying protective mechanisms of preconditioning on hepatic I/R injury in normal and steatotic livers. New insights into the mechanisms of failure of fatty livers and those involved in the benefits of ischemic preconditioning could result in new surgical and/or pharmacological strategies to effectively protect normal as well as steatotic livers from I/R injury.

Materials and Methods

Experimental Animals

All experiments were performed in male Zucker rats (Iffa-Credo, L'Abresle, France). Zucker rats constitute a well-characterized model of nutritionally induced obesity. As previously reported,²¹ steatosis in Zucker rats is not associated with inflammation, as in other models of steatosis using ethanol ingestion or a choline-deficient diet. Homozygous Zucker rats (Obese, Ob) lack the cerebral leptin receptor and develop obesity at the age of 8 weeks because of markedly increased food intake and decreased energy expenditure. In contrast, heterozygous Zucker rats (Lean, Ln) have cerebral leptin receptors and maintain a lean phenotype throughout life. Homozygous (Ob) and heterozygous (Ln) Zucker rats 16 to 18 weeks of age were used for the experiments. The difference of steatosis in the Ob *versus* Ln Zucker rats has been determined by using specific lipid staining such as red oil staining. Ob Zucker rats showed severe and macrovesicular and microvesicular fatty infiltration in hepatocytes (between 60% and 70% steatosis). In contrast, Ln Zucker rats show no evidence of steatosis (Figure 1). Animals were fed a laboratory diet containing 12% fat, 28% protein, and 60% carbohydrates (5001 rodent diet; PMI Inc., Brentwood, MO) with water and food *ad libitum* until use and were kept under constant environmental conditions with a 12-hour light-dark cycle.

Figure 1. Difference of steatosis in the Ob *versus* Ln Zucker rats using red oil staining. **A:** Ln Zucker rats have no evidence of steatosis. **B:** Ob Zucker rats showed fatty infiltration in hepatocytes. (3- μ m frozen sections; original magnifications, $\times 214$).

Hepatic Ischemia

A model of segmental (70%) hepatic ischemia was used. The animals were anesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg).²⁹ After a midline laparotomy, the hepatic artery and portal vein to the left and median liver lobes were occluded for the period of ischemia under study. This method of partial ischemia prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Reperfusion was initiated by removal of the clamp. Experiments to assess animal survival were performed by resecting the nonischemic lobes (30%) at the time of reperfusion.^{17,21}

This study was performed in concordance with the European Union regulations (Directive 86/609 EEC) for animal experiments.

Experimental Design

Protocol 1: Effectiveness of Preconditioning in Steatotic Livers Subjected to Hepatic I/R

We evaluated whether preconditioning periods demonstrated to be effective against hepatic I/R injury in normal livers,^{24,25,30} could also confer protection in presence of steatosis. For this purpose, the following experi-

mental groups were studied: group 1: control (C) ($n = 12$), lean (Ln) and obese (Ob) animals (six in each group) were subjected to anesthesia and laparotomy. Group 2: I/R ($n = 36$), a group of animals ($n = 12$, 6 Ln and 6 Ob) were subjected to 60 minutes of partial ischemia followed by 2 hours of reperfusion. A second group of animals ($n = 12$, 6 Ln and 6 Ob) were subjected to 60 minutes of partial ischemia followed by 6 hours of reperfusion. A third group of animals ($n = 12$, 6 Ln and 6 Ob) were subjected to 60 minutes of partial ischemia followed by 24 hours reperfusion. Group 3: Preconditioning plus I/R (PC + I/R): This group is subdivided in three subgroups of 36 animals in each group, based on the different preconditioning periods applied. Animals subjected to I/R (as in group 2) were subjected to previous preconditioning induced by: 10 minutes of ischemia and reperfusion periods (10 + 10) (group 3.1),²⁴ 10 minutes of ischemia followed by 15 minutes of reperfusion (10 + 15) (group 3.2),²⁵ or 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10) (group 3.3).³⁰ Evaluation of hepatic injury was performed by determinations of alanine aminotransferase (ALT) and α -glutathione S-transferase (α -GST). The effect of ischemic preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion on survival in both Ln and Ob animals subjected to 60 minutes of total ischemia ($n = 10$ in each group) was evaluated. As in previous studies,^{21,25} survival was considered permanent if the rats were alive 30 days after surgery.

