

RAPID COMMUNICATION

Ischemic Preconditioning Attenuates the Oxidant-Dependent Mechanisms of Reperfusion Cell Damage and Death in Rat Liver

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In an in vivo rat model of liver ischemia followed by reperfusion a consistent appearance of necrosis and activation of biochemical pathways of apoptosis was reproduced and monitored after 30 minutes reperfusion. Preconditioning by application of a short cycle of ischemia-reperfusion (10 minutes + 10 minutes) positively conditioned recovery of the organ at reperfusion, attenuating both necrotic and apoptotic events. Preconditioning at least halved cell oxidative damage occurring early at reperfusion, and as a major consequence, the increase of cytolysis and apoptosis occurring at reperfusion was about 50% less. The attenuation of both pathways of cell death by preconditioning appeared at least partly related to its modulate action on H₂O₂ and 4-hydroxy-2,3-trans-nonenal production. The overall data point to a marked diminished oxidant generation and oxidative reactions as one major possible mechanism through which ischemic preconditioning exerts protection against necrotic and apoptotic insult to the postischemic liver. (*Liver Transpl* 2002;8:990-999.)

Oxidative stress (i.e., the prevalence of oxidizing species over the tissue antioxidant potential) has been repeatedly reported to occur in organs undergoing ischemia-reperfusion.¹⁻³ Particularly in the liver, the postischemic oxidative stress and cell damage observed during the first minutes of reperfusion is interpreted as

an overproduction of oxygen free radicals in activated Kupffer cells,^{4,5} and in the mitochondria of hepatocytes and sinusoidal endothelial cells, where oxygen reflow encounters severely reduced respiratory chains.² Further, there is agreement that the inflammatory reaction triggered during and after blood reflow through a specific organ induces a paradoxically more harmful phase of damage than that occurring during the preceding sustained ischemic period.^{6,7}

Another consistent consequence of ischemia-reperfusion is the stimulation of programmed cell death. Also, it has been confirmed recently that apoptosis contributes to tissue damage in models of liver reperfusion injury. The number of apoptotic cells are correlated with the duration of ischemia and also with survival. Consistently, the inhibition of apoptotic effectors like caspases or calpain-like proteases reduces the extent of the injury.⁸⁻¹⁰ As with postreperfusion necrosis, the pathogenesis of which implies oxidative mechanisms of cell damage,¹¹ postischemia/reperfusion apoptosis may to some extent be dependent on oxidative stress. Indeed, an increasing body of literature supports the hypothesis of oxidative damage acting as an efficient apoptotic stimulus.¹²

Because of the harmful side effects of organ reperfusion, procedures able to counteract this kind of damage are actively planned and developed. Ischemic preconditioning (IP) (i.e., the brief ischemic pretreatment of an organ that, for various surgical reasons, undergoes long-term ischemia followed by reperfusion) is considered a promising approach to the problem.¹⁴

IP has been shown to attenuate the tissue injury observed after reperfusion of the heart,^{14,15} brain and skeletal muscle.^{16,17} Quite recently this procedure has been extended to experimental models of hepatic vessel interruption and liver resection.¹⁸⁻²⁰ Moreover, in a recent study carried out on experimental liver ischemia-reperfusion, IP has been shown to confer protection against prolonged ischemia, by inhibiting apoptosis through down-regulation of caspase 3 activity.²¹

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Although the exact mechanisms of protection are yet to be defined, various effects have been attributed to preconditioning (i.e., improvement of energy metabolism),^{22,23} modulation of oxidative stress,²⁴ increase of the steady-state levels of vasoactive mediators like adenosine, nitric oxide, or both.^{18,20}

The data reported here indicate that the protective effect of IP against liver postischemic damage involves prevention of the ischemic/reperfusion-dependent exacerbation of oxidative stress and lipid peroxidation. Indeed, IP strongly, though not completely, inhibited the enhancement of H₂O₂ and 4-hydroxy-2,3-trans-nonenal (HNE) production induced by ischemia-reperfusion. This molecular mechanism of protection appears, at least in part, responsible for the marked attenuation both of necrosis and programmed cell death observed during the initial phase of blood reflow.

Materials and Methods

Induction of Long-Term Hepatic Ischemia Followed by Reperfusion

Male Sprague-Dawley rats (314 ± 21 g body weight) were used. They had free access to standard chow diet and water. After anesthesia with 10% ketamine chlorhydrate saline solution, rats were positioned under a heat lamp to maintain their body temperature constant and after laparotomy and subjected to selective inflow occlusion of the median and left lateral lobes of the liver by placing a bulldog clamp on the portal triad. Reperfusion was achieved by removing the clamp. Tissue samples from the median and left lateral lobes were taken at the end of the reperfusion phase for biochemical and morphologic analysis. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Experimental Groups

The rats were subdivided into five groups of five rats each.

- Group 1. Sham-operated: animals subjected to anesthesia and laparotomy only.
- Group 2. Standard Ischemia (Isch): the medial and left lateral lobes were subjected to 90 minutes of ischemia (selective lobar ischemia)
- Group 3. Standard Ischemia-Reperfusion (Isch/Rep): after 90 minutes of selective lobar ischemia, reperfusion was allowed for 30 minutes.
- Group 4. Ischemic Preconditioning plus Ischemia (Prec + Isch): before longterm ischemia, the medial and left lateral lobes were subjected to 10 minutes ischemia followed by 10 minutes reperfusion.
- Group 5. Ischemic Preconditioning plus Ischemia-Reper-

fusion (Prec + Isch/Rep): before the cycle of ischemia/reperfusion, the medial and left lateral lobes were subjected to preconditioning procedure as for group 4.

Biochemical Assessment of Liver Cytolysis

Irreversible hepatic damage was evaluated by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a commercial kit from Sigma Chemical Co. (St. Louis, MO). Blood samples were obtained from the posterior cava vein at the end of the reperfusion period.

