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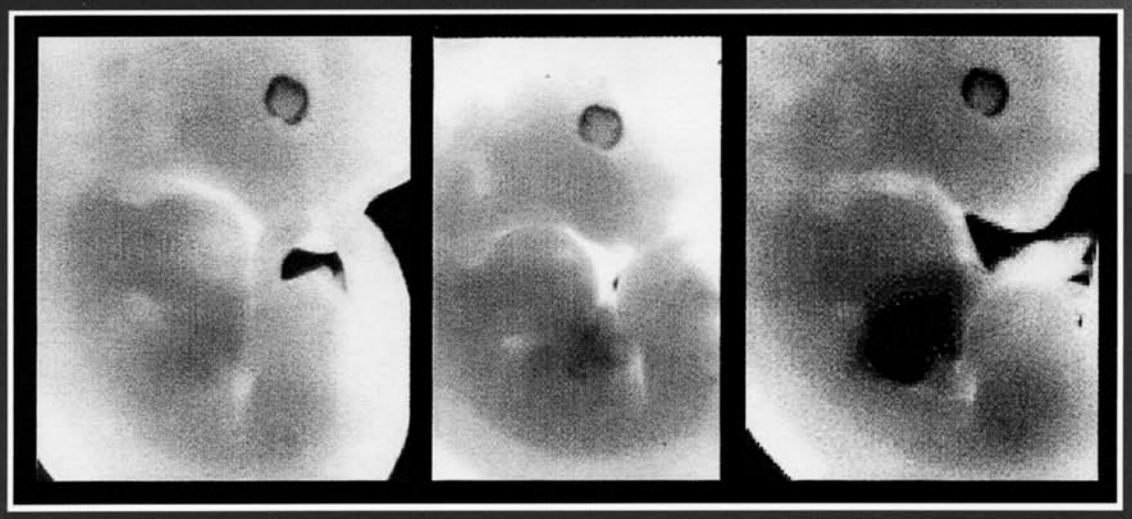

CONTROL OF SOMATIC MUTAGENESIS:
HEPATIC-INDUCIBLE CHIMERIC MICE

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W.B. SAUNDERS

Extended Preservation of Rat Liver Graft by Induction of Heme Oxygenase-1

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Livers can be preserved only for a short period without jeopardizing the transplantation outcome. Heat shock proteins (HSPs) protect against ischemia and reperfusion injury. We studied whether their induction and, in particular, the induction of heme oxygenase 1 (HO-1), improves transplantation survival after an extended time of cold storage. Rats were subjected to heat preconditioning (42°C for 20 minutes). Livers were harvested 24 hours later, preserved in cold University of Wisconsin solution for 44 hours, and transplanted in isogeneic rats (arterialized transplantation). HO-1 was specifically induced and inhibited by cobalt protoporphyrin and tin protoporphyrin, respectively. All animals receiving a graft without preconditioning and subjected to 44 hours of cold preservation died within 3 days, whereas 89% of rats who received a graft exposed to heat survived for 3 weeks ($P = .0004$). Preconditioning reduced serum aspartate transaminase (AST) and lactate dehydrogenase activities after reperfusion, improved bile flow, and decreased the histologic lesions of reperfusion injury. These significant effects of heat preconditioning were prevented by administration of tin protoporphyrin and could be reproduced by administration of cobalt protoporphyrin. In grafts without preconditioning, only a small fraction (<5%) of hepatocytes were positive with the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay, and even less expressed activated caspase 3. Preconditioning tended to reduce the number of positive cells and to stimulate the expression of antiapoptotic Bcl-X_L. In conclusion, heat preconditioning and, specifically, overexpression of HO-1 improve posttransplantation survival and graft function after prolonged cold ischemia preservation. The mechanism underlying these beneficial effects does not appear to be prevention of apoptosis. (HEPATOLOGY 2002;35:1082-1092.)

It is of paramount importance to avoid primary graft dysfunction and even nonfunction as a result of post-reperfusion injury. The risk for cold ischemia and reperfusion injury limits the time of preservation to only a few hours. The introduction of the University of Wisconsin solution more than 10 years ago extended the time of cold storage between harvesting and transplantation.[1,2] Preservation of livers before transplantation for more than 12 to 18 hours still carries the risk for compromising the function of the future graft,[3-6] and favors posttransplantation infection.[7]

One of the most promising strategies that lengthens the time of cold ischemia without risking reperfusion injury is preconditioning. The future graft is subjected to an acute sublethal stress for a limited period of time before harvesting. This preconditioning confers resistance to subsequent lethal stress by inducing the expression of the heat shock proteins (HSPs). HSPs protect the cellular machinery of many organs from a wide variety of insults such as anoxia, ischemia, and oxidative stress.[8-10] Hepatic reperfusion injury after cold preservation involves the release of oxygen radicals, and the loss of viability of endothelial cells and hepatocytes.[11,12] Recently, apoptosis has been identified as a mechanism of hepatic injury after ischemia and reperfusion[13,14] and proposed to be critical

Abbreviations: HSP, heat shock protein; HO-1, heme oxygenase 1; HP, heat preconditioning; SnPP, tin-protoporphyrin; CoPP, cobalt-protoporphyrin; AST, aspartate aminotransferase; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling.

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in liver graft after transplantation. Apoptosis involves the interplay of several proapoptotic factors such as caspases and antiapoptotic factors such as members of the Bcl family. HSPs interfere with several steps of the apoptotic program,[15] providing a rationale for their involvement in preventing ischemia and reperfusion injury.

HSP 32, better known as heme oxygenase 1 (HO-1),[16] catalyzes the breakdown of heme into biliverdin, carbon monoxide, and iron.[17] Biliverdin is converted into bilirubin, a potent antioxidant, and carbon monoxide functions as a signaling molecule. Many of the protective effects of the HSPs have been attributed to HO-1[18-23] and expression of HO-1 is amenable to pharmacologic manipulations. Its pharmacologic induction attenuated oxidative injury in a rat kidney ischemia-reperfusion model[24] and improved survival after transplantation of fatty livers in rats.[25] We wondered whether this beneficial effect could allow longer cold preservation of liver grafts.

An experimental rat model of syngeneic, vascularized liver transplantation was designed to examine (1) whether heat shock preconditioning could extend the period of cold ischemia; (2) whether pharmacologic induction of HO-1, an approach clinically feasible, reaches the same results; and (3) whether such a preconditioning reduces reperfusion-induced apoptosis.