Protocol 2: Protective Mechanisms of Ischemic Preconditioning Induced by 5 Minutes of Ischemia Followed by 10 Minutes of Reperfusion

Effect of Preconditioning on the Mechanisms Potentially Involved in the Vulnerability of Fatty Livers to Hepatic I/R (Lipid Peroxidation, Neutrophil Infiltration, Alterations in Hepatic Microcirculation, and TNF Release): For this purpose, malondialdehyde (MDA), as an index of lipid peroxidation, and myeloperoxidase (MPO) activity, as an index of neutrophil accumulation, were measured in liver corresponding to groups 1, 2, and 3.3 (protocol 1). TNF levels were also evaluated in liver and plasma samples. Hepatic blood perfusion measurement in liver during hepatic reperfusion was analyzed. Lung damage after hepatic reperfusion was evaluated by MPO, MDA, and edema formation. Histological analysis of liver and lung were performed.

Effect of Ischemic Preconditioning on Reactive Oxygen Species (ROS) Antioxidant Systems: To evaluate the effect of preconditioning on glutathione (GSH) and superoxide dismutase (SOD) during hepatic ischemia, the following experimental groups were studied: group 4: ischemia (I) ($n = 6$), animals subjected to 60 minutes of partial hepatic ischemia (as in group 2). Group 5: Preconditioning plus ischemia (PC + I) ($n = 6$), same as group 4 but with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion. SOD and GSH levels were analyzed in liver after the sustained ischemia. These antioxidant systems were also measured after hepatic reperfusion in liver samples corresponding to groups 1, 2, and 3.3 (protocol 1).

To evaluate the role of GSH on postischemic ROS generation, the following experimental group was studied: group 6: I/R plus glutathione ester (I/R + GSH) ($n = 36$), same as group 2, but with administration of GSH ester (5 mmol/kg, i.v.) 5 minutes before reperfusion.^{31,32} At the end of reperfusion, blood samples were collected to determine ALT and α -GST. Liver samples were processed to determine MDA levels.

Effect of Ischemic Preconditioning on TNF: To test the role of TNF, an additional experimental group was studied: group 7: I/R plus anti-TNF ($n = 36$), same as group 2, but with previous administration of rabbit anti-TNF- α polyclonal antibody directed against rat TNF (3 mg/kg, i.v.) 30 minutes before ischemia.³³ Control experiments with rabbit IgG that is not specific to any antigen were performed. After hepatic reperfusion, MPO levels in liver and ALT and α -GST levels in plasma were evaluated. MPO, MDA, and edema formation in lung was also analyzed. To evaluate if ischemic preconditioning could confer protection by reducing the deleterious effects of TNF on hepatic I/R injury, hepatic and plasma TNF levels were measured after hepatic reperfusion in experimental groups 1, 2, and 3.3 (protocol 1).

Role of NO in Hepatic Preconditioning: To evaluate the role of NO, the following experimental groups were set up: group 8: preconditioning plus I/R plus L-NAME (PC + I/R + NAME) ($n = 36$), animals subjected to I/R (as in group 2) were subjected to previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion and treated with L-NAME, inhibitor of NO synthesis (10 mg/kg i.v.), 5 minutes before preconditioning.²⁴ Group 9: I/R plus NO donor ($n = 36$), animals subjected to I/R (as in group 2) were treated with the NO donor spermine NONOate (10 mg/kg i.v.) 5 minutes before ischemia.²⁴

Evaluation of hepatic injury was performed by determinations of ALT and α -GST in plasma. MDA, and MPO activity were evaluated in liver. Hepatic blood perfusion measurement in liver during hepatic reperfusion was analyzed. Histological analyses of liver were performed. The whole experimental design is summarized in Figure 2.

The dose and pretreatment times of GSH ester administration has been reported to be effective to evaluate the effects of GSH depletion on hepatic I/R injury.^{31,32} GSH itself is not suitable because it is not taken up by cells in its intact form, because it is degraded into its constituent amino acids before entering the cells. The GSH ester is readily permeable to cells. Once inside the cell cytosol the ester is hydrolyzed by an esterase to yield GSH. The ester is most effective in increasing hepatic GSH levels.³¹ The dose and pretreatment times of anti-TNF antibody used in the present study have been reported to be effective in evaluating the role of TNF in different experimental models of inflammation.^{33,34} The dose and pretreatment times of L-NAME and NO donor have been reported to be effective in evaluating the role of NO in ischemic preconditioning in normal livers.^{24,35}

