

Kazuhiko Yamagami
Georg Enders
Rolf Josef Schauer
Rosemarie Leiderer
Jörg Hutter
Yuzo Yamamoto
Yoshio Yamaoka
Claus Hammer
Konrad Messmer

Heat-shock preconditioning protects fatty livers in genetically obese Zucker rats from microvascular perfusion failure after ischemia reperfusion

Received: 4 April 2002
Revised: 27 September 2002
Accepted: 11 October 2002
Published online: 16 April 2003
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K. Yamagami is a recipient of the E.S.O.T. Fujisawa Study and Research Grant 2000 (2000–2001)

K. Yamagami · Y. Yamamoto
Y. Yamaoka
Department of Gastroenterological
Surgery, Kyoto University Graduate
School of Medicine, Kyoto, Japan

G. Enders · R. Leiderer · J. Hutter
C. Hammer · K. Messmer (✉)
Institute for Surgical Research,
Klinikum Grosshadern,
Marchionistrasse 27,
81377 Munich, Germany
E-mail: icfsek@icf.med.uni-muenchen.de
Tel.: +49-89-70954400
Fax: +49-89-70958897

R.J. Schauer
Department of Surgery,
University of Munich, Munich, Germany

Abstract Reduced tolerance of steatotic livers to ischemic injury is considered to correlate with impaired microcirculation. The aim of this study was to investigate the impact of heat-shock preconditioning (HSPC) on microcirculatory failure after ischemia/reperfusion (I/R) in steatotic livers by means of intra-vital fluorescence microscopy. Obese Zucker rats were used. In the HS group, rats underwent whole-body hyperthermia followed by 60-min partial liver ischemia. In group IR, rats were exposed only to ischemia. Microcirculation parameters (sinusoidal perfusion rate, sinusoidal diameter, leukocyte–endothelial interaction) were significantly better preserved in the HS group than in the IR group. Liver enzymes, oxygenated glutathione/reduced glutathione (GSSG/GSH) ratio, and electron microscopy showed less

damage in the HS group. A marked expression of heat shock protein 72 (HSP72) and heme oxygenase (HO-1) was found only in the livers of group HS. HSPC mitigated the I/R injury of steatotic livers by preventing post-ischemic failure of microcirculation. This beneficial effect was found to be associated with the induction of HSP72 and HO-1.

Keywords Heat-shock preconditioning · Steatotic liver · Liver microcirculation · Intra-vital fluorescence microscopy · Ischemia/reperfusion · Heat shock proteins

Introduction

Existence of abundant fat droplets in the liver has been connected to poor outcome after liver transplantation [22]. Steatosis is a common disorder of the liver, with an incidence of 16–26% [26, 27]. It can be caused by obesity, alcohol drinking, and a variety of metabolic disorders [2, 10]. Liver steatosis is clinically classified into three grades according to the proportion of hepatocytes with fatty infiltrates: mild (<30%), moderate (30–60%), and severe steatosis (60%) [7].

After liver transplantation, liver grafts with mild steatosis yield results comparable to those obtained from

normal livers, but severe steatosis is usually associated with a high risk of primary non-function (PNF) and should, therefore, not be accepted for liver grafts [7]. Livers with moderate steatosis have been used only for emergency transplantations [1]; however, due to serious organ shortage they have recently also been used in elective, low risk, non-urgent recipients who were able to tolerate a phase of liver graft dysfunction. The solving of the problem of PNF that is attributable to liver grafts with moderate steatosis would contribute to the enlargement of the donor pool. Therefore, the salvaging of livers with moderate steatosis is considered to be an important goal in liver transplantation.

Steatotic livers are susceptible to some stressful events, especially to ischemia/reperfusion (I/R) during surgical intervention [22]. The vulnerability of steatotic livers to normothermic I/R has also been proven experimentally [13]. We have previously reported that heat-shock preconditioning (HSPC) provides the liver with ischemic tolerance, even in steatotic livers [32]. As a possible mechanism for the acquisition of ischemic tolerance, reduction of microcirculatory failure was suggested. Rat livers that underwent HSPC showed less congestion after I/R in hematoxylin-eosin samples. However, there is no direct evidence so far that hepatic microcirculatory failure after I/R is ameliorated in the steatotic livers that received HSPC. The aim of this study was to investigate, by intra-vital fluorescence microscopy, the impact of HSPC on microcirculatory failure after I/R in steatotic livers.