Material and Methods

Animals and Treatments. Male Lewis rats (Harlan, Horst, Netherlands) weighing 250 to 320 g were allowed free access to chow and water. Animal experiments were performed in accordance with the regulations for laboratory animals and were approved by local authorities. Donor animals but not recipients underwent hyperthermia preconditioning (HP). Donor animals were anesthetized by intraperitoneal injection of pentobarbitone sodium (50 mg/kg body weight) and body temperature was monitored with a rectal thermometer. Under free and secured ventilation conditions, anesthetized animals were placed into a 42°C water bath and body temperature was slowly elevated to 42°C and maintained for 20 minutes. In the control groups, donor animals were subjected to anesthesia without heat stress. Before HP and sham HP, all animals were given 5 mL of isotonic saline intraperitoneally to avoid dehydration. Harvesting of the liver was performed 24 hours after preconditioning. In some animals, tin protoporphyrin (SnPP; Porphyrin Products, Logan, UT) was used to inhibit the activity of HO-1. It was prepared as described[18] and administered intraperitoneally to the transplant donor 23 hours after HO-1 induction, meaning 1 hour before graft harvesting. The dose used (10 μmol/kg body weight) has been shown to effectively inhibit the activity of HO-1 without toxicity.[18] To selectively induce HO-1, animals received intraperitoneally 5 mg/kg of cobalt protoporphyrin (CoPP; Porphyrin Products), as described by Amersi et al.,[25] 24 hours before harvesting, in place of HP.

Surgical Procedures. A midline laparotomy was performed. The liver was flushed first with chilled lactated Ringer's solution and thereafter for 5 minutes with 50 mL of 4°C cold University of Wisconsin solution via the hepatic artery. The liver was then removed and stored at 4°C. Orthotopic liver transplantation was performed with hepatic artery reconstruction. The suprahepatic vena cava was anastomosed with 8-0 Prolene continuous suture (Ethicon, Somerville, NJ). Portal vein, infrahepatic vena cava, and hepatic artery reconstruction was performed by using the cuff technique. The bile duct connection was made with an intraluminal polyethylene splint. Anhepatic time ranged from 11 to 13 minutes. All transplant procedures and preconditioning interventions were performed by a single investigator (Y.-H.T.), who was not involved in subsequent analysis.

Study Groups. Ten rats were dedicated to the study of expression of HSPs after HP and CoPP. Survival studies were performed on 45 animals observed until death or 3 weeks after transplantation. Twenty-eight rats were subjected either to sham HP (controls), preconditioning with CoPP, HP, or HP with administration of SnPP. Survivors were killed 1 hour after liver transplantation (survival was 6 of 7, 7 of 7, 6 of 7, and 4 of 7, respectively). Twenty-eight rats were subjected to the same treatments and the survivors were killed 8 hours after transplantation (survival was 3 of 7, 6 of 7, 6 of 7, and 0 of 7, respectively). Serum aspartate transaminase (AST), lactate dehydrogenase activities, and serum bilirubin concentration were measured in jugular venous blood by standard methods (Central Laboratory of the University Hospital, Berne). To measure the bile flow, the common bile duct was cannulated and the bile collected for 15 minutes. The volume of bile was determined gravimetrically. The grafts were harvested for Western blot and morphologic analysis. For transmission electron microscopy studies, 19 rats were divided into 2 groups, sham HP and HP, and killed 1 and 8 hours after transplantation; survival was 4 of 4 and 4 of 4, respectively, after 1 hour, and 4 of 7 and 4 of 4, respectively, after 8 hours.

Western Blot Analysis for HSP 90, HSP 72, HO-1, and Bcl-X_L. Livers were minced and suspended in 4 volumes of 0.25 mol/L sucrose at 4°C and homogenized 2 times for 30 seconds with a Polytron (Janke + Kunkel, IKA-Werke, Staufen, Germany). Protein concentration was determined according to Lowry et al.[26] Proteins from whole-liver homogenate were separated

by sodium dodecyl sulphate–polyacrylamide gel electrophoresis by using a 5% polyacrylamide gel and transferred to Protean nitrocellulose membranes (Protean, Schleicher and Schuell, Dassel, Germany). The membranes were blocked at 4°C overnight and probed for 2 hours with the primary antibody. Antibodies against HSP 32 (1:2,000), HSP 72 (1:1,000), and HSP 90 (1:1,000) were rabbit polyclonals from Stressgen (Victoria, British Columbia, Canada), and the polyclonal antibody against Bcl-X_L (1:1,000) was from Transduction Laboratories (Lexington, KY). The membranes were washed and incubated for 1 hour with peroxidase-conjugated immunoglobulin G donkey-anti-rabbit 1:50,000 (Pierce, Lausanne, Switzerland). Signals were detected by enhanced chemiluminescence (Pierce) and quantified with a CCD camera (Fujifilm LAS-1000; Raytest, Urdorf, Switzerland) and the software AIDA 2.1 (Raytest). All the gels were routinely stained with Coomassie blue and membranes were stained with Ponceau to check for equal loading of protein and transfer, respectively. Positive controls were recombinant HO-1, HSP 72, and HSP 90 provided by Stressgen and endothelial lysate for Bcl-X_L (provided as positive control by Transduction Laboratories).

Histologic Evaluation of Ischemia-Reperfusion Injury. To determine the histologic degree of liver injury, specimens from grafts were fixed in buffered 4% formalin and embedded in paraffin. Tissue sections (4-μm thick) were routinely stained with hematoxylin-eosin. Light microscopic changes characteristic of ischemia-reperfusion injuries were graded according to the criteria proposed by Camargo et al.[27] Liver sections were evaluated with a 1-mm square grid. The severity of injury on each square was scored: 0 for minimal or no evidence of injury, 1 for mild injury (cytoplasmic vacuolation, focal nuclear pycnosis), 2 for moderate injury (extensive nuclear pycnosis, cytoplasmic hypereosinophilia, loss of intercellular borders), and 3 for severe injury (disintegration of hepatic cords, hemorrhage, and neutrophil infiltration).[27] Per graft, 1,000 squares on 2 slides were graded by an investigator blinded to the treatment groups.

Transmission Electron Microscopy. Livers were rinsed with 20 mL Ringer's solution and immediately perfused and fixed with Karnovsky's solution (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 mol/L sodium phosphate, Soerensen buffer, pH 7.4). Whole livers were removed and randomly chosen blocks comprising about 1 mm^3 of tissue were postfixed in OsO_4, dehydrated, embedded in Spurr's low viscosity resin, and sectioned in 1-μm sections for screening by light microscopy. No obvious intragroup differences were noted. Blocks were sectioned and contrasted with uranyl acetate and lead citrate. More than 20 digital transmission electron microscopy images were recorded for each group at nominal magnifications of 1,500 and 3,700.