PROTOCOL 1

PROTOCOL 2

Figure 2. Experimental protocols set up in both normal and fatty livers. Protocol 1 (effectiveness of preconditioning). I/R, Animals subjected to 60 minutes of hepatic ischemia followed by 2, 6, and 24 hours of reperfusion. PC + I/R, I/R with previous preconditioning induced by: 10 minutes of ischemia and reperfusion periods (10 + 10); 10 minutes of ischemia followed by 15 minutes of reperfusion (10 + 15); or 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10). Protocol 2 (protective mechanisms of preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion). I, Animals subjected to 60 minutes of hepatic ischemia; PC + I, I with previous preconditioning; I/R + GSH, I/R treated with GSH-ester; I/R + anti-TNF, I/R treated with anti-TNF antibody; PC + I/R + NAME, PC + I/R treated with L-NAME; I/R + NO, I/R treated with NO donor.

Biochemical Determinations

ALT and α -GST

Evaluation of hepatocyte damage was performed by enzymatic determinations of ALT and α -GST^{36,37} in plasma using commercial kits from Boehringer Mannheim (Munich, Germany) and Biotrin Int. (Dublin, Ireland), respectively.

Lipid Peroxidation Assay

Lipid peroxidation has been used as an indirect measurement of oxidative damage induced by ROS.³⁸ Lipid peroxidation in liver samples was determined by the thiobarbiturate reaction measuring the formation of MDA.³⁹ Briefly, 0.5 ml of 0.5% butylated hydroxytoluene was

added to 2 ml of liver homogenate to prevent lipid auto-oxidation. For protein precipitation, 2 ml of 20% trichloroacetic acid was added to 2 ml of homogenate. After mixing and centrifuging, 1 ml of 0.67% thiobarbiturate-water solution was added to the supernatant and boiled for 60 minutes. After cooling, optical density at 530 nm was assayed.

SOD Assay

For SOD determination, liver samples were homogenized in 100 mmol/L of Tris (hydroxymethyl) aminomethane (Tris)-HCl buffer with 0.2 mmol/L of phenylmethylsulfonyl fluoride and 0.5 mmol/L of dithiothreitol (pH 8.1). SOD activity was assayed by determining the ability of the enzyme to inhibit the superoxide anion-mediated reduction of nitro blue tetrazolium (25 μ mol/L) to formazan, according to the method of Sun and colleagues.⁴⁰ The latter was determined spectrophotometrically at 560 nm. The superoxide anion required for this reaction is generated by xanthine (0.1 mmol/L) and xanthine oxidase (200 U/L).

Glutathione Measurement

For the analysis of GSH, liver samples were homogenized in 1.1% KCl. Proteins were precipitated with 1 N perchloric acid. After centrifugation, samples were neutralized with 10% K₂CO₃.⁴¹ The amount of GSH was measured using glutathione transferase and 1-chloro-2,4-dinitrobenzene.⁴² Fifty μ l of the previous treated sample were mixed with 225 μ l of 0.1 mol/L potassium phosphate buffer, pH 7.0, and 10 μ l of 10 mmol/L 1-chloro-2,4-dinitrobenzene in ethanol. The reaction was started with 5 μ l of glutathione transferase solution (12 U/L) and monitored at 340 to 400 nm reaching the end point 5 minutes after enzyme addition.

MPO Assay

MPO has been used as a marker of neutrophil infiltration and activation.^{43,44} MPO activity was measured photometrically using 3,3',5,5'-tetramethylbenzidine as a substrate.⁴⁵ Liver and lung samples were macerated with 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L of phosphate buffer, pH 6. Homogenates were then disrupted by sonication for 30 seconds and subsequently snap-frozen in dry ice and thawed three times before a final 30-second sonication. Samples were incubated at 60°C for 2 hours and then spun down at 4000 $\times$ g for 12 minutes. Supernatants were collected for MPO assay. The assay mixture consisted of 20 μ l of supernatant, 10 μ l of tetramethylbenzidine (final concentration, 1.6 mmol/L) dissolved in dimethyl sulfoxide, and 70 μ l of H₂O₂ (final concentration, 3.0 mmol/L) diluted in 80 mmol/L of phosphate buffer, pH 5.4. An enzyme unit is defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute.