Hydrogen Peroxide Analysis

Tissue from sham-operated and reperfused livers were homogenized (10%, w/v) in a buffer containing 0.033 mol/L Na₂HPO₄ and 0.9% KCl, pH 7.4 at 4°C. The homogenates were centrifuged at 40,000 g for 45 minutes and the supernatant was used for H₂O₂ analysis. The hydrogen peroxide-mediated oxidation of Fe²⁺ to Fe³⁺ ion, under acidic conditions and in the presence of xylenol orange dye, was quantified as elsewhere described.²⁵ For the working reagent, 1 mL solution A containing ammonium ferrous sulfate (25 mmol/L) and sulfuric acid (2.5 mmol/L) was mixed with 100 mL of solution B containing sorbitol (100 mmol/L) and xylenol orange (125 μmol/L) in water. A 0.1 mL volume of supernatant was added to 1 mL of working reagent, mixed and incubated at room temperature for 15 minutes. The absorbance was read at 560 nm. The values were plotted against a standard curve with 1 to 50 μmol/L concentrations of H₂O₂. The absolute values of H₂O₂ were expressed in μmol/g wet tissue.

Determination of Free 4-Hydroxy-2,3-trans-nonenal

Hepatic tissue levels of free HNE were determined with the method described by Esterbauer et al.²⁶ Briefly, determination was performed on fresh cytosolic fractions by high performance liquid chromatography with a Symmetry C₁₈ column. The mobile phase used was acetonitrile:bidistilled water (42%, v:v). The concentration of free HNE was calculated by comparison with a standard solution of HNE at a known concentration.

Determination of 4-Hydroxy-2,3-trans-nonenal and Malondialdehyde Protein Adducts By Fluorescence Spectroscopy

Aliquots of 10% homogenates were mixed with cooled 10% trichloroacetic acid for 60 minutes and then centrifuged at 2,000 rpm/10 min/4°C. The pellets were extracted 3 times with 6 mL of ethanol-diethylether (3:1, v/v). The proteins thus precipitated were dried under nitrogen stream and resuspended in 3 mL of distilled water plus 0.5% sodium dodecyl sulphate. Detection by fluorescence spectroscopy was carried out by recording the excitation spectra of the samples from 280 to 420 nm, with a fixed emission of 460 nm. Using standard adducts of aldehydes with bovine serum albumin,

the excitation maxima for HNE and MDA-protein adducts were found at 355 and 390 nm, respectively.

Caspase 3 Activity Assay

Caspase 3 activity was determined by measuring proteolytic cleavage of the specific substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA, CaspACE Assay System, Colorimetric, Promega, Madison, WI, USA). Liver tissue was quickly excised and sonicated in assay buffer (1 mmol/L ethylenediaminetetraacetic acid, 145 mmol/L NaCl, 100 mmol/L Tris, 0.1 mmol/L dithiothreitol, 0.1% CHAPS, 10% glycerol), the cytosolic fraction was obtained and protein content determined. The samples were diluted and incubated overnight at 37°C on Ac-DEVD-pNA substrate in the presence or absence of the specific caspase 3 inhibitor Z-VAD-FMK. The chromophore pNA, which is released from the substrate on cleavage, was monitored by a photometer at 405 nm. The amount of yellow color produced is proportional to the amount of caspase activity present in the sample. The results were expressed as pmol pNA liberated per μg protein.

Morphologic Studies

Fragments of reperfused medial lobe of approximate size $1.0 \times 0.5 \times 0.3$ cm were placed overnight in 4% formaldehyde, 50 mmol/L phosphate buffer solution, pH 7.4. Paraffin wax sections of 5 μm were stained with Harris hematoxylin and eosin. Necrosis was semiquantitatively evaluated in 10 nonconsecutive, randomly chosen $\times 250$ histologic fields, scored as follows: 0, absence of necrosis; 1, spotty necrosis (scattered necrotic hepatocytes); 2, focal necrosis (periportal or perivenular or midacinar necrosis); 3, multifocal necrosis (necrosis in more than one acinar zone); and 4, necrosis bridging between vascular inflow and outflow structures. Morphologic criteria such as increased cytoplasmic eosinophilia, vacuolization, cell disruption, loss of architecture, and nuclear changes consisting in pycnosis, karyorrhexis, or karyolysis were used to determine necrosis.

Samples from both sham-operated and postischemic livers were fixed in 2.5% glutaraldehyde solution in 0.1 mol/L sodium phosphate buffer, pH 7.4, for 3 hours at 4°C, postfixed in a 2% osmium tetroxide solution, dehydrated in graded ethanol solutions, and embedded in Epon-Araldite. Ultrathin sections stained with uranyl acetate and lead citrate were examined through a 410 Philips transmission electron microscope (Philips Electronic Instruments Inc, Eindhoven, Holland) with an 80-kV acceleration voltage.

Immunofluorescence of Cytochrome *c*

Fragments of liver were fixed in 4% paraformaldehyde 154 mmol/L Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH 7.5 overnight at 4°C, washed in rinse water and treated with 0.1 mole Tris, pH 7.5 with 0.2% glycine. Sections were then dewaxed and embedded in paraffin wax. Rehydrated sections were washed twice in modified Shield's medium-PIPES (18 mmol/L MgSO_4 , 5 mmol/L CaCl_2 , 40 mmol/L

KCl, 24 mmol/L NaCl, 0.5% Triton X-100, 0.5% Igepal, 5 mmol/L PIPES, pH 6.8) and incubated for 10 minutes at 37°C with 100 mmol/L sodium cyanoborohydride in 140 mmol/L NaCl, 10 mmol/L phosphate-buffered saline (PBS), pH 7.4. Washed sections in MSM-PIPES were preblocked (3% bovine serum albumin, 5% normal rabbit serum, and 0.3% Tween 20) for 30 minutes before incubation with cytochrome *c* monoclonal antibody (PharMingen) at room temperature for 90 minutes followed by rinse in PBS. The secondary antibody, a fluorescein isothiocyanate-labeled rabbit antimouse immunoglobulin G (IgG), was diluted in PBS and sections incubated for 45 minutes at room temperature. Cytochrome *c* fluorescence at 490 excitation and 525 nm emission wave lengths was analyzed with a 510 Laser Scanning Microscope (Zeiss, Jena, Germany).