In Situ *Detection of Apoptosis Using the Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling Assay.* Paraffin-embedded sections (4 μm) were deparaffinized by soaking twice in xylol 100% for 10 minutes, then treated for 30 minutes with 4% diethylpyrocarbonate in ethanol at 4°C to avoid false positivity with the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay.[28] The slides were successively soaked in ethanol 100% (2 × 10 min), ethanol 94% (5 min), ethanol 70% (5 min), ethanol 50% (5 min), and double-distilled water (5 min). After treatment with Proteinase K (10 μg/mL) at 37°C for 60 minutes in 5 mmol/L ethylenediaminetetraacetic acid in Tris 20 mmol/L (pH 8.1), and 0.3% H_2O_2 in methanol for 30 minutes to block the endogenous peroxidases, the TUNEL assay was performed by using a commercial kit (Roche Diagnostic, Basel, Switzerland). The sections were treated with terminal nucleotidyl transferase in the presence of fluorescein-dUTP and d-NTP and incubated with horseradish peroxidase–conjugated antifluorescein antibody (Roche) and developed by using 3-amino-9-ethylcarbazol (Envision System kit; DAKO, Zug, Switzerland). Tissues treated with DNAse I served as positive controls and sections stained without terminal nucleotidyl transferase served as negative controls. The specimens were counterstained with hematoxylin and mounted with Aquamount (DAKO). The degree of apoptosis was estimated by point counting. Each section with its label blinded was counted at a final magnification of 40× by using a point-to-point distance of 50 μm. Each point was classified as overlaying a positive cell or not.[29]

Immunohistochemistry for Activated Caspase 3. Paraffin-embedded sections were deparaffinized and hydrated by successive soaking in xylol 100% (2 × 10 min), ethanol 100% (2 × 10 min), ethanol 94% (5 min), ethanol 70% (5 min), ethanol 50%, and double-distilled water (5 min). Endogenous peroxidases were blocked for 30 minutes in methanol containing H_2O_2 0.6%. Slides were placed for 5 minutes twice in Tris-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and for 30 minutes with normal goat serum (Kirkegard & Perry, Gaithersburg, MD) before incubation overnight at 4°C with a rabbit polyclonal antibody recognizing activated caspase 3 (1:2,000 CM1 Ab; IDUN Pharmaceuticals, Inc., La Jolla, CA). After washing with Tris-buffered saline containing bovine serum albumin, the sections were incubated for 30 minutes at room temperature with

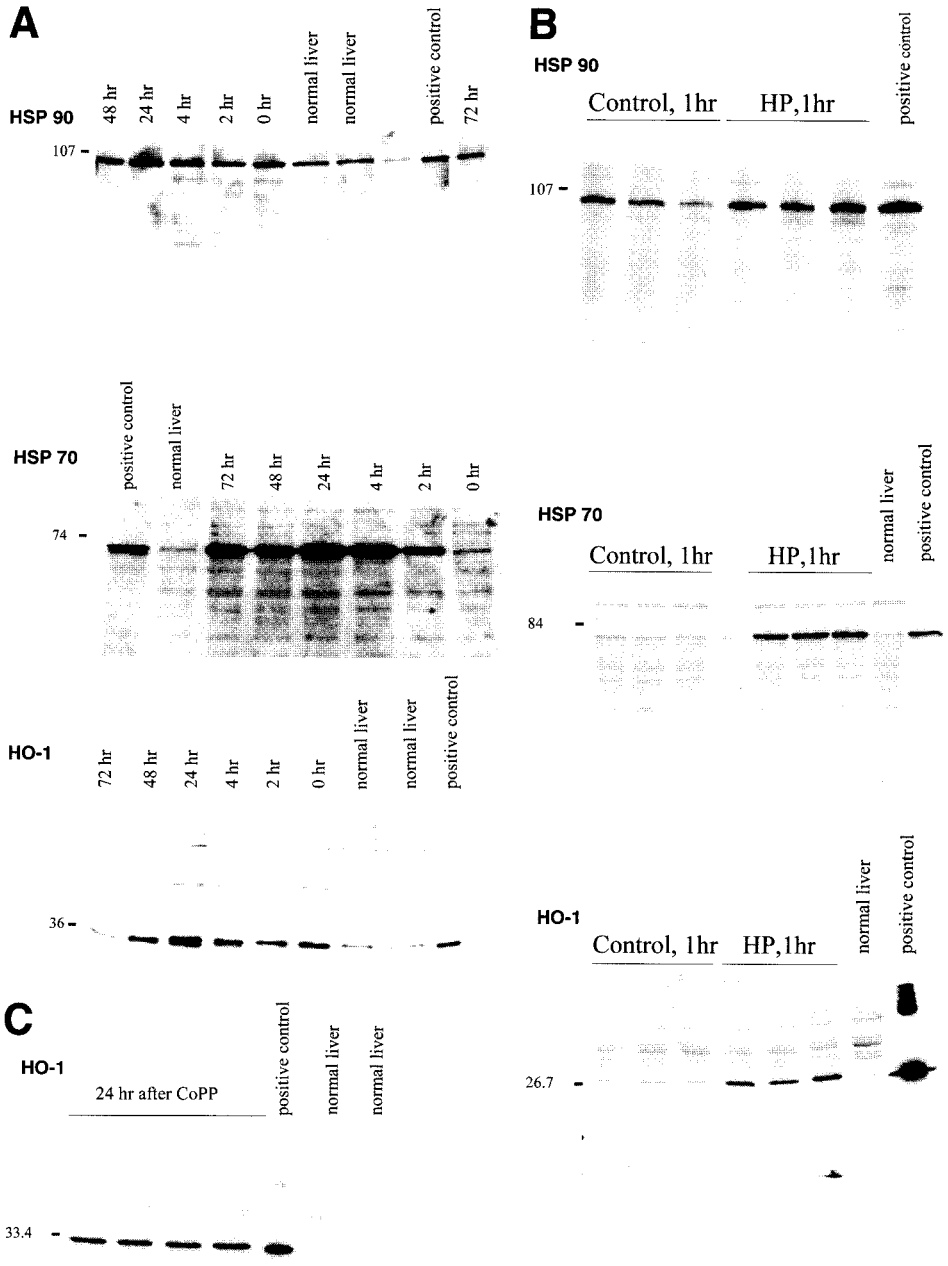

Fig. 1. Hepatic expression of HSP 90, HSP 72, and HO-1 (HSP32) assessed by Western blots. (A) Time course after HP without ischemia. The expression of HO-1 was maximal 24 hours after HP. (B) Hepatic expression 1 hour after transplantation. The expression of the HSPs was higher in livers subjected to HP before harvesting (n = 3 different animals) compared with control livers (n = 3 different animals). (C) Hepatic expression of HO-1 24 hours after administration of CoPP (n = 4 different animals).

peroxidase-labeled goat-anti-rabbit immunoglobulin (DAKO kit K4008) and exposed for 15 minutes to 3-amino-9-ethylcarbazol. Sections from mammary glands in involution served as positive controls and the first antibody was omitted in negative controls. The sections were counterstained with hematoxylin and mounted with Aquamount. The activation of the caspase 3 was assessed by point counting (2,000 points per graft) as described in the previous paragraph.