Edema Formation

After resection, lung samples were weighed and then placed in an oven at 55°C until a constant weight was

obtained. In this determination, edema is represented by an increase in the wet-to-dry weight ratios.⁴⁶

TNF Assay

Liver and plasma TNF- α levels were measured using a commercial immunoassay kit for mouse TNF- α from Biosource (Camarillo, CA).⁴⁷

Hepatic Tissue Blood Flow

The hepatic tissue blood flow was measured using a laser Doppler flowmeter (model LD5000; Transonic Systems Inc., Ithaca, NY). This has been considered as a suitable technique for estimation of hepatic microvascular perfusion.^{48–51} Significant correlation between the results obtained by intravital microscopy and those obtained by laser Doppler flowmeter have been found in experimental models of hepatic I/R.⁵⁰ In each animal the probe of the flowmeter was placed on the same points on the surface of the median lobe and the left lateral lobe of the liver. The means of measurements at five sites on the surface of the median lobe and the left lateral lobe were considered.^{50,52} Values of hepatic blood flow were expressed as percentage of the preischemia value. Separate measurements of blood flow in the hepatic portal vein at the left and median liver lobes were performed with an ultrasonic transit time volume flowmeter (T206; Transonic Systems, Inc.) using a miniature flow probe of 1 mm that was placed around this vessel. This has been considered as another method to evaluate hepatic blood flow in different experimental models of hepatic I/R.^{53,54}

Histology

For the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method using an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting in cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. Forty random sections were investigated per slide to determine the percentage of necrotic cells.⁵⁵ Steatosis in liver was evaluated using red oil staining on frozen specimens according to standard procedures.

Statistics

Data are expressed as means $\pm$ SEM. Statistical comparison was performed with analysis of variance, followed by Student-Newman-Keuls tests. An associated probability of $P < 0.05$ was considered to be significant.

A)

B)

Figure 3. A: ALT and α -GST levels in plasma from Ln and Ob Zucker rats subjected to 60 minutes followed by 2, 6, and 24 hours of reperfusion. Steatotic rats had higher ALT and α -GST levels compared with the lean (# indicates $P < 0.05$). **B:** Percentage of grade 3 necrosis in the following experimental groups: I/R, 60 minutes of ischemia followed by 24 hours of reperfusion; PC + I/R, I/R with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10); PC + I/R + NAME, PC + I/R treated with L-NAME; I/R + NO, I/R treated with NO donor. +, $P < 0.05$ versus I/R; °, $P < 0.05$ versus PC + I/R.

Results

All fatty rats died after 60 minutes of total hepatic ischemia within 3 days after liver surgery, whereas all lean

animals survived the same ischemic challenge for 30 days after surgery. A significant improvement in survival was found in fatty rats subjected to hepatic ischemia with previous preconditioning, because 30% of animals died during 5 days and 70% survived for 30 days after surgery. ALT and α -GST levels from lean (Ln) and obese (Ob) Zucker rats subjected to 60 minutes of partial hepatic ischemia followed by 2, 6, and 24 hours of reperfusion are shown in Figure 3A. These parameters of hepatic injury were higher in fatty rats at each time point. Thus, at 6 hours of reperfusion, reperfusion times included into the range in which maximum levels in the parameters of hepatic injury have been shown,^{55,56} the values of ALT and α -GST were at least three times higher in fatty than in normal livers. The percentage of grade 3 necrosis in normal and steatotic livers at 24 hours of reperfusion was 17% and 74%, respectively (Figure 3B). We evaluated whether ischemic preconditioning could be effective in the presence of steatosis. For this purpose, we considered the studies reported in the literature on the different ischemic preconditioning periods (10 + 10, 10 + 15, or 5 + 10 minutes), demonstrated to be effective against hepatic I/R injury in normal livers.^{24,25,30} These studies of ischemic preconditioning have been centered in the hepatic injury induced by 90 minutes of sustained ischemia. The results of the present study indicate that these preconditioning periods could also confer protection in both normal and steatotic livers, after 60 minutes of ischemia and 2 hours of reperfusion (Figure 4). We next evaluated whether these beneficial effects on hepatic injury are transient or by contrast are maintained up for extended reperfusion times. At 24 hours of reperfusion, the 10 + 10-minute preconditioning period resulted in parameters of hepatic injury similar to those found in unpreconditioned group. In regards to the 10 + 15- and 5 + 10-minute preconditioning periods, the latter conferred the most protection against hepatic injury. The protection conferred by the 5 + 10-minute preconditioning period was reflected in histological changes (see Figure 6). The histological findings in liver at 2 hours of reperfusion showed no significant lesions in either Ln or Ob animals. Only slight incipient patchy necrosis distributed throughout the hepatic parenchyma was found (grade 1) (data not shown). At 24 hours of reperfusion, the histological study in normal liver showed moderate and multifocal areas of coagulative necrosis and neutrophilic infiltration, randomly distributed throughout the parenchyma (see Figure 6A), whereas severe, extensive, and confluent areas of coagulative necrosis with neutrophilic infiltration were observed in fatty livers (see Figure 6B). By contrast, when preconditioning consisting of 5 minutes of ischemia followed by 10 minutes of reperfusion was performed, the extent and number of necrosis areas at 24 hours after hepatic reperfusion was markedly reduced in normal and fatty livers with respect to I/R. In the case of normal liver, these areas were mainly of incipient hepatocyte necrosis (see Figure 6C) whereas in fatty livers patchy areas of hepatocyte incipient necrosis and scattered multifocal areas of coagulative hepatocyte necrosis were observed (see Figure 6D). As shown in Figure 3, the percentage of necrosis in both normal and