Immunohistochemical Evaluation of HNE-Protein Adducts

Paraffin-embedded sections were deparaffinized, rehydrated, then immunostained for the presence of an *in vivo* marker of lipid peroxidation (i.e., the HNE-protein adducts). To this purpose, sections were incubated with an anti-HNE-histidine polyclonal antibody (Alpha Diagnostic International, San Antonio, TX) in PBS, pH 7.4, containing 1% Tween 20 and 1% bovine serum albumin, as elsewhere described.²⁷ Peroxidase-linked secondary antibody and diaminobenzidine were used to detect specific binding. No positive staining was detected when tissue from the same animals were processed without primary antibody, indicating the absence of not specific binding under the conditions used.

Protein Determination

The protein content of liver homogenates was determined by the spectrophotometric method described by Peterson²⁸ with bovine serum albumin as control.

Statistical Analysis

Data were expressed as means \pm SE. The statistical significance of differences between groups was analyzed using the one-way analysis of variance (ANOVA). The *P* values were corrected by the Bonferroni method.

Results

Ischemic preconditioning attenuates the generation of H_2O_2 and lipid peroxidation end-products induced by ischemia/reperfusion

Among the various biologic effects of oxidative stress, the peroxidation of membrane lipids is by far the most expressed. The oxidative breakdown of polyunsaturated fatty acids, which are present in significant quantities in all cell membranes, leads to the production of a variety of aldehydes, in particular HNE and MDA. Thus, detection of free HNE and HNE and MDA-

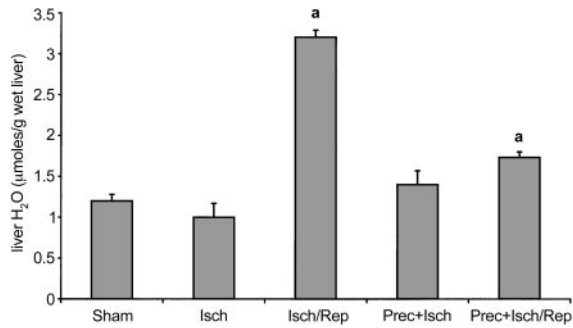


Figure 1. Steady-state concentration of H₂O₂ after long-term ischemia/reperfusion. Results are expressed as mean values \pm SE of 5 animals per experimental group. Sham, rats subjected to laparotomy only; Isch, rats subjected to 90 minutes of ischemia; Isch/Rep, rats subjected to 90 minutes ischemia and 30 minutes reperfusion; Prec + Isch: rats subjected to ischemic-preconditioning procedure (10 minutes ischemia followed by 10 minutes reperfusion) before long-term ischemia-reperfusion treatment; Prec + Isch/Rep, rats subjected to the ischemic-preconditioning procedure (10 minutes ischemia followed by 10 minutes reperfusion) before undergoing the long-term ischemia-reperfusion treatment. The significant differences between groups are as follows: a, $P < 0.05$: Isch/Rep versus Sham, Prec+Isch/Rep versus Sham.

protein appears to be one of the most reliable analyses presently available to monitor lipid peroxidation (e.g., in the reperfusion syndrome).^{26,28}

Steady-state concentration of H₂O₂ and production of free HNE were not modified by 90 minutes of ischemia, as the values at that time point were quite similar to those observed in the liver of sham-operated animals (Figs. 1 and 2). However, a different pattern was

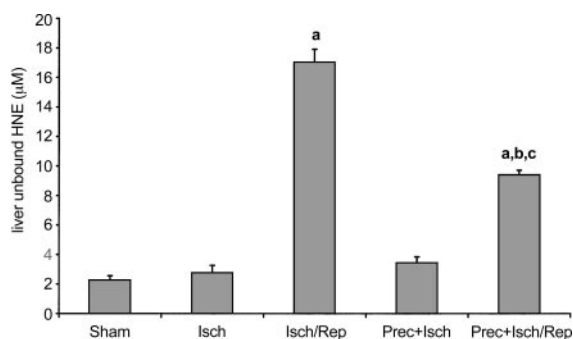


Figure 2. Levels of free HNE after 90 minutes ischemia and 30 minutes reperfusion. Results are expressed as mean values \pm S.E. of 5 animals per experimental group. Experimental groups are as in Figure 1. The significant differences between groups are as follows: a, $P < 0.05$: Isch/Rep versus Sham, Prec+Isch/Rep versus Sham; b, $P < 0.05$: Prec+Isch/Rep versus Isch/Rep; c, Prec+Isch/Rep versus Prec Isch

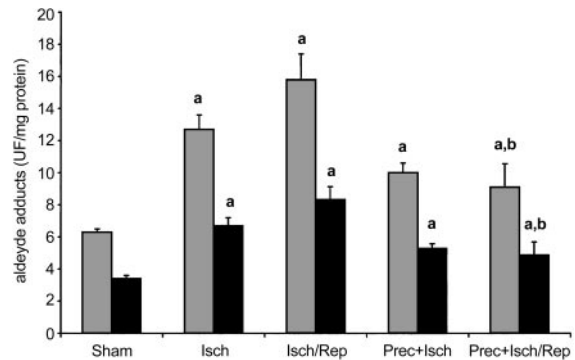


Figure 3. Levels of 4-hydroxy, 2, *trans*-nonenal (HNE)/malondialdehyde (MDA) protein adducts after 90 minutes ischemia and 30 minutes reperfusion. Results are expressed as median values \pm SE of 5 animals per experimental group. Experimental groups as in Figure 1. The significant differences between groups are as follows: a, $P < 0.05$: Isch versus Sham, Isch/Rep versus Sham, Prec+Isch versus Sham, Prec + Isch/Rep versus Sham; b, $P < 0.05$: Prec + I/R versus Isch Rep. □, HNE; ■, MDA.