Statistics. Graft survival was calculated with the Kaplan-Meier product limit estimator. Differences in survival rates between the groups were tested with the log rank test in a statistical package program (SPSS Statistical Software, Chicago, IL). Results are expressed as mean ± SD. The results of the control grafts were compared with the results of the grafts pretreated with CoPP and the results of the grafts subjected to HP were compared with the results of grafts subjected to HP and SnPP treatment by unpaired Student's *t* test, normality was assessed by the Shapiro Wilk's W test (Statistica; StatSoft, Tulsa, OK). A *P* value of less than .05 was considered statistically significant.

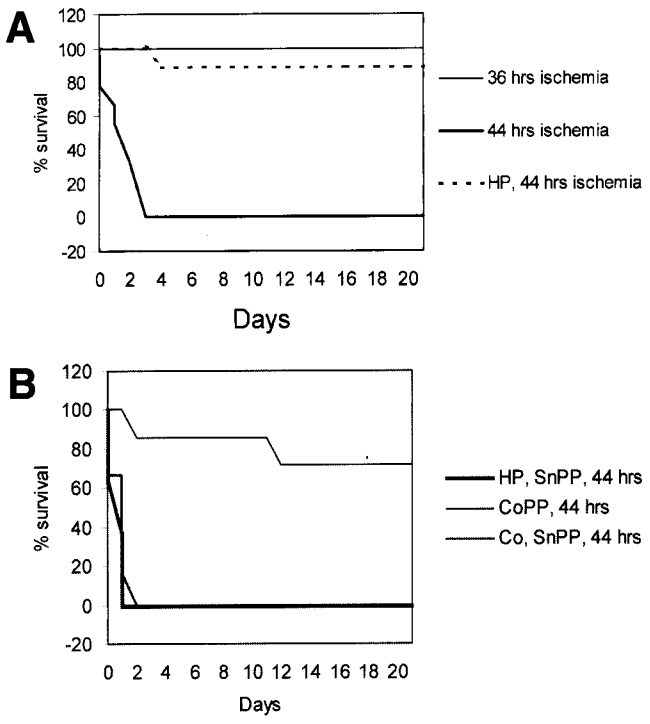

Fig. 2. Survival after liver transplantation. (A) Cold ischemia for 36 hours did not jeopardize the liver transplantation outcome (n = 6). When the period of cold ischemia lasted for 44 hours, all the experimental animals died in the 3 days after the transplantation (n = 9). This poor survival was significantly improved by HP (n = 9; log rank test 0.0004). **Thin line**, 36 hours ischemia; **bold line**, 44 hours ischemia; **dotted line**, 44 hour ischemia. (B) Induction of HO-1 with CoPP before the 44 hours of cold ischemia (n = 7) resulted in an improvement of survival comparable with the one achieved with HP. The survival was significantly reduced by the inhibition of HO-1 by SnPP (n = 6; log rank test 0.0005). The beneficial effect of HP on the survival of rat recipients of a liver graft subjected to 44 hours of cold ischemia was also abolished by SnPP (n = 8). The survival curve of animals treated with HP + SnPP and the survival curve of animals treated with CoPP + SnPP overlap. **Thin line**, CoPP, 44 hours; **bold line**, Co, SnPP, 44 hours.

Results

Induction of HSPs After Preconditioning. After hyperthermia, HSP 90, HSP 72, and HSP 32 (HO-1) were induced as assessed by Western blotting (Fig. 1A). Their expression peaked 24 hours after preconditioning. The expression of the HSPs was higher in grafts subjected to HP 1 hour after transplantation than in grafts not subjected to HP (Fig. 1B). Treatment with CoPP, in place of HP, resulted in the induction of HO-1 (Fig. 1C).

Effect of Preconditioning and HO-1 Inhibition on Survival. Cold ischemia of the harvested liver for 36 hours before transplantation did not lead to recipient death. In contrast, 44 hours of cold ischemia was inevitably associated with death. As shown in Fig. 2A, this poor outcome could be overcome by HP 24 hours before harvesting. The beneficial effect of HP on survival was completely blocked by administration of the HO-1 inhibitor, SnPP, 23 hours after heat shock, corresponding to 1 hour before harvesting. This effect was reproduced by pharmacologic induction of HO-1 with CoPP given 24 hours before harvesting in place of HP. SnPP abolished the survival benefit conferred by CoPP, showing that induction of HO-1 was responsible for this effect (Fig. 2B).

Effect of Preconditioning and HO-1 Inhibition on Liver Function. Serum AST and lactate dehydrogenase activities and serum bilirubin concentration were determined 1 and 8 hours after transplantation. Serum AST activity was lower in animal recipients of a preconditioned graft (Fig. 3A) and the difference increased 8 hours after reperfusion. The same was observed with lactate dehydrogenase activity (data not shown). The bilirubin concentration was as high in the recipients of a preconditioned graft as in the other groups (Fig. 3B). This reflects the induction of HO-1, which catalyzes the formation of biliverdin, subsequently converted to bilirubin. The bile flow was severely reduced in animals without preconditioning (Fig. 3C). The bile flow was low 1 hour after transplantation in grafts subjected to HP or treated with CoPP, but improved at 8 hours. At both time points it was significantly higher than in grafts not preconditioned.

Effect of Preconditioning on Histology. The morphometric assessment of the ischemia-reperfusion injury using an appropriate score revealed a significant reduction in the morphologic lesions 1 and 8 hours after transplantation, in grafts subjected to HP or treated with CoPP (Fig. 4). This protective effect was overcome by administration of SnPP after HP to inhibit HO-1. The structural abnormalities were further defined by electron microscopy (Fig. 5). Some alterations such as small vacuoles, retraction or loss of endothelial lining of sinusoids, and trapping or adhesion of leukocytes and platelets in sinusoids were found in all specimens. HP specimens tended, however, to show a better preservation of the endothelial lining without apoptotic figures (Fig. 5A). HP apparently prevented hepatocyte damage at 1 hour when shrinkage of hepatocytes, pycnosis, and widening of endothelium-deprived sinusoids was evident in control animals (Fig. 5B). Advanced or irreversible hepatocyte damage was widespread in control livers and virtually absent in HP livers from transplants that survived for 8 hours (Fig. 5C and D).