Materials and methods

Animals

All experiments were performed in accordance with the German legislation on the protection of animals. Genetically, obese (fa/fa) male Zucker rats (300–350 g) were used (Charles River Wiga, Sulzfeld, Germany) because this species of rat is a well-characterized model of nutritionally induced obesity. The mechanism producing steatosis in this animal is closely related to the most common cause of steatosis in humans and is not associated with inflammation [14]. Animals were fed with standard rat chow and tap water in chip-bedded cages in an air-conditioned room. They were allotted to three groups: a control group (group C, $n=6$), an I/R group (group IR, $n=6$) and an HSPC group (group HS, $n=6$).

Histology

Moderate-grade steatosis was histologically confirmed in the liver. Tissue samples were fixed in Bouin's solution [15], embedded in paraffin, stained with hematoxylin-eosin, and examined under the light microscope.

Heat shock exposure

Animals were anesthetized by intramuscular injection of atropine sulfate (0.05 mg/body weight) and intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The rats in the HS group were placed in a temperature-controlled water bath to keep their rectal temperature at 42 °C for 15 min. The rats in groups C and IR underwent the same procedure, but the rectal temperature was maintained at 37 °C instead of 42 °C. The heat-exposure period of 15 min was determined from the minimal period necessary to induce heat shock protein 72 (HSP72) in our previous study [33].

Surgical procedures

The animals were anesthetized again 48 h after each pretreatment, placed on a heating pad to maintain rectal temperature at 37 °C, tracheotomized, and ventilated mechanically. Polyethylene catheters (ID 0.58 mm; Portex, Kent, UK) were inserted into the left

carotid artery and left jugular vein for the monitoring of mean arterial pressure, sampling of blood, infusing of Ringer's lactate solution (2 ml/h per 100 g body weight) continuously, and injecting of fluorescent dyes. PaO_2 and PaCO_2 were maintained at 100–120 mmHg and 35–40 mmHg, respectively, by adjustment of the inspiratory O_2 fraction (32–38%), tidal volume (0.8–1.2 ml/100 g body weight), and respiratory rate (45–55 frequencies/min). After laparotomy, ligamentous attachments of the liver were dissected to mobilize the left lobe, and the left lateral lobe of the liver was carefully exteriorized to position on an adjustable stage. Before the induction of ischemia, we performed intra-vital fluorescence microscopy to obtain baseline data. Rats in the IR and HS groups underwent 60-min ischemia of the median and left lateral lobes by the clamping of the left hepatic artery and the left branch of the portal vein, followed by 120-min reperfusion. Sham-operation without ischemia was performed in rats of group C, which served as controls.

Intra-vital fluorescence microscopy

The microvascular blood flow in the left lobe of the liver was studied with a modified fluorescence microscope (Leitz, Germany) with a 100-W HBO mercury lamp for epi-illumination. The microscope images were transferred by CCD camera (FK6990, Cohu, Prospective Measurements, San Diego, Calif., USA) to a video system (S-VHS, AG7330, Panasonic, Tokyo, Japan) for off-line analysis. Sodium fluorescein (200 µg/kg body weight; Merck, Darmstadt, Germany) and rhodamine 6G (48 µg/kg body weight; Merck) were given intravenously to stain plasma and leukocytes, respectively. Two filters, 450–490 nm/> 515 nm (excitation/emission) and 530–560 nm/> 580 nm, were used to visualize sodium fluorescein and rhodamine 6G, respectively. In each liver, we randomly selected seven–ten acini and seven–ten post-sinusoidal venules, to examine microcirculation parameters at baseline conditions at 60 and 120 min after reperfusion.

Measurement of liver related enzyme and serum glutathione levels

Serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactic dehydrogenase (LDH) 120 min after reperfusion were measured with a Hitachi 917 automatic analyzer. Glutathiones (reduced glutathione, GSH; oxygenated glutathione, GSSG) in the serum 120 min after reperfusion were measured as previously described [4].

Electron microscopy

Following the last blood sampling, the liver was perfused with Karnovsky solution (25% glutaraldehyde, 8% paraformaldehyde, and Soerensen buffer pH 7.4) via the aorta [9]. Specimens were cut into 1-mm cubes and fixed in Karnovsky solution, post-fixed with 2% osmium tetroxide, dehydrated, and embedded in Araldite. Ultra-thin sections were cut on an ultra-microtome (Ultracut, Reichert-Jung/Leica, Munich, Germany), contrasted with uranyl acetate and lead citrate. Finally, the specimens were viewed in a Zeiss EM900 (Oberkochen) electron microscope operating at 80 kV.