observed after liver reperfusion in the presence or absence of preconditioning: In the reperfused, nonpreconditioned liver, steady-state concentrations of H₂O₂ and free HNE were respectively three and eight times higher than those detected in the sham-operated group (Figs. 1 and 2). Preconditioning was able to reduce the postreperfusion level of H₂O₂ to values similar to those of sham-operated, whereas free HNE production results were halved (Figs. 1 and 2).

As far as the levels of HNE and MDA are concerned, a net significant increase was observed, after 90 minutes ischemia, in both nonpreconditioned and preconditioned livers in regard to the sham-operated group (Fig. 3). However, after 30 minutes of reperfusion, the levels of HNE and MDA maintained an increasing trend only in the nonpreconditioned group, whereas in the preconditioned livers they remained steady, and significantly lower compared to that in the nonpreconditioned group (Fig. 3). Accordingly, an intense immunohistochemical staining for cytoplasmic HNE-modified proteins was observed in the nonpreconditioned reperfused livers. Some hepatocytes also exhibited nuclei positive for HNE-protein. Epithelial duct cells also showed a cytoplasmic positive reaction (Fig. 4A). In contrast, HNE-protein was markedly decreased when IP was applied before ischemia-reperfusion. In this case, only a slight reaction was observed on scattered periportal and perivenular hepatocytes, as well as in the epithelial duct cells (Fig. 4B). No HNE-modified proteins were detected in the sham-operated group of animals (Fig. 4C).



Figure 4. Immunostaining for HNE-modified proteins. Numerous periportal hepatocytes with an intense positive staining were seen in the Isch/Rep group (A), whereas in the Prec + Isch/Rep group, a slight positive reaction was present on scattered hepatocytes (B). In the sham group, no HNE-modified proteins were detected. (C) (hematoxylin counterstained, $\times 400$). Experimental groups as in Figure 1.

Table 1. Hepatocellular Necrosis Score

Groups	Score
Sham	0
Isch/Rep	$2.5 \pm 0.2^*$
Prec + Isch/Rep	$1.6 \pm 0.3^\dagger$

NOTE. Results are expressed as mean values \pm SE of 5 animals per experimental group.
Abbreviations: Isch/Rep, rats submitted to 90 minutes of ischemia followed by 30 minutes of reperfusion; Prec + Isch/Rep, rats submitted to ischemic preconditioning procedure (10 minutes of ischemia followed by 10 minutes of reperfusion) before entering the same long-term ischemia-reperfusion treatment.
* $P < .05$, Isch/Rep and Prec + Isch/Rep *v* Sham.
 $^\dagger P < .05$, Prec + Isch/Rep *v* Isch/Rep.

Ischemic Preconditioning Markedly Protects Rat Liver Against Necrosis Early After Reperfusion

Long-term ischemia followed by reperfusion induced hepatic necrosis in both preconditioned and nonpreconditioned animals. However, IP greatly reduced the extension of irreversible cell injury, detectable 30 minutes after declamping of hepatic artery and portal vein. The light microscopy analysis of nonpreconditioned livers showed multiple and extensive areas of hepatocellular necrosis. On the contrary, in the livers of the animals previously subjected to IP, only scattered groups of necrotic hepatocytes were observed (data not shown). Accordingly, in preconditioned livers, the necrosis score was approximately half that found in nonpreconditioned livers (Table 1). Electron microscopy analysis clearly revealed a marked degree of mitochondrial degeneration in nonpreconditioned versus preconditioned livers. In the former, the mitochondria were characterized by intense swelling with loss of cristae and marked decrease of matrix density; further, some mitochondria showed blebs with rupture of the external membrane (Fig. 5A). In the preconditioned liver, on the contrary, mitochondrial alterations were limited to a mild degree of swelling (Fig. 5B). Consistently, ALT and AST plasma levels at 30 minutes after reperfusion were approximately halved in preconditioned versus nonpreconditioned animals (Fig. 6).

Ischemic Preconditioning Counteracts Reperfusion-Dependent Apoptosis Through Down-Regulation of Cytochrome *c* Release and Caspase-3 Activity

Cytochrome *c* is one of the upstream signals for caspase-3 activation. The effect of IP on hepatic cyto-