Effect of Preconditioning and of HO-1 Inhibition on Hepatic Apoptosis (TUNEL). We used the TUNEL assay to determine the histologic extent of apoptosis. Fig. 6 (upper panel) shows a histologic section from a graft 1 hour after transplantation without HP (left panel), with HP (middle panel), and with HP and SnPP administration (right panel). The bar graph (Fig. 7) gives the results

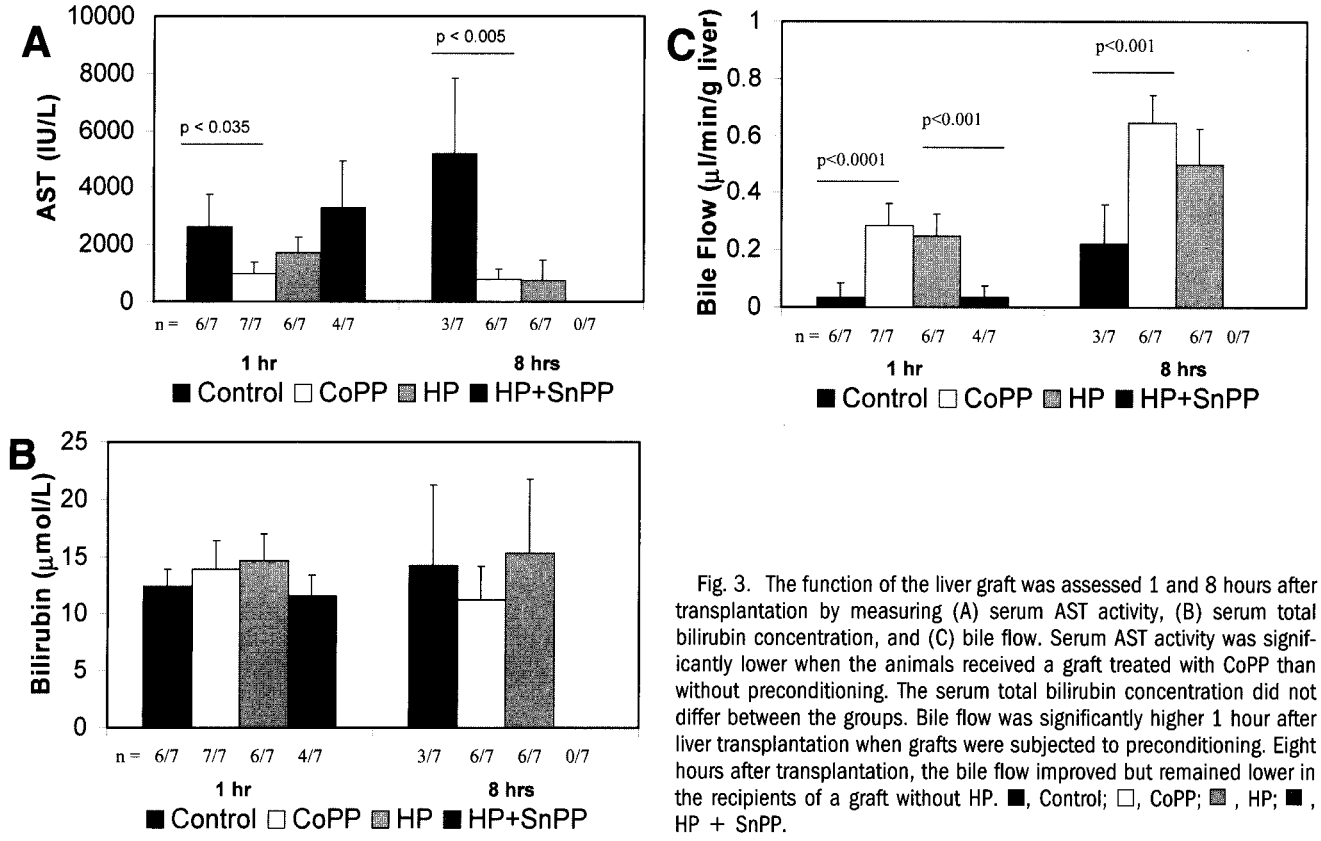

Fig. 3. The function of the liver graft was assessed 1 and 8 hours after transplantation by measuring (A) serum AST activity, (B) serum total bilirubin concentration, and (C) bile flow. Serum AST activity was significantly lower when the animals received a graft treated with CoPP than without preconditioning. The serum total bilirubin concentration did not differ between the groups. Bile flow was significantly higher 1 hour after liver transplantation when grafts were subjected to preconditioning. Eight hours after transplantation, the bile flow improved but remained lower in the recipients of a graft without HP. ■, Control; □, CoPP; ▩, HP; ■, HP + SnPP.

of the morphometric analysis. Less than 5% of the cells were positive with the TUNEL method 1 hour after reperfusion in grafts subjected to 44 hours of cold ischemia without preconditioning. We found a significant reduction in grafts preconditioned with CoPP. The effect of SnPP in HP-treated grafts was not significant. In normal livers, less than 1/500 points were positive for this assay (data not shown).

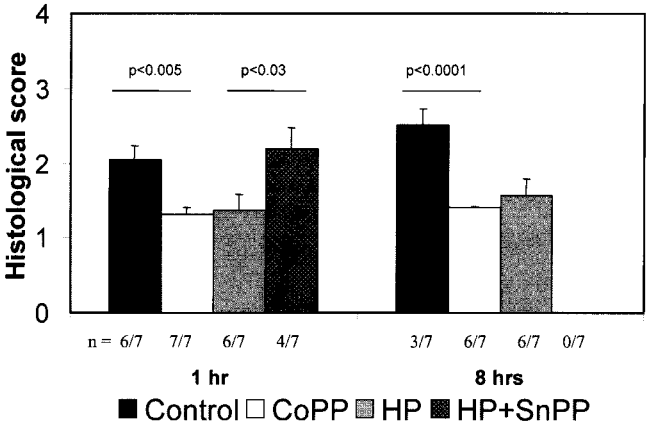

Fig. 4. Histologic lesions caused by ischemia-reperfusion injury were assessed blindly according to an appropriate grading system.[27] The mean score was significantly lower 1 and 8 hours after liver transplantation in the grafts that were treated with CoPP than in the controls and in the grafts subjected to HP than in those subjected to HP + SnPP. ■, Control; □, CoPP; ▩, HP; ■, HP + SnPP.

Effect of Preconditioning and of HO-1 Inhibition on Activated Caspase 3. The activation of the executioner caspase 3 was determined by immunohistochemistry by using an antibody specific for the activated caspase. Fig. 6 (lower panel) shows the histology of a graft 1 hour after transplantation without HP (left panel), with HP (middle panel), and with HP and SnPP administration (right panel). Less cells (10-fold) were positive for this marker than for the TUNEL assay and most of the positive cells appeared sinusoidal. The results of the morphometric analysis by point counting could not detect a significant reduction of the activation of caspase 3 in preconditioned grafts (Fig. 8). In sections from normal livers no staining could be detected (data not shown).