Western blot analysis

To detect HSP72 and heme oxygenase (HO-1) expression, we sampled liver tissue (before heat exposure, and 24, 48, and 72 h after heat exposure in separate animals). Western blot analysis was

employed for HSP72 and HO-1 as described elsewhere (anti-HSP70 monoclonal antibody: SPA-810; anti-HO-1 monoclonal antibody: OSA-111; Stress Gen Biotechnologies, Victoria, Canada) [25]. Densitometric analysis was performed with BioID-software (Vilber-Lourmat/LTF, Wasserburg, Germany).

Statistical analysis

Values were expressed as means \pm SD. A Kruskal-Wallis rank test was used for the comparison of different groups. A Student-Newman-Keuls test was employed as a post-hoc test. $P=0.05$ was considered statistically significant.

Results

Histological examination of steatotic livers

Approximately 40–60% of the hepatocytes included fatty droplets. This finding was equivalent to moderate hepatic steatosis in humans (Fig. 1).

Mean arterial pressure

Mean arterial pressure (MAP) was not significantly different between the groups (group C, group IR, and group HS) at any time period (Fig. 2).

Hepatic microcirculation

At baseline, sinusoidal perfusion rate was above 96% in all groups (Fig. 3A). The sinusoidal perfusion rate 60 min after reperfusion was significantly better in the HS group than in the IR group (HS: $81.3 \pm 12.2\%$; IR: $62.9 \pm 10.6\%$; $P < 0.05$). Although the differences between the IR and the HS groups were not significant at the end of perfusion (120 min), the sinusoidal perfu-

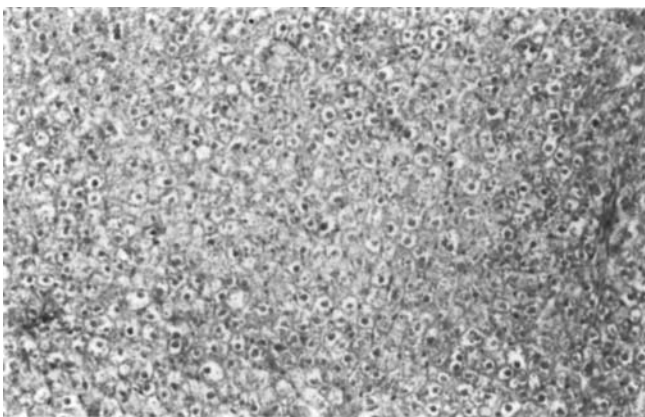


Fig. 1 Micrograph of the liver tissue of Zucker fat rats (300–350 g) revealed that between 40–60% of hepatocytes had deposits of fatty droplets. Hematoxylin and eosin stain (original magnification; $\times 40$)

sion rate in the HS group recovered to $85.4 \pm 8.1\%$, however, it remained at $78.9 \pm 9.2\%$ in group IR. As shown in Fig. 3B, the diameter of sinusoids was significantly narrowed after ischemia. However, this post-ischemic narrowing of the sinusoidal diameter was significantly better attenuated in the HS than in the IR group.

Considerable increases in the number of stagnant leukocytes in hepatic sinusoids and adherent leukocytes in post-sinusoidal venules were observed after ischemia. As shown in Fig. 3C, the number of stagnant leukocytes in the sinusoids was increased to 37.3 ± 9.36 (cells/lobule) and 48.1 ± 15.6 at 60 min and 120 min after reperfusion, respectively, in group IR. In the HS group, however, the number of stagnant leukocytes was slightly reduced (60 min: 27.0 ± 9.82 (cells/lobule), 120 min: 24.8 ± 10.2). The number of stagnant leukocytes in the HS group was significantly lower than that of the IR group 120 min after reperfusion ($P < 0.05$). Figure 3D shows the number of adherent leukocytes in post-sinusoidal venules. The values in the IR group were significantly higher than those in the HS group (60 min, IR: 331.7 ± 139.7 (cells/ mm^2), HS: 130.2 ± 78.3 ; 120 min, IR: 357.8 ± 74.9 , HS: 175.8 ± 79.0 , $P < 0.05$ each time).