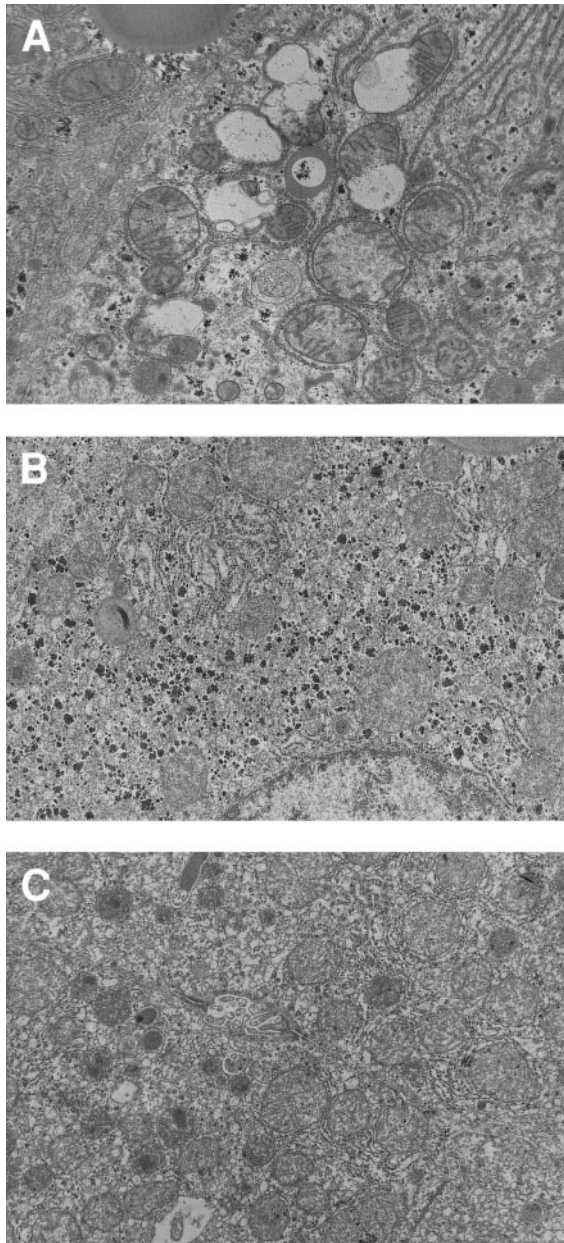


Figure 5. Electron microscopy images of hepatocytes after long-term ischemia/reperfusion \pm preconditioning. Typical images of ultrastructural alterations were chosen from the different experimental groups. (A) Isch/Rep: swollen mitochondria with decreased matrix density, blebbing and rupture of the external membrane. The rough endoplasmic reticulum shows some degree of swelling. (B) Prec+Isch/Rep: mitochondria with mild swelling. The rough endoplasmic reticulum appears normal. (C) Sham: mitochondria and the other organelles exhibit a normal structure (uranyl acetate and lead citrate \times 12,300). Experimental groups as in Figure 1.

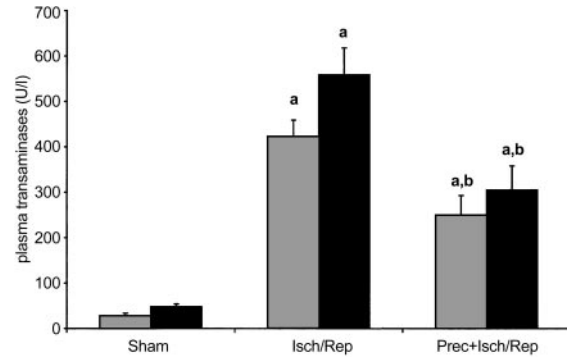


Figure 6. ALT and AST plasma levels as biochemical markers of irreversible hepatic injury after long-term ischemia/reperfusion \pm preconditioning. Results are expressed as mean values \pm SE of 5 animals per experimental group. Experimental groups as in Figure 1. The significant differences between groups are as follows: a, $P < 0.05$: Isch/Rep and Prec+Isch/Rep versus Sham; b, Prec+Isch/Rep versus Isch/Rep. \square , ALT; \blacksquare , AST.

chrome *c* was monitored by immunofluorescence. In sham-operated animals, cytochrome *c* appeared in the cytoplasm in a punctate pattern indicative of mitochondrial localization (Fig. 7A). When livers were examined after a cycle of ischemia/reperfusion, hepatocytes showed dramatic panfluorescence, consistent with massive release of cytochrome *c* into the cytoplasm (Fig. 7B). On the contrary, most hepatocytes in preconditioned livers did not show this pattern of staining but rather retained the punctate pattern of fluorescence, suggesting that cytochrome *c* release into the cytoplasm was markedly reduced (Fig. 7C).

Consistent with the release of cytochrome *c* into the cytoplasm, a net stimulation of one of the effector caspases was observed. Indeed, the activity of caspase-3 in nonpreconditioned livers undergoing 90 minutes ischemia followed by 30 minutes reperfusion was about one order of magnitude higher than that measurable in sham-operated rats. On the other hand, the reperfusion-dependent increase of caspase-3 was much less dramatic in preconditioned liver, being only 2 to 3 times higher than in sham operated rats (Table 2).

Discussion

In the *in vivo* experimental model of liver reperfusion injury employed here, a brief episode of vascular occlusion has been confirmed to make the organ highly resistant to cell damage consequent to prolonged ischemia followed by tissue reoxygenation. Both necrotic and apoptotic pathways of cell death, clearly evident soon after reperfusion, were markedly though not com-