Effect of Preconditioning and of HO-1 Inhibition on Expression of Bcl-X_L. We assessed the expression of the antiapoptotic protein Bcl-X_L by Western blotting. Its expression was not higher in control grafts than in grafts pretreated with CoPP and this corresponded to the level found in normal liver. Bcl-X_L was expressed at higher levels in the grafts subjected to HP and this was significantly reduced by administration of SnPP after HP (Fig. 9).

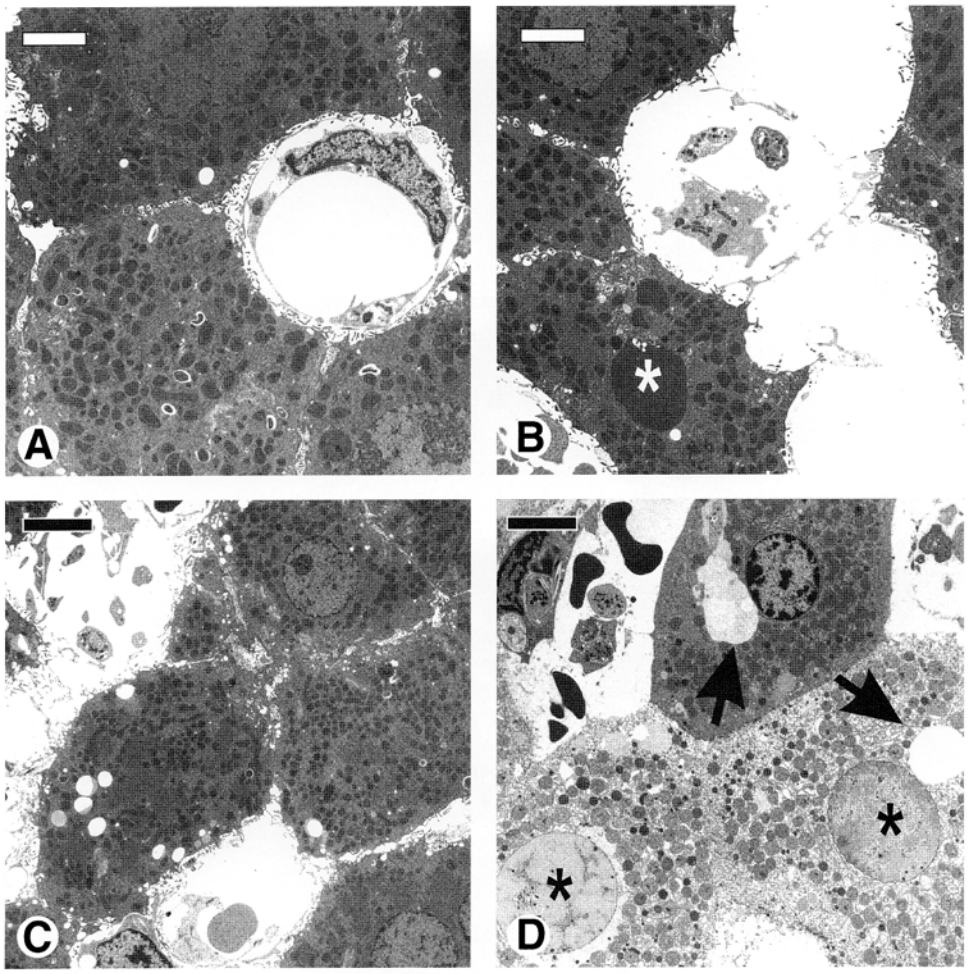

Fig. 5. Electron microscopy images of perfusion fixed livers explanted 1 hour (top row) or 8 hours (bottom row) after transplantation. Heat-treated grafts (left column) and controls (right column). Widening of sinusoidal spaces, loss of endothelial lining, and apparent loss of fluid from hepatocytes, which appear smaller and darker, characterize early damage in controls (B), whereas in heat-treated transplants the endothelial lining appears more intact. (A) The **asterisk** denotes a pycnotic nucleus. At the later time tissue damage progresses in heat-treated and control livers and trapping of platelets and other blood cells is evident. Although heat-treated hepatocytes appear to show potentially reversible alterations such as (D) vacuolization, (C) irreversible cell damage is evident in the controls such as homogenization of chromatin (**asterisks**) and presence of large vacuoles (**arrows**). (A and B) **Bars** represent 3 μm, (C and D) **bars** represent 8 μm.

Discussion

Our results, using a model of syngeneic, vascularized liver transplantation in the rat, suggest that (1) HP permits longer cold storage of the harvested liver without compromising survival, (2) pharmacologic induction of HO-1 achieves the same beneficial effect, (3) apoptosis is not extensive after cold ischemia and reperfusion, and (4) preconditioning and induction of HO-1 partially reduces apoptosis in the hepatic graft.

Currently, a greater availability of harvested human livers is precluded by the short period of cold ischemia tolerated by this organ. We found that cold ischemia for 44 hours, which, without preconditioning, leads inevitably to graft failure and death of the experimental animals, was well tolerated after HP. In agreement with the documented hepatic expression of HO-1 in response to several stress signals[30] and with a recent report implying that HO-1 carries most of the protective effect of preconditioning before warm hepatic ischemia,[23] our results with CoPP and SnPP point to a central role for HO-1 in the protective effect of HP. This suggests that pharmacologic induction of HO-1 might be of clinical value to permit longer cold storage of harvested livers before transplantation. Amersi et al.[25] documented the beneficial effect of HO-1 in the particular situation of fatty liver transplantation by using obese rats treated with CoPP or with adenoviral HO-1. Fatty livers are exquisitely sensitive to ischemia-reperfusion injury and therefore routinely discarded for transplantation. Overexpression of HO-1 improved survival and liver functions. Here, we show that a similar strategy can be successfully used to extend the duration of cold storage of normal liver. Kato et al.[31] recently reported a prolongation of liver isograft survival after 40 hours of cold preservation with CoPP preconditioning. The effect was less substantial than shown here, perhaps because of the lower dose of CoPP than in our studies (1.5 mg/kg vs. 5 mg/kg intraperitoneally).