Liver enzyme release and glutathiones in the blood

In Table 1, the serum concentrations of AST, ALT, and LDH 120 min after reperfusion are listed. In the HS group, concentrations of these enzymes were much lower than those of the IR group ($P < 0.05$). The GSSG/GSH ratio in the serum of the IR group was significantly higher than that of the HS group 120 min

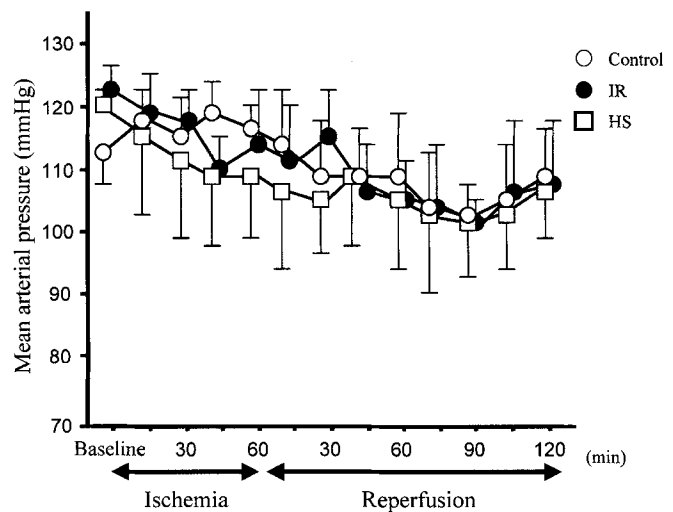


Fig. 2 Changes in MAP. No significant differences were observed among the three groups. All data are expressed as means \pm SD for six animals in each group. Open circles group C, closed circles group IR, squares group HS