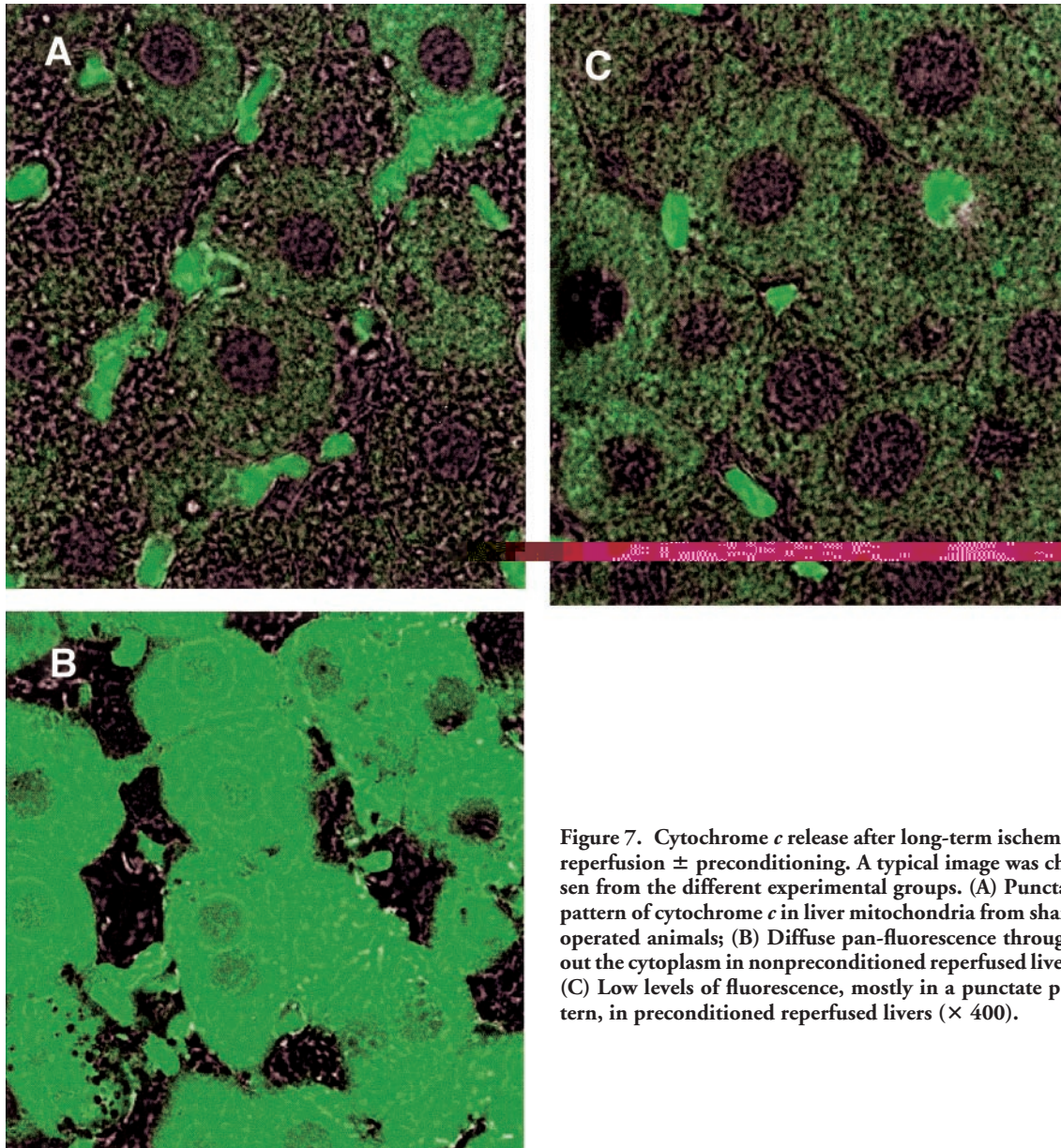


Figure 7. Cytochrome *c* release after long-term ischemia/reperfusion \pm preconditioning. A typical image was chosen from the different experimental groups. (A) Punctate pattern of cytochrome *c* in liver mitochondria from sham-operated animals; (B) Diffuse pan-fluorescence throughout the cytoplasm in nonpreconditioned reperfused livers; (C) Low levels of fluorescence, mostly in a punctate pattern, in preconditioned reperfused livers ($\times 400$).

pletely prevented by the ischemic preconditioning procedure. However, the molecular mechanisms underlying the protective effect of preconditioning are still largely unclear. The reported results may significantly contribute to further understanding the protection mechanisms that IP triggers against necrosis and apoptosis in the reperfused liver.

In this study, oxidative stress was assessed by measuring hepatic steady-state concentrations of H_2O_2 and of aldehydic end products of lipid peroxidation, namely free HNE, as well as HNE and MDA bound to protein. We observed a direct correspondence between the levels

Table 2. Caspase-3 Activity

Groups	Specific Activity (pmol pNA/mg Protein)
Sham	1.45 \pm 0.43
Isch/Rep	11.60 \pm 2.15*
Prec + Isch/Rep	4.53 \pm 0.77†

NOTE. Results are expressed as mean values \pm SE of 5 animals per experimental group. Experimental groups were those reported in Table 1.
* $P < .05$, Isch/Rep *v* Sham.
† $P < .05$, Prec + Isch/Rep *v* Isch/Rep.

of pro-oxidants and oxidation products and the degree of postreperfusion damage, the latter being expressed in terms of mitochondrial alterations, activation of apoptosis process and hepatocellular necrosis. A key effect of preconditioning was the capacity to downregulate the production of H_2O_2 , as well as that of free HNE and HNE/MDA-protein occurring during the reperfusion phase. As a result of this better balanced redox situation, the preconditioned livers showed a better morphologic preservation of mitochondria, which also was evident in terms of attenuated levels of plasma aminotransferases.

In recent years, several laboratories have reported evidence of apoptotic cell death during hepatic ischemia and reperfusion.²⁹⁻³² Activation of caspases, especially the effector caspase-3, is considered an early biochemical event essential for the progression of the apoptotic processes. Indeed, activation of caspase-3 mediated by mitochondrial cytochrome *c* release has been confirmed in various cell types, in response to different death stimuli.³² In the present study, we showed a massive release of cytochrome *c* into the cytoplasm after a sustained cycle of ischemia/reperfusion monitored by confocal microscopy (Fig. 7B). Simultaneously, a dramatic increase of caspase-3 activity was observed (Table 2). Our data are in agreement with the results of a recent elegant publication by Soeda et al,³² who showed that, after 90 minutes of warm ischemia of rat liver, progressive release of mitochondrial cytochrome *c* and activation of caspase-3 occur early at reperfusion, with morphologic manifestation of apoptosis. However, our data are evidently contrary to the previous research by Gujral et al³¹ who did not find either morphologic or biochemical data to support the hypothesis that apoptosis is a quantitatively relevant mechanism of cell death during warm hepatic ischemia/reperfusion. Such discrepancy could be mainly explained by the actual difference in the length of sustained ischemia in the reperfusion models (i.e., 60 minutes ischemia in the Gujral study against 90 minutes ischemia adopted by us and by Soeda et al³²).

