Heat stress enhances recovery of hepatocyte bile acid and organic anion transporters in endotoxemic rats by multiple mechanisms

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Abstract Heat stress (HS) reduces the many sequelae of lipopolysaccharide (LPS)-induced endotoxemia. Without HS, endotoxins have been shown to induce a transcriptional down-regulation of hepatocyte transport proteins for bile acids and organic anions. We performed experiments in isolated perfused rat livers at various times after LPS administration with and without HS pretreatment to determine whether HS would correct deficient transport of bromosulfophthalein (BSP). Possible mechanisms involved were investigated in livers from intact animals. In isolated perfused livers, LPS injection reduced BSP excretion to 48% compared with saline-injected controls ($P < 0.01$). When HS was applied 2 hours prior to LPS, BSP excretion increased to 74% of controls ($P < 0.05$ vs LPS and controls). Expression of the basolateral (*Oatp1a1*) and canalicular (*Mrp2*) organic anion transporter involved in the transport of BSP recovered more rapidly when HS preceded LPS application. Recovery of mRNA levels of these transporters occurred also earlier. Coimmunoprecipitation experiments and immunoelectron microscopy using a double immunogold labeling of heat shock protein 70 (HSP70) and various hepatocyte transporters suggested colocalization with HSP70 for the canalicular bile acid transporter (*Bsep*) in the subcanalicular space. In contrast, no colocalization was shown for *Ntcp* and anion transporters. In conclusion, we could show that HS enhances recovery of organic anion transporters and bile acid transporters following endotoxemia. Faster recovery of mRNA seems to be a key mechanism for anion transporters, whereas physical interaction with HSP70 plays a role in preservation of bile acid transporters. This interaction of HSP70 and canalicular transporters occurs only in pericanalicular vesicles but not when the protein is integrated into the plasma membrane.

INTRODUCTION

In the septic state, transcriptional down-regulation of the basolateral sodium taurocholate cotransporting protein (*Ntcp*) and the canalicular bile salt export pump (*Bsep*) leads to a reduced bile flow and cholestasis (Green et al 1996; Moseley et al 1996; Lee et al 2000). In addition, the basolateral organic anion transporting protein 1a1 (*Oatp1a1*), previously known as *Oatp1* (Hagenbuch and Meier 2004), and the canalicular multidrug resistance protein 2 (*Mrp2*) are decreased in endotoxemic animal

models (Kullak-Ublick et al 1994; Paulusma et al 1996; Trauner et al 1997, 1999).

These studies gave a rationale for previous findings describing defective excretion of sulfobromophthalein (BSP), an organic anion likely to be transported by hepatocyte organic anion transporters (Utili et al 1976, 1977).

Previously, our group described preserved bile acid transport after heat stress preconditioning in a rat model of endotoxemic cholestasis (Bolder et al 2002). The protective effect coincided with the induction of heat shock protein 70 (HSP70). The natural function of HSP70 as a molecular chaperone and the fact that transporter mRNA levels were unaffected led to the conclusion of a direct protective interaction of HSP70. Because endotoxemia re-

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sults in a down-regulation of organic anion transport in humans and animals, a protective effect of heat stress would be of clinical relevance. In this study, we aimed to test whether heat stress has protective effects on organic anion transport.

Here, we present experiments elucidating the effect of heat stress on Oatp1a1 or Mrp2 following endotoxemia. Our results show that heat stress enhanced recovery of both transporters as mRNA signals recovered earlier after a nadir and preceded the course of the respective anion transport proteins. Monitoring key cytokines showed reduced levels of proinflammatory and enhanced levels of anti-inflammatory mediators.

MATERIALS AND METHODS

Transport of BSP in the isolated perfused rat liver

The animal experiments were approved by the Committee on Animal Studies of the University of Regensburg. Male Spague Dawley rats (Charles River, Sulzfeld, Germany), weighing 250–300 g were housed at a 12-hour light cycle at 21°C. The animals had free access to water and rat chow. The maximum transport rate ($T_{\max}$) was tested according to the established $T_{\max}$ method in the isolated perfused rat liver (IPRL) (Kitani et al 1986; Bolder et al 1997).