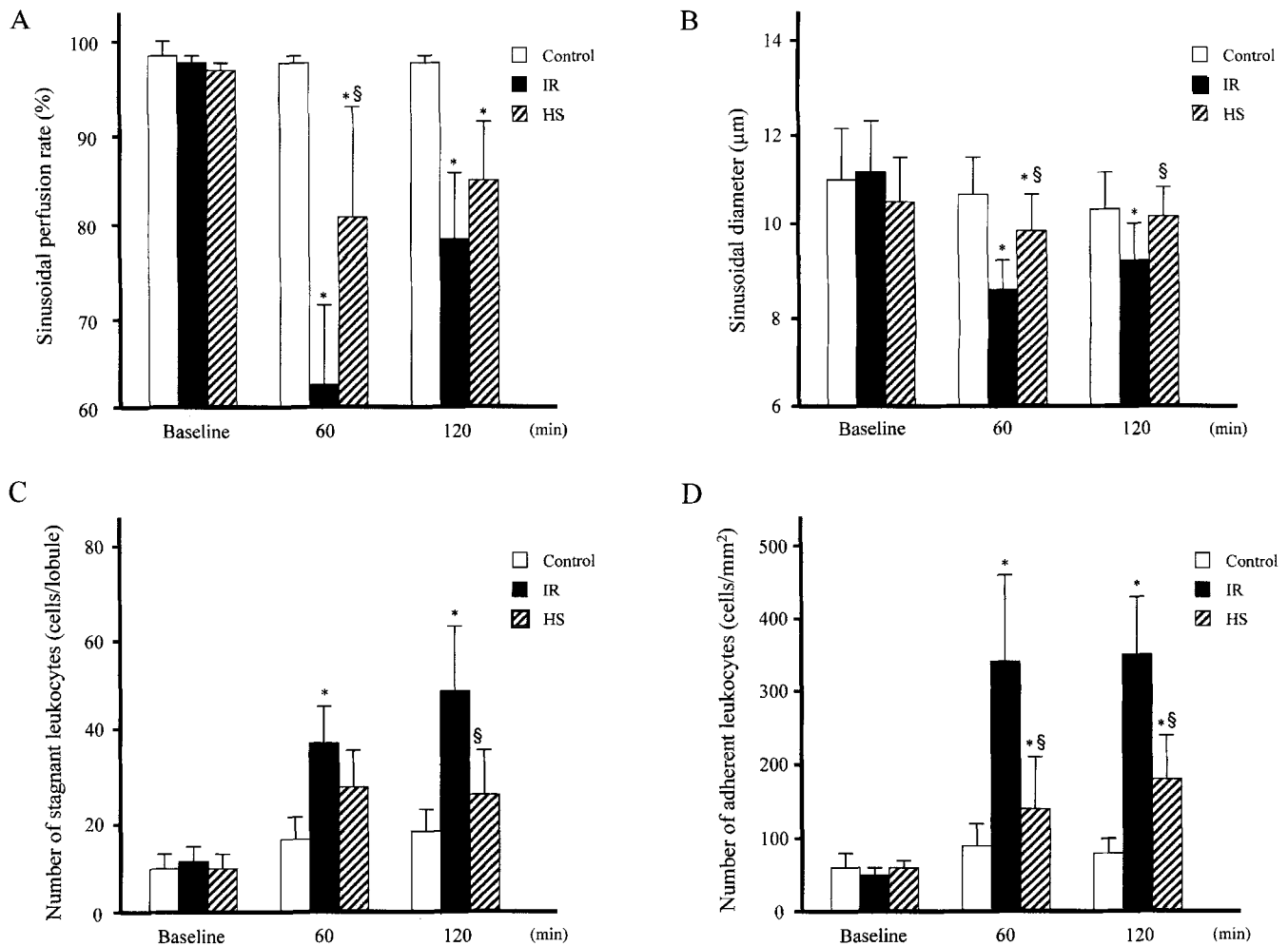


Fig. 3 A Sinusoidal perfusion rate. B Sinusoidal diameter given as the average sinusoidal diameter in the mid-zonal area of each individual acinus. C Number of stagnant leukocytes in the cells in hepatic sinusoids. D Number of adherent leukocytes in post-sinusoidal venules. All data are expressed as means \pm SD for six animals in each group. * $P < 0.05$ compared with group C, § $P < 0.05$ compared with group IR

after the onset of reperfusion, being indicative of higher oxidative stress in the animals in group IR ($P < 0.05$, Table 1).

Electron microscopy

Animals in the IR group showed severe swelling of endothelial cells and a narrowing of the sinusoidal lumen. The space of Disse was not clearly visible. The microvilli of the hepatocytes were reduced both in size and number. The mitochondrial cristae of the hepatocytes were not visible. Neutrophilic granulocytes adhered to the sinusoidal endothelial cells. On the other hand, there was no sinusoidal endothelial swelling in the HS group. A large number of microvilli of the hepato-

Table 1 Liver-related enzyme activities and the redox state of glutathione in serum at 120 min after reperfusion. Data are expressed as means \pm SD ($n = 6$ in each group)

Parameter	Control	IR	HS
AST (IU/ml)	61.0 \pm 17.6	394.8 \pm 126.5*	233.3 \pm 46.4*§
ALT (IU/ml)	44.3 \pm 13.0	545.2 \pm 226.7*	230.8 \pm 81.7*§
LDH (IU/ml)	339.2 \pm 195.1	5225.7 \pm 2635.2*	2696.7 \pm 1158.6*§
GSSG/GSH $\times 10^{-2}$	3.2 \pm 2.7	9.7 \pm 3.5*	4.5 \pm 2.9§

* $P < 0.05$ compared with control; § $P < 0.05$ compared with IR

cytes was projecting into the space of Disse. The cristae of the mitochondria were clearly visible (Fig. 4).

HSP72 and HO-1/HSP-32 expression

Figure 5 illustrates the time course of expression for the inducible type of HSP70 (HSP72) and of HO-1 in the steatotic liver tissue in response to a 15-min heat exposure. HSP72 was not detectable in the control liver, but an intense specific band for HSP72 was clearly detected

24 and 48 h after heat exposure. HSP72 expression was diminished 72 h after HSPC. HO-1 was detectable even in the control liver, but its expression was stronger after heat exposure (24 h, 48 h).

Discussion

Protection of liver function from the detrimental effects of I/R injury in steatotic livers is a serious problem in liver transplantation. It has been documented that im-

paired microcirculation is a considerable factor in the reduced tolerance of steatotic livers to I/R injury. Hepatocytes with fat deposits increase their cellular volume, compress sinusoidal vascular linings irregularly, and narrow the sinusoidal lumina [12]. These morphologically unfavorable conditions decrease tissue blood flow at the microvascular level and consequently result in an insufficient oxygen supply to the hepatocytes in steatotic livers [20]. These early changes in microcirculation are suspected to amplify the negative effects of additional ischemic insults [22]. In the early stages of