steatotic livers was significantly less in preconditioned than in unpreconditioned group.

We also evaluated the role of NO in the protection conferred by preconditioning against liver damage. The inhibition of NO synthesis in the preconditioned group resulted in parameters of hepatic injury and histological lesions similar to those observed after hepatic reperfusion (Figures 5 and 6). No differences in the percentage of necrosis with respect to that observed in the I/R group were found (Figure 3). The administration of a NO donor resulted in ALT and α -GST values and histological lesions comparable to those seen in preconditioned group.

The effect of the 5 + 10-minute preconditioning period on lipid peroxidation, neutrophil infiltration, alterations in hepatic microcirculation, and TNF release was evaluated. MDA levels after hepatic reperfusion showed only a slight tendency to be increased in normal livers, whereas MDA levels were significantly increased at each time point of reperfusion in fatty livers (Figure 7). Ischemic preconditioning reduced the increases in MDA found in fatty livers after hepatic reperfusion. We evaluated if preconditioning could confer resistance against ROS damage, by preventing the depletion or inactivation of antioxidant mechanisms, such as GSH and SOD, which could occur during hepatic I/R.^{32,57,58} As shown in Figure 8A, SOD activity levels found in fatty livers after ischemia (I) were of the same order as in the control group and no significant decreases in SOD levels were observed after hepatic reperfusion (I/R). With regard to GSH, ischemia leads to a significant reduction in GSH levels in fatty livers with respect to the control group that was not modified after hepatic reperfusion. However, when ischemia was preceded by preconditioning, GSH levels were similar to those of the control group. We evaluated whether the maintenance of GSH content after hepatic ischemia induced by preconditioning could contribute to attenuate the injuring effects of ROS in I/R processes. The administration of GSH-ester to I/R (I/R + GSH) resulted in MDA and transaminase levels significantly lower than those obtained after hepatic reperfusion (Figure 8B). These values remained unchanged with increasing doses of GSH-ester (data not shown). The effect of preconditioning on neutrophil accumulation after hepatic reperfusion was assessed by MPO level determination (see Figure 7). Both, normal and fatty livers showed an increase in neutrophil accumulation up to 6 hours of reperfusion, this increase being similar in both groups. When preconditioning preceded ischemia, hepatic MPO values comparable to those in the control group were found. The inhibition of NO synthesis abolished the benefits of preconditioning on lipid peroxidation and neutrophil accumulation. The administration of a NO donor resulted in values of MDA and MPO similar to those observed in the preconditioned group.

With respect to changes in blood flow using the Laser Doppler flowmeter, the recovery of hepatic blood flow after hepatic reperfusion was similar in fatty and nonfatty livers (Figure 9). A significant recovery of hepatic blood perfusion was observed in both groups when preconditioning was performed. The inhibition of NO synthesis in the preconditioned groups resulted in values of hepatic

Figure 4. Effect of ischemic preconditioning on ALT and α -GST levels in plasma from Ln and Ob Zucker rats subjected to 60 minutes of ischemia followed by 2, 6, and 24 hours of reperfusion. Control, anesthesia and laparotomy; I/R, IR; PC + I/R, I/R with previous preconditioning induced by 10 minutes of ischemia and reperfusion periods (10 + 10); 10 minutes of ischemia followed by 15 minutes of reperfusion (10 + 15); or 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10). *, $P < 0.05$ versus control; +, $P < 0.05$ versus I/R.