Also, morphologic analyses support cytochrome *c* release from damaged mitochondria as shown by electron microscopy of nonpreconditioned liver, which showed that mitochondria were swollen with formation of blebs. On the other hand, a much better preserved mitochondrial structure was observed in the preconditioned livers. In the later group of animals, 1. cytochrome *c* immunofluorescence was only slightly higher than it was in the sham-operated group, with a distribution mostly in reticular and punctate patterns, indicative of a quite normal mitochondrial structure; 2.

caspase-3 activity was steadily decreased to values statistically not different as to those obtained in the liver of sham animals; and 3. the release of transaminase, as well as the score of necrosis were significantly attenuated.

The reported results well support the opinion that oxidative stress might be an important condition for promoting cell death (necrosis, apoptosis, or both) in response to postischemic injury. In particular, H_2O_2 and HNE appear to be reactive species actually able to mediate both necrotic and apoptotic responses to an ischemia-reperfusion cycle because they can spread through the cell, oxidize critical cellular thiol groups, and lead to the disruption of lipid membranes at a distance from the original site of free radical formation.^{33,34} Concerning the apoptosis pathway of cell death induced by reactive oxygen species (ROS), a straight relationship between H_2O_2 concentration and manifestation of apoptosis expressed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) positivity, cytochrome *c* delocalization, caspases activation, poly (ADP-ribose) polymerase cleavage, and dissipation of mitochondrial membrane potential was recently reported.³⁵⁻³⁸ Moreover, HNE was also shown to exert a proapoptotic effect, through activation of caspases,^{38,39} as well as by thiol oxidation/derivatization and lipid peroxidation reactions on the core of the mitochondrial permeability transition pore complex (i.e., adenine nucleotide translocator [ANT]), causing mitochondrial permeability transition, release of cytochrome *c* and apoptosis.⁴⁰

Our results allow us to hypothesize that ischemic preconditioning exerts beneficial effects already during the early phase of reperfusion most probably through modulation of one of the major hepatocellular sources of ROS generation after ischemia and reperfusion (e.g., the auto-oxidation rate of components of the respiratory chain), namely nicotinamide adenine dinucleotide (reduced)-ubiquinone oxidoreductase (complex I) and ubiquinone cytochrome *c* reductase (complex III).² To support the proposed mechanism of ischemic tolerance to ischemic injury, Peralta et al²⁴ recently showed that preconditioning conferred protection to the liver against ischemia/reperfusion damage by blocking the xanthine oxidase pathway of ROS generation. Taken together, the present experimental data suggest that the beneficial effects of ischemic preconditioning could be of clinical interest, because of its ability to prevent the formation of ROS early after a warm cycle of ischemia and reperfusion.

Similar protection was obtained against liver ischemia/reperfusion injury by heat shock preconditioning procedure.⁴¹ In that paper, the authors showed a good

correlation among the levels of heat shock protein 72, the improvement of hepatic microcirculation and the decrease of HNE-protein. Whether the surgical procedure of ischemic preconditioning is able to upregulate the inducible forms of heat shock proteins is yet to be investigated, and certainly is a target of our future research.

Preconditioning appears to induce intrinsic protective events which are responsible for prevention of cell death by apoptosis and necrosis. Delineating these intervening cellular events will aid in the prevention of ischemia/reperfusion damage after transplantation, hepatic resection, and different shock states.