The functions of the reperfused liver graft after extended cold storage were significantly improved by preconditioning. The serum activities for AST and lactate dehydrogenase were lower in recipients of a graft that was subjected to HP or CoPP treatment. In contrast, the serum bilirubin concentration was slightly higher 1 hour after transplantation in pretreated animals, which is in

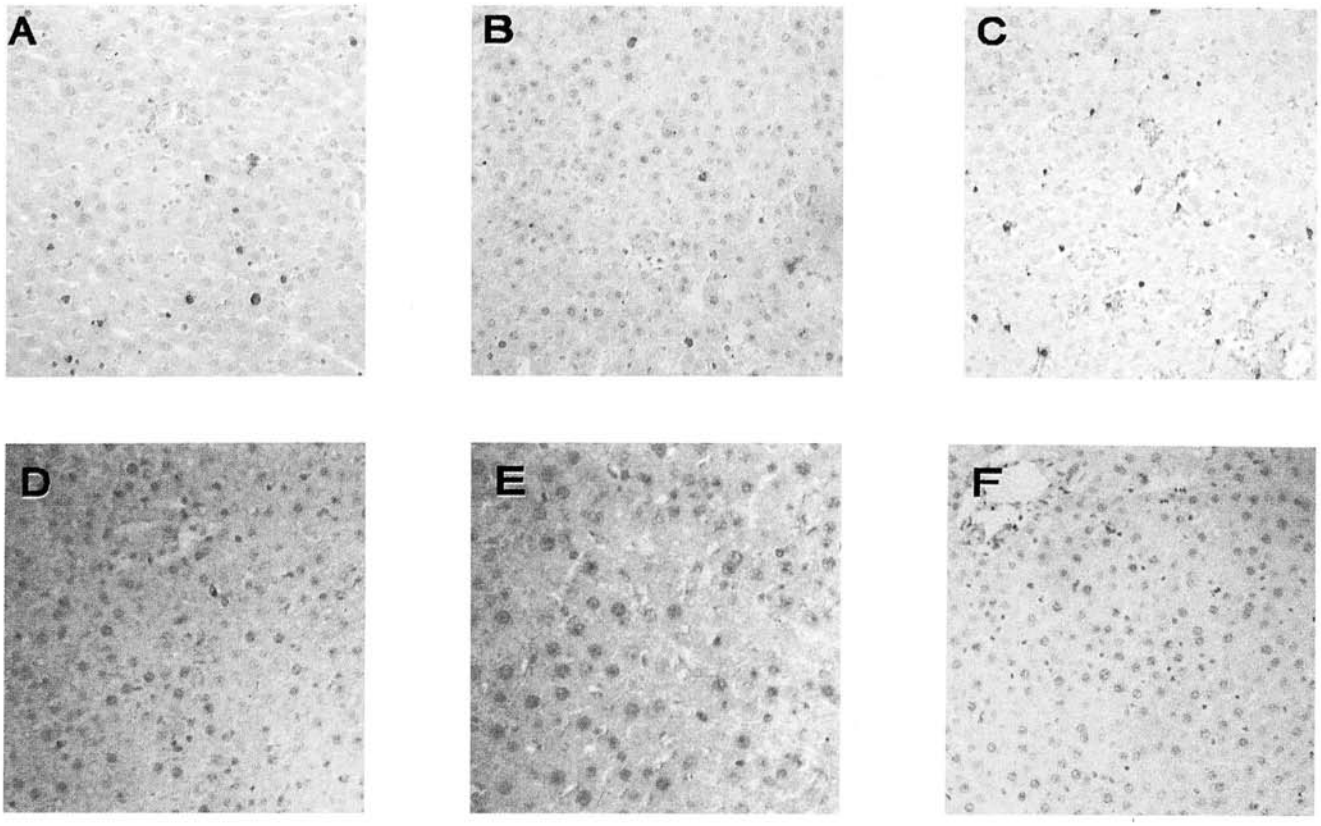

Fig. 6. The pictures of the upper panel show apoptotic cells by the TUNEL assay and the pictures of the lower panel cells positive by immunohistochemistry for activated caspase 3. A/D, B/E, and C/F show, respectively, histologic sections from a graft 1 hour after transplantation without HP, with HP, and with HP and SnPP administration (original magnification ×100).

line with the induction of HO-1. This protection of hepatic functions correlated histologically with less severe ischemia-reperfusion damages.

Apoptosis was recently recognized to be a feature of hepatic ischemia-reperfusion injury.[13,14,32,33] Sinusoidal endothelial cells are particularly vulnerable to apoptosis[13] and damage to endothelial cells leads to secondary hepatocellular injury and death.[34,35] Electron microscopy

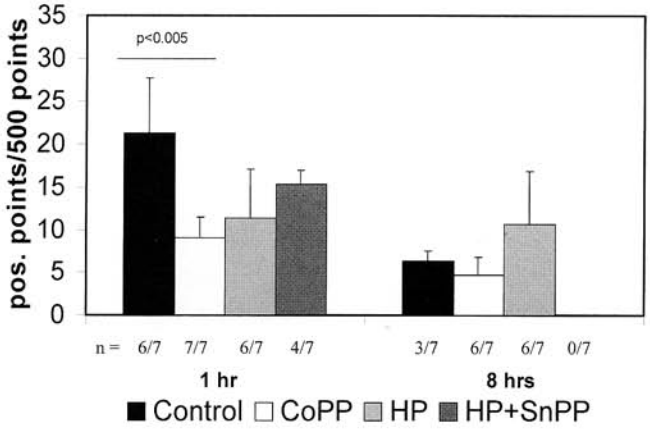

Fig. 7. Blinded morphometric analysis by point counting of the TUNEL assay. Less than 5% of the counted points overlay positive cells. More cells were positive 1 hour after reperfusion in the graft without preconditioning than in the graft subjected to HP or treated with CoPP. The effect of preconditioning with CoPP was significant. Eight hours after transplantation there was no difference. ■, Control; □, CoPP; ▩, HP; ▩, HP + SnPP.

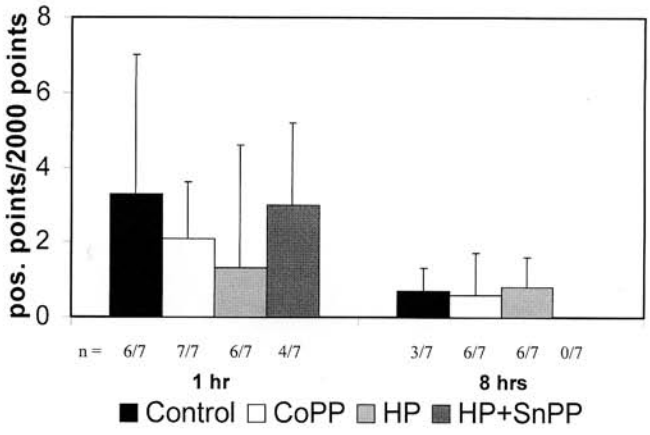

Fig. 8. The activation of the executioner caspase 3 was determined by immunohistochemistry by using an antibody specific for the activated caspase followed by morphometric analysis in a blinded fashion. Few cells only were positive for activated caspase 3, less than 0.5%. There was a trend toward less positive cells in the grafts subjected to preconditioning, but it did not reach statistical significance. ■, Control; □, CoPP; ▩, HP; ▩, HP + SnPP.