Figure 5. Role of NO implicated in preconditioning on ALT and α -GST levels in plasma from Ln and Ob Zucker rats subjected to 60 minutes of hepatic ischemia followed by 2, 6, and 24 hours of reperfusion. Control, anesthesia and laparotomy; I/R, PC + I/R, I/R with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10); PC + I/R + NAME, PC + I/R treated with L-NAME; I/R + NO, I/R treated with NO donor. *, $P < 0.05$ versus control; +, $P < 0.05$ versus I/R; °, $P < 0.05$ versus PC + I/R.

Figure 6. Histological lesions in liver after 24 hours of reperfusion. **A:** I/R (Ln): small area of coagulative hepatic necrosis (arrows) with neutrophil infiltration. **B:** I/R (Ob): widespread coagulative hepatic necrosis with neutrophil infiltration (arrows). **C:** PC + I/R (Ln): irregular area of incipient necrosis (asterisk). **D:** PC + I/R (Ob): moderate area of coagulative necrosis (arrows). **E:** PC + I/R + NAME (Ln): small area of coagulative hepatic necrosis (arrows) with neutrophil infiltration. **F:** PC + I/R + NAME (Ob): widespread coagulative hepatic necrosis with hemorrhage and neutrophil infiltration (arrows). **G:** I/R + NO (Ln): irregular area of incipient necrosis (asterisk). **H:** I/R + NO (Ob): moderate area of coagulative necrosis (arrows). I/R, ischemia-reperfusion; PC + I/R, I/R with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion before I/R; PC + I/R + NAME, PC + I/R treated with L-NAME; I/R + NO, I/R treated with NO donor. (H&E; original magnifications, $\times 500$).

Figure 7. MDA and MPO in liver from Ln and Ob Zucker rats subjected to 60 minutes of ischemia followed by 2, 6, and 24 hours of reperfusion. Control, anesthesia and laparotomy; I/R, ischemia-reperfusion; PC + I/R, I/R with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10); PC + I/R + NAME, PC + I/R treated with L-NAME; I/R + NO, I/R treated with NO donor. *, $P < 0.05$ versus control; +, $P < 0.05$ versus I/R; °, $P < 0.05$ versus PC + I/R.

A)

B)

Figure 8. A: SOD and GSH content in fatty livers. No significant differences in SOD activity were found. A significant decrease in GSH content was observed in unpreconditioned group (*, $P < 0.05$ versus control). **B:** Effect of GSH-ester on MDA and hepatic injury (ALT and α -GST). I, 60 minutes of ischemia; I/R, 60 minutes of ischemia followed by 6 hours of reperfusion; PC + I, I with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10); PC + I/R, I/R with previous preconditioning induced by 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10); I/R+GSH, I/R treated with GSH-ester. *, $P < 0.05$ versus control; +, $P < 0.05$ versus I/R.

blood perfusion on the same order of those observed after hepatic reperfusion (Figure 9). NO donor pretreatment simulated the effects of preconditioning on hepatic blood flow. These results of hepatic blood flow were confirmed with measurements of blood flow in portal vein.

We considered the possibility that the greater degree of injury observed in fatty livers might be related to a lower blood flow recovery at the early time points, but this was ruled out because the recovery of blood flow throughout the 2 hours of reperfusion was similar in normal and fatty

Figure 9. Blood hepatic perfusion in liver from Ln and Ob Zucker rats subjected to 60 minutes of ischemia followed by 2, 6, and 24 hours of reperfusion. I/R, ischemia-reperfusion; PC + I/R, I/R with previous preconditioning, induced by 5 minutes of ischemia followed by 10 minutes of reperfusion (5 + 10); PC + I/R + NAME, PC + I/R treated with L-NAME; I/R + NO, I/R treated with NO donor. *, $P < 0.05$ versus control; ⁺, $P < 0.05$ versus I/R; ^o, $P < 0.05$ versus PC + I/R. Values are expressed as percentages of the initial blood flow.

livers (data not shown). Preconditioning resulted in similar values of hepatic blood perfusion in both groups of livers, fatty and nonfatty, after 2 hours of reperfusion.