References

- Adkinson D, Hoellwarth ME, Benoit JN, Parks DA, Mc Cord JM, Granger DN. Role of free radicals in ischemia-reperfusion injury to the liver. *Acta Physiol Scand Suppl* 1986;40:548-590.
- Gonzalez Flecha B, Cutrin JC, Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *J Clin Invest* 1993;91:456-464.
- Mathews WR, Guido DM, Fisher MA, Jaeschke H. Lipid peroxidation as molecular mechanism of liver cell injury during reperfusion after ischemia. *Free Radic Biol Med* 1994;16:763-770.
- Jaeschke H, Farhood A. Neutrophil and Kupffer cell induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991;260:G335-G342.
- Cutrin JC, Llesuy S, Boveris A. Primary role of Kupffer cell-hepatocyte communication in the expression of oxidative stress in the post-ischaemic liver. *Cell Biochem Funct* 1998;16:65-72.
- Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in the rat liver in vivo. *FASEB J* 1990;4:3355-3359.
- Cutrin JC, Boveris A, Zingaro B, Corvetto G, Poli G. In situ determination by surface chemiluminescence of temporal relationships between evolving warm ischemia-reperfusion injury in rat liver and phagocyte activation and recruitment. *Hepatology* 2000;31:622-632.
- Kohli V, Gao W, Camargo CA Jr, Clavien P-A. Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. *Proc Natl Acad Sci* 1997;94:9354-9359.
- Gao W, Bentley RC, Madden JF, Clavien P-A. Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation. *Hepatology* 1998;27:1652-1660.
- Sindram D, Kohli V, Madden J, Clavien P-A. Calpain inhibition prevents sinusoidal endothelial cell apoptosis in the cold ischemic rat liver. *Transplantation* 1999;68:136-140.
- Poli G, Cutrin JC, Biasi F. Lipid peroxidation in the reperfusion injury of the liver. *Free Rad Res* 1998;28:547-551.
- von Harsdorf R, Li P-F, Dietz R. Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis. *Circulation* 1999;99:2934-2941.
- Chandra J, Orrenius S. Apoptosis and oxidative stress: promotion, modulation and abrogation. In: Yoshikawa T, Toyokuni S, Yamamoto Y, Naito Y, eds. *Free Radicals in Chemistry, Biology and Medicine*. England: OICA International (UK) Limited; 2000: pp 285-298.
- Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74:1124-1136.
- Murry CE, Richard VJ, Jennings RB, Reimer KA. Myocardial protection is lost before contractile function recovers from ischemic preconditioning. *Am J Physiol* 1991;260:H796-H804.
- Pérez-Pinzón MA, Xu GP, Dietrich WD, Rosenthal M, Sick TJ. Rapid preconditioning protects rats against ischemic neuronal damage after 3 but not 7 days of reperfusion following global cerebral ischemia. *J Cereb Blood Flow Metab* 1997;17:175-182.
- Pang CY, Yang RZ, Zhong A, Xu N, Boyd B, Forrest CR. Acute ischaemic preconditioning protects against skeletal muscle infarction in the pig. *Cardiovasc Res* 1995;29:782-788.
- Peralta C, Hotter G, Closa D, Gelpi E, Bulbena O, Roselló-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: Role of nitric oxide and adenosine. *Hepatology* 1997;25:934-937.
- Yoshizumi T, Yanaga K, Soejima Y, Maeda T, Uchiyama H, Sugimachi K. Amelioration of liver injury by ischaemic preconditioning. *British J Surg* 1998;85:1636-1640.
- Peralta C, Hotter G, Closa D, Prats N, Xaus C, Gelpi E, et al. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A₂ receptors. *Hepatology* 1999;29:126-132.
- Yadav SS, Sindram D, Perry DK, Clavien P-A. Ischemic preconditioning protects the mouse liver by inhibition of apoptosis through a caspase-dependent pathway. *Hepatology* 1999;30:1223-1231.
- Yabe KI, Nasa Y, Sato M, Iijima R, Takeo S. Preconditioning preserves mitochondrial function and glycolytic flux during and early period of reperfusion in perfused rat hearts. *Cardiovasc Res* 1997;33:677-685.
- Peralta C, Bartrons R, Riera L, Manzano A, Xaus C, Gelpi E, et al. Hepatic preconditioning preserves energy metabolism during sustained ischemia. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G163-G171.
- Peralta C, Bulbena O, Xaus C, Prats N, Cutrin JC, Poli G, et al. Ischemic preconditioning: A defense mechanism against the reactive oxygen species generated after hepatic ischemia reperfusion. *Transplantation* 2002;73:1203-1211.
- Wolff SP. Ferrous ion oxidation in presence of ferric ion indicator xylene orange for measurement of hydroperoxides. *Methods Enzymol* 1994;233:182-189.
- Estebauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods Enzymol* 1990;186:407-421.
- Kono H, Arteel GE, Rusyn I, Sies H, Thurman RG. Ebselen prevents early alcohol-induced liver injury in rats. *Free Radic Biol Med* 2001;30:403-411.
- Peterson GL. A simplification of the protein assay method of Lowry et al. Which is more generally applicable? *Anal Biochem* 1977;83:346-356.
- Natori S, Selzner M, Valentino KL, Fritz LC, Srinivasan A, Clavien P-A, et al. Apoptosis of sinusoidal endothelial cells occurs during liver preservation injury by a caspase-dependent mechanism. *Transplantation* 1999;68:89-96.
- Sindram D, Porte RJ, Hoffman MR, Bentley RC, Clavien P-A. Platelets induces sinusoidal endothelial cells apoptosis upon reperfusion of the cold ischemic rat liver. *Gastroenterology* 2000; 118:183-191.

31. Gujral JS, Bucci TJ, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: Apoptosis or necrosis? *Hepatology* 2001;33:397-405.
32. Soeda J, Miyagawa S, Sano K, Masumoto J, Taniguchi S, Kawasaki S. Cytochrome *c* release into cytosol with subsequent caspase activation during warm ischemia in rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001;281:G1115-G1123.
33. Rice-Evans C, Burdon R. Free radical-lipid interactions and their pathological consequences. *Prog Lipid Res* 1993;32:71-110.
34. Poli G, Schaur RJ. 4-hydroxynonenal in the pathomechanisms of oxidative stress. *IUBMB Life* 2000;50:315-321.
35. Tan S, Sagara Y, Liu Y, Maher P, Schubert D. The regulation of reactive oxygen species production during programmed cell death. *J Cell Biol* 1994;141:1423-1432.
36. Jones BE, Lo CR, Liu H, Pradhan Z, Garcia L, Srinivasan A, et al. Role of caspases and NF- κ B signaling in hydrogen peroxide and superoxide-induced hepatocytes apoptosis. *Am J Physiol Gastrointest Liver Physiol* 2000;278:G693-G699.
37. Akao M, Ohler A, O'Rourke B, Marbán E. Mitochondrial ATP-sensitive potassium channels inhibit apoptosis induced by oxidative stress in cardiac cells. *Circ Res* 2001;88:1267-1275.
38. Choudhary S, Zhang W, Zhou F, Campbell GA, Chan LL, Thompson EB, et al. Cellular lipid peroxidation end-products induce apoptosis in human lens epithelial cells. *Free Radic Biol Med* 2002;32:360-369.
39. Liu W, Kato M, Akhand A, Hayakawa A, Suzuki H, Miyata T, et al. 4-hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. *J Cell Sci* 200;113:635-641.
40. Vieira HLA, Belzacq A-S, Haouzi D, Bernassola F, Cohen I, Jacotot E, et al. The adenine nucleotide translocator: A target of nitric oxide, peroxynitrite, and 4-hydroxynonenal. *Oncogene* 2001;20:4305-4316.
41. Yamagami K, Yamamoto Y, Kume M, Kimoto S, Yamamoto H, Ozaki N, et al. Heat shock preconditioning ameliorates liver injury following normothermic ischemia-reperfusion in steatotic rat livers. *J of Surg Res* 1998;79:47-53.