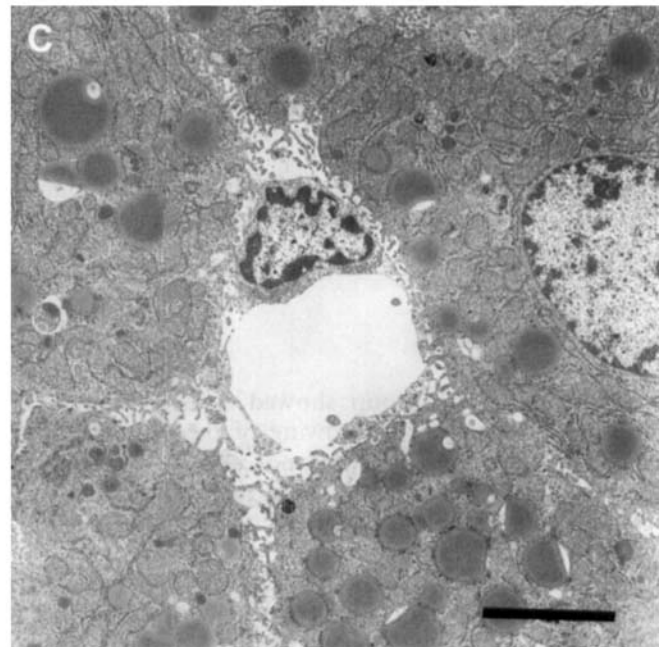
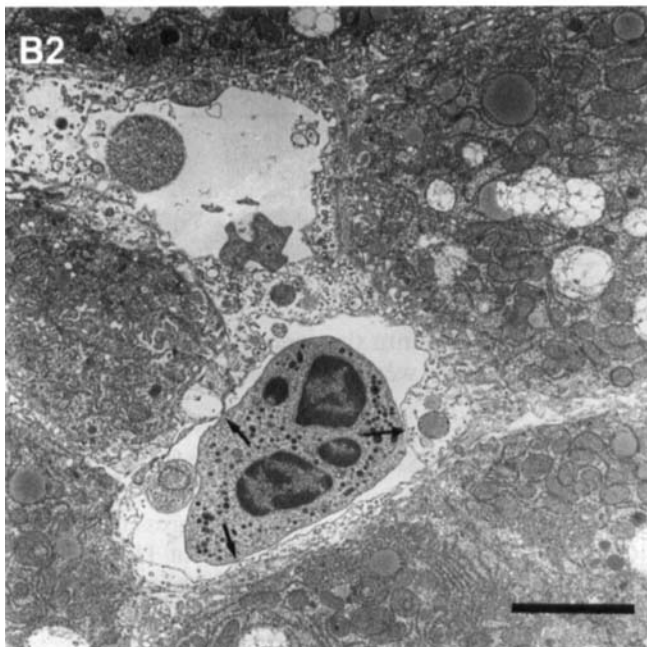
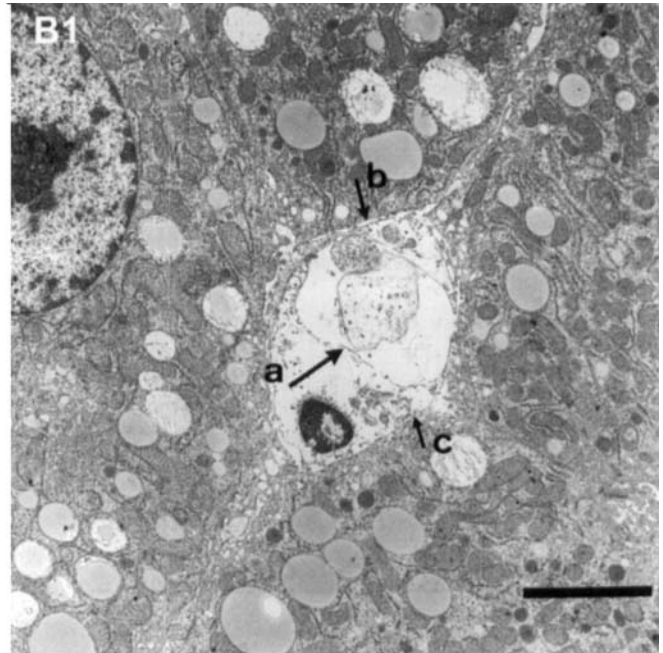
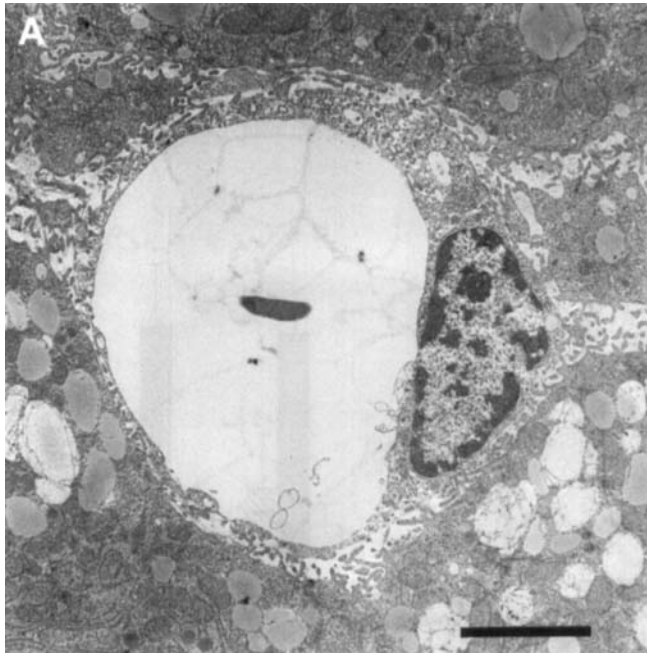
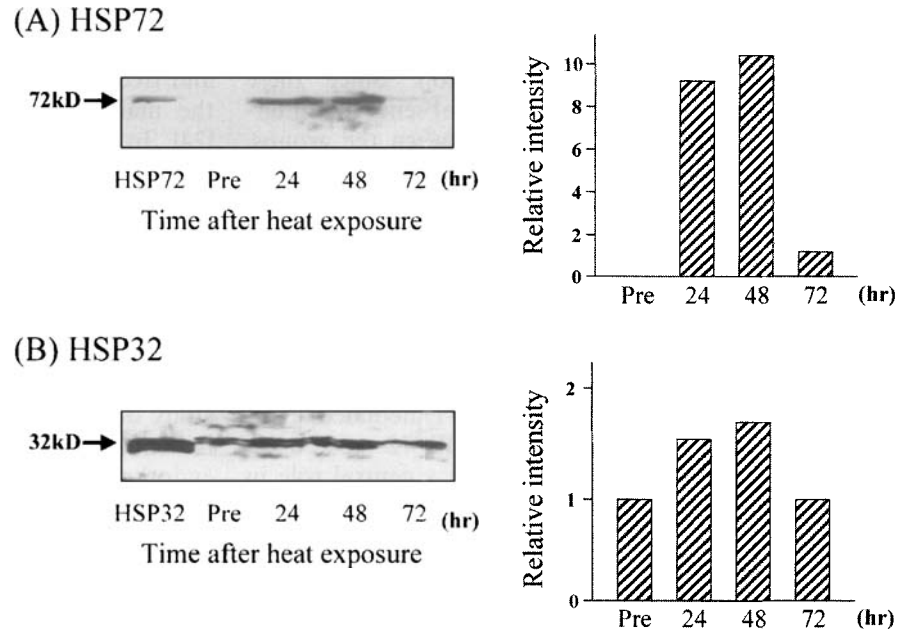