The role of TNF in the benefits of preconditioning on hepatic I/R injury was also evaluated. No significant increases in TNF levels were observed in normal and fatty livers undergoing 60 minutes of hepatic ischemia. The administration of the antibody anti-TNF resulted in transaminase and hepatic MPO levels similar to those found after hepatic reperfusion. All of these results seem to indicate that TNF could not be responsible for the neutrophil accumulation and liver damage observed after hepatic reperfusion in livers subjected to 60 minutes of ischemia. The histopathological findings showed no apparent pulmonary damage consequent to hepatic I/R in either Ln or Ob animals. In line with these results, the biochemical findings indicated neither neutrophil accumulation and oxidative stress nor edema formation in lung after reperfusion in both types of livers (data not shown).

Discussion

Steatotic livers seem to be more vulnerable to hepatic I/R damage. Fatty animals showed decreased survival after 60 minutes of total ischemia compared with lean animals. In addition, the biochemical and histological results indicated that steatotic livers tolerated poorly hepatic damage induced by 60 minutes of partial hepatic ischemia. The results obtained from previous studies^{24,25,30} and those obtained in the present work seem to indicate that preconditioning periods effective against hepatic damage from 90 minutes of ischemia could be unable to confer protection from 60 minutes of hepatic ischemia. Ischemic preconditioning consisting of 5 minutes of ischemia followed by 10 minutes of reperfusion conferred the strongest protection against hepatic I/R after 60 minutes of partial ischemia in both normal and steatotic livers. This also increased the animal survival in fatty livers after 60 minutes of total ischemia.

Production of ROS has been invoked as a key mechanism of I/R injury in several organs, including the liver.⁵⁹⁻⁶¹ One of the important effects of uncontrolled production of ROS is the peroxidation of membrane and other cellular lipids.^{60,61} Previous studies in orthotopic rat liver transplantation suggested that the greater lipid peroxidation after reperfusion in steatotic livers induced by alcohol may lead to cell death.²⁰ The present work showed an increased lipid peroxidation in steatotic livers from Zucker rats subjected to 60 minutes of normothermic ischemia, whereas no lipid peroxidation was observed in nonfatty livers. These results provide additional data about the differences in the mechanisms of hepatic I/R injury between fatty and nonfatty livers, since previous studies have reported differences in the mechanisms of cell death between both types of livers.²¹ In the case of fatty livers, it has been postulated that the hepatocytes are more susceptible to lipid peroxidation either because of the presence of excessive fat and/or greater production of ROS.¹⁰ In our hands, ischemic preconditioning

reduced the lipid peroxidation observed in fatty livers after hepatic reperfusion. Considering that preconditioned and unpreconditioned livers showed a similar degree of steatosis (all animals were randomized for group assignments), the effect of ischemic preconditioning on lipid peroxidation could be explained by a reduced post-ischemic ROS generation. This endogenous protective mechanism could probably be more effective against ROS than pharmacological strategies based on the administration of antioxidants. To be effective, antioxidants need to reach the site of action in adequate concentrations.⁶² Until now, data about the effectiveness of the administration of antioxidants on the deleterious effects of ROS in fatty livers was controversial. Recent studies in obese Zucker rats, indicated that the administration of tocopherol, which possesses antioxidant properties, resulted in amelioration and improved tolerance to warm ischemia.⁶² However, other experimental studies in steatotic livers, induced by a choline-methionine-deficient diet, show that the administration of GSH precursors, such as *N*-acetylcysteine, could help restore hepatocellular integrity in the steatotic liver but does not scavenge free radical.¹² In addition, both dietary high fat and alcohol exposure produced SOD/catalase-insensitive free radical species that may be involved in the mechanism of failure of fatty livers after orthotopic liver transplantation.²⁰ The results of the present work indicate that preconditioning prevented GSH depletion during hepatic ischemia and this endogenous protective mechanism was more effective against ROS and hepatic damage than GSH-ester pretreatment.

Neutrophils have been involved in the increased vulnerability of fatty livers to hepatic I/R injury, especially in alcoholic fatty livers.^{13,63} However, they do not account for the differentially greater injury in the nonalcoholic fatty liver during the early or late hours of reperfusion. The results of the present study show practically the same degree of neutrophil accumulation in normal and fatty livers. These findings are in line with those obtained previously in other experimental models of nonalcoholic fatty livers, including cholesterol-induced fatty livers.¹³ These observations could be of clinical interest because pharmacological strategies that could be effective in alcoholic fatty livers by reducing the neutrophil infiltration may be not sufficient to reduce the hepatic I/R injury in nonalcoholic fatty livers. Probably, a combination of more pharmacological strategies or other strategies that do not exert their action exclusively by reducing neutrophil infiltration, including ischemic preconditioning may be required to effectively protect against the hepatic I/R injury.