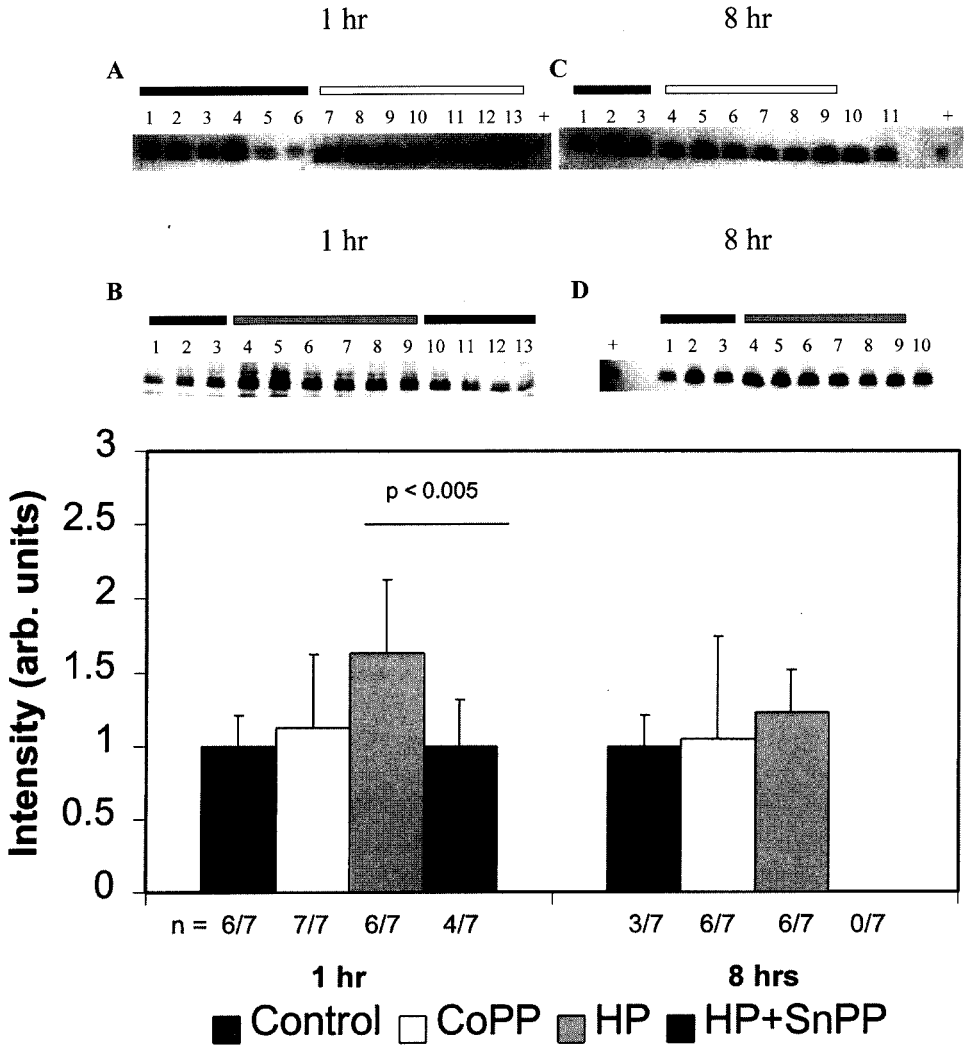

Fig. 9. The graft expression of the antiapoptotic protein Bcl-X_L was assessed by Western blotting analysis. The lines above the blots are in the same color than in the bar graph, that is, control, CoPP, HP, HP + SnPP. The controls 1, 2, and 3 are the same in A and B and they are also the same in C and D. The sign + is for positive control. Lanes 10 and 11 in blot C and lane 10 in the blot D represent normal liver. Bcl-X_L was not induced in the graft treated with CoPP, but its expression was significantly higher 1 hour after transplantation in grafts that were subjected to HP in comparison with those subjected to HP + SnPP.

analysis of the hepatic graft documented preservation of the sinusoidal integrity in HP grafts (Fig. 5). Several HSPs interact directly with the machinery of programmed cell death. Carbon monoxide suppressed *in vitro* endothelial cell apoptosis.[36] The proteolytic maturation of procaspase 3 can be hindered by HSP 70[37] and HSP 90.[38] Therefore, we investigated whether HP could prevent apoptosis in grafts after extended cold ischemia. We found that apoptosis was quantitatively a minor phenomenon in hepatic grafts after transplantation. Less than 5% of cells were positive with the TUNEL assay and less than 0.2% stained positive for activated caspase 3. False positives that are known to occur in liver tissue were carefully avoided by additional treatment with diethyl pyrocarbonate.[28] Moreover, this assay also may stain necrotic cells,[39] explaining why we observed less cells positive for activated caspase 3. These results suggest that apoptosis is not massive in the hours after transplantation despite prolonged cold ischemia. They are in line with a recent reevaluation of the importance of apoptosis in the hepatic ischemia-reperfusion injury.[40] Consequently, we observed only a modest effect of preconditioning on apoptosis. One hour after reperfusion there was a trend toward fewer positive cells for activated caspase 3 in the preconditioned grafts and an increase in the expression of the antiapoptotic protein Bcl-X_L after HP that was significantly blocked by SnPP. Prevention of apoptosis appears not to be the reason for the beneficial effects of preconditioning.

Several other mechanisms of action can explain the protective effect of HO-1. HO-1 serves as an inducible cellular barrier against oxidative stress.[41,42] Bilirubin has antioxidant properties.[43,44] However, it probably does not mediate the effect of HO-1 in the graft because its concentration is not different between the groups. Carbon monoxide stimulates the formation of cyclic guanosine monophosphate. Cyclic guanosine monophosphate has been shown to protect livers against Kupffer cell–induced oxidative injuries[45] and to enhance the production of bile,[46] which is consistent with the higher bile flow we observed in grafts subjected to an effective induction of

HO-1. The carbon monoxide–cyclic guanosine monophosphate pathway has important effects on the vasculature and platelet functions. Stimulation of vasodilatation and inhibition of platelet aggregation by carbon monoxide[47,48] could prevent hepatic perfusion disturbances. Moreover and particularly relevant in this context of transplantation, HO-1 has immunosuppressive properties. Its induction inhibits lymphoproliferative alloresponse and prolongs heterotopic heart allograft survival.[49] Soares et al.[50] reported that expression of HO-1 ensured long-term survival in a model of cardiac xenotransplantation.

In conclusion, the results presented support a beneficial role for HO-1 in liver transplantation by permitting longer cold ischemia. Pharmacologic induction of HO-1 before organ harvesting is an attractive approach to minimize preservation injury in liver grafts. Its beneficial effects occur probably through other mechanisms than reduction of apoptosis.

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