Fig. 5 Expression of HSP72 and HO-1 production in steatotic livers after heat exposure. HSP72: the *first lane* shows positive control (recombinant human HSP70). In the liver without heat exposure (*Pre*), HSP72 was not detectable. At 24 and 48 h after HSPC, the band of HSP72 was clearly visible. HO-1: the *first lane* shows positive control (recombinant rat HO-1/HSP32). In the liver without heat exposure (*Pre*) HO-1 protein was expressed only weakly. At 24 and 48 h after HSPC, HO-1 protein was induced strongly



reperfusion, endothelial cell swelling [28], vasoconstriction [17], leukocyte entrapment [31], and platelet aggregation within the sinusoids [6] are observed. They are thought to lead steatotic livers to severe injury in conjunction with a pre-existing reduced sinusoidal perfusion. Our results demonstrate that partial (70%) hepatic ischemia of 60 min and subsequent reperfusion cause severe swelling of endothelial cells, as shown by electron microscopy and significant narrowing of the sinusoids in the mid-zonal area. Other established parameters are jeopardized sinusoidal perfusion and enhanced leukocyte-endothelial interaction in the hepatic sinusoids and post-sinusoidal venules [18]. In the present study, I/R injury of the liver caused a significant reduction of sinusoidal perfusion rate and a progressive increase in the numbers of leukocyte stagnation in hepatic sinusoids and leukocyte adherence in post-sinusoidal venules.