Experimental observations in warm and cold ischemia in fatty livers induced by a choline-deficient diet have demonstrated disruption in the sinusoidal microcirculation.^{14,15} However, it should be pointed out that in other experimental models of obesity including the genetic abnormality in lipid metabolism seen in obese Zucker rats, there is not an excessive alteration to the microcirculation.²¹ The authors found no significant changes in portal pressure between Ln and Ob Zucker rats and suggests that a lack of reperfusion was probably not a major contributing factor in fatty livers. In line with these

observations, the results of the present work show similar hepatic blood flow during reperfusion in normal and fatty livers. However, it cannot be excluded that disturbances of the microcirculation could be involved in the mechanism of injury in steatotic livers and that strategies including ischemic preconditioning could be useful in both normal and fatty livers to reduce the microcirculatory alterations associated with hepatic I/R, at least after 60 minutes of hepatic ischemia. Previous studies indicated that hepatic blood flow was not affected by preconditioning in normal livers.²⁴ However, the differences in the times of sustained ischemia and preconditioning periods used in that study (90 minutes and 10 minutes of ischemia and reperfusion periods) could explain, at least partially, the potential differences in the underlying protective mechanisms of ischemic preconditioning.

The effectiveness of anti-TNF therapy in the clinical practice to reduce the hepatic I/R injury in steatotic livers subjected to 30 minutes of normothermic ischemia has been reported.¹⁶ Experimental results indicate that alcoholic fatty livers exposed to 30 minutes of normothermic ischemia showed an increase in TNF, whereas, the non-alcoholic fatty liver did not show cytokine induction after hepatic I/R.¹³ Taking the experimental results of the present study into account, TNF does not seem to play a key role in the hepatic I/R injury in normal and fatty livers subjected to 60 minutes of ischemia, suggesting that anti-TNF therapy could not be an effective strategy to reduce hepatic I/R in these conditions. However the possibility that this treatment could be effective at longer ischemic periods should be not excluded. Previous studies indicated that the administration of anti-TNF antibodies was able to confer protection against both liver and lung damage after hepatic I/R in normal livers subjected to 90 minutes of hepatic ischemia.

The multiple and different mechanisms of I/R injury between normal and steatotic livers, as well as between different types of steatosis suggest the inherent difficulties in the effective prevention of hepatic I/R injury using pharmacological strategies. Thus, we postulated that ischemic preconditioning could be of clinical interest because it is an effective strategy to reduce the hepatic I/R injury in both normal and fatty livers. Our results show that ischemic preconditioning was able to control the mechanisms involved in the hepatic I/R injury during the reperfusion of fatty livers, including the increases in lipid peroxidation, neutrophil accumulation, and the failures in hepatic microcirculation. We also evaluated if these beneficial effects could be mediated by NO.

NO is considered to be one of the most likely candidates for mediating preconditioning.²³ Along this line, the implication of NO in the protective effects of preconditioning on hepatic I/R injury has been previously demonstrated in normal livers in experimental models of warm and cold ischemia.^{24,27} However, although its beneficial effects on hepatic injury are well established, the mechanisms by which it confers protection remain to be elucidated. The administration of NO donors as well as inhibitors of NO synthesis in different experimental models of hepatic I/R indicate that the beneficial effects of NO could be related to an improvement of the hepatic micro-

circulation, as well as a reduced neutrophil accumulation and oxidative stress.^{64–66} Taking these observations into account, we evaluated whether ischemic preconditioning, through NO generation, could modulate the mechanisms involved in hepatic I/R and the ensuing hepatic injury associated with this process. The results of the present study indicated that the inhibition of NO synthesis in both normal and fatty livers abolished the benefits of preconditioning on hepatic blood flow, neutrophil accumulation, and oxidative stress. Biochemical parameters of hepatic injury and histological results similar to those observed after hepatic reperfusion were found. In addition, NO donor pretreatment simulated the benefits of preconditioning, resulting in biochemical and histological results similar to those observed when preconditioning was performed.

The present experimental results indicating that ischemic preconditioning is able to confer protection in steatotic livers could be interesting in liver surgery. The potential application of ischemic preconditioning in clinical practice could improve the tolerance of fatty livers to I/R injury in normothermic conditions, including hepatic resections. It could also improve the initial conditions of donor livers with low steatosis that are available for transplantation but with deficient postsurgical results, and could increase as well the use of donor livers with severe steatosis that are presently discarded for transplantation.

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