Leukocytes are thought to be important players in the manifestation of microvascular and tissue injury. The degree of tissue damage during I/R strongly correlates

with the number of recruited leukocytes [11]. A multi-step process mediated by a variety of cell surface molecules enables the adhesion of leukocytes to the endothelial cells. Nakano et al. found that adhesion of neutrophils was increased in fatty livers and that the liver could be protected from oxidative stress by the blocking of neutrophil adhesion with intercellular adhesion molecule-1 (ICAM-1) antibodies [19]. In the present study, we showed that expression of HSPs after HSPC was associated with attenuation of I/R-induced leukocyte-endothelial interaction. The mechanism by which HSPC induces this phenomenon remains unclear. Stokes et al. demonstrated that thermotolerance decreased I/R-induced up-regulation of ICAM-1 on the surface of renal endothelium [23]. The mechanical trapping of leukocytes to sinusoids due to severe swelling of the endothelial cells after I/R is also considered a significant cause for leukocyte stasis. Attenuation of swelling and a better preservation of endothelial cells in the HS group could have contributed to the inhibition of post-ischemic leukocyte stagnation. Recently, we reported that HSPC suppressed the release of TNF-alpha and cytokine-induced neutrophil chemoattractant (CINC) during I/R [36]. This phenomenon is a possible mechanism of reduced neutrophil infiltration because TNF-alpha stimulates production of proinflammatory cytokines, adhesion molecules [5, 21] and chemokines [29]. CINC has been reported to play an important role in neutrophil transmigration [30]. The significantly lower number of stagnant leukocytes after HSPC is most likely responsible for diminished oxidative stress and amelioration of liver injury.

Although the unfavorable morphological characteristics of steatosis were not altered by HSPC (data not shown), the microcirculation parameters such as

Fig. 4 Electron microscopy of rat livers after 120 min of reperfusion. **A** Group C. The endothelium without swelling completely surrounds the sinusoid. The microvilli of the hepatocytes project into the space of Disse. The cristae of the mitochondria can be clearly seen. **B** Group IR. (1) Sinusoidal endothelial cells are severely swollen (*arrow a*). The lumen of the sinusoid is narrowed. The number of microvilli of the hepatocytes is reduced. Microvilli are partly or completely missing (*arrow b*) and the space of Disse is hardly visible (*arrow c*). The cristae of the mitochondria are not clearly visible. (2) A neutrophilic granulocyte adheres to swollen endothelial cells (*arrow*). **C** Group HS. Absence of the swelling of sinusoidal endothelium. Microvilli project abundantly into the space of Disse. The cristae of the mitochondria are clearly visible. The black bar in each picture represents 5 μ m

sinusoidal perfusion rate and sinusoidal diameter showed significantly better values in the HS group than in the IR group during reperfusion. Since these improvements were observed in the absence of differences in systemic arterial pressure between the groups, the improved microvascular perfusion after I/R appeared to be a direct organ-specific effect of HSPC on the liver. Improvement of microcirculation seems to be associated with a lower degree of oxidative damage in the blood as well as lower release of liver-related enzymes, leading to less liver damage.

Western blot analysis demonstrated intensified bands of HSP72 and HO-1 24 h and 48 h after heat stress. The mechanism behind HSP72- and HO-1-mediated cytoprotection is poorly understood. HSP72 acts as a molecular chaperon, thus it may play a central role in capturing denatured proteins and in correcting their structure [8]. We have shown that over-expression of HSP72 in the liver tissue suppressed the formation of 4-hydroxy-2-nonenal (HNE) modified proteins, which are denatured proteins produced by oxidative stress, after reperfusion [34]. HO-1 is a microsomal enzyme, known also as heat shock protein 32, and shows antioxidative

properties in the liver [35]. HO-1 catalyzes the conversion of heme into biliverdin, carbon monoxide (CO), and free iron [16]. HO-1-derived CO may contribute to the maintenance of hepatic sinusoidal vasodilatation [24]. In an ex-vivo I/R-injury model, up-regulation of HO-1 improved portal venous blood flow [3]. Deterioration of portal blood flow is the cause of lobular ballooning, hepatocyte swelling, and sinusoidal congestion, and thereby directly triggers microcirculatory failure. Therefore, over-expression of HO-1 after HSPC may be involved in the improvement of hepatic microcirculation after I/R.

In conclusion, intra-vital fluorescence microscopy study directly demonstrated that HSPC improved post-ischemic sinusoidal perfusion and inhibited leukocyte-endothelial cell interaction in steatotic livers. This beneficial effect of HSPC on hepatic microcirculation is most likely the cause of attenuation of post-ischemic injury in steatotic livers.

Acknowledgment We thank Mrs. Alke Schropp, Institute for Surgical Research, Klinikum Grossharden, for her excellent technical assistance in histology and electron microscopy.

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