Endotoxemia was induced by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (0.6 mg/100 g body weight) serotype 0111:B4 (Sigma, Deisenhofen, Germany) suspended in 1 mL of 0.15 mol/L NaCl (LPS group). Control animals received 1 mL of 0.15 mol/L NaCl. Heat stress (HS) was applied 2 hours prior to LPS injection by external warming of animals on a heat pad (HS/LPS group) (Bolder et al 2002). Rectally measured body temperature was kept at 42°C for 10 minutes before the animals were allowed to cool down to their normal temperature. Effects of heat stress without LPS injection were investigated 26 hours after hyperthermia (HS group).

Because previous experiments had shown maximum impairment of transport between 12 and 24 hours after induction of endotoxemia, transport studies with BSP were performed at these time points.

Chemical analysis

Biliary excretion

BSP excretion was measured photometrically at a wavelength of 580 nm as described previously (Bolder et al 1997).

Tumor necrosis factor- α /interleukin-10 assay

Determination of tumor necrosis factor- α (TNF α) and interleukin-10 (IL-10) by enzyme-linked immunoassay (ELISA) was performed in plasma after LPS or HS/LPS treatment according to the instructions of the supplier (R&D Systems, Inc., Minneapolis, MN, USA).

Immunofluorescence

Rat livers were fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Semithin tissue sections (0.5–1 μ m) were cut using an Ultracut E equipped with a F4-cryo attachment (Leica, Glattbrugg, Switzerland) and incubated for indirect 2-channel fluorescence. Images were taken from the perivenous area (zone 3), which is most susceptible to induction of HSP expression. Inducible HSP70 was detected with a monoclonal antibody with specificity for HSP70 only (SPA-810; clone C92f3A-5; StressGen Corp., Victoria, BC, Canada), whereas membrane proteins were probed in the same section with antisera against Oatp1a1 (Dumont et al 1997; Eckhardt et al 1999) or Mrp2 (Madon et al 2000), kindly provided by Dr. B. Stieger (University Hospital Zurich, Switzerland). Primary antibodies were visualized using a secondary antibody conjugated to Cy2 (for HSP70) or Texas Red (for Oatp1a1 and Mrp2). Micrographs were taken with an Axiophot epifluorescence microscope (Zeiss, Zürich, Switzerland).

Electron microscopy

Small tissue blocks were cryoprotected with sucrose/poly(vinylpyrrolidone) (PVP) (Tokuyasu 1989), mounted on stubs, and frozen in LN₂. Sections (100 nm) were cut on a ultracryomicrotome (Ultracut UCT equipped with an EM FCS attachment; Leica). For immunocytochemistry, the 2 antibodies listed above (immunofluorescence) were used simultaneously. Sections were incubated for 30 minutes at room temperature. Bound antibodies were detected by incubation for 30 minutes in gold of different size (5 nm anti-mouse for HSP70, and 10 nm anti-rabbit for transport proteins). Finally, sections were embedded in methyl cellulose containing 2.5% uranyl acetate. Controls included sections that were not incubated into primary antibody and grids having absorbed gold label. The former showed little if any labeling; gold solutions containing aggregates were discarded.

Immunoblot analyses

Immunoblot analyses for Oatp1a1 and Mrp2 were performed using liver plasma membranes (LPM) prepared as described previously (Bossard et al 1993). Analyses

were carried out after treatments specified in the figure legends.

Detection of HSPs was carried out in cell lysates prepared by homogenization of liver tissue in a glass homogenizer as described previously (Bolder et al 2002). The blots were probed with antisera against Oatp1a1 or Mrp2. Bound antibody was detected using horseradish peroxidase-conjugated mouse or rabbit IgG by the ECL kit (Amersham, Little Chalfont, UK). Quantification of blots was performed in relation to actin.

Northern blot analyses

Total RNA was extracted from liver tissue by a guanidinium thiocyanate method. Twenty micrograms of RNA were electrophoresed in an agarose formaldehyde gel and transferred to a nylon membrane. Prehybridization for 1 hour was carried out in a commercially available solution (QickHyb; Stratagene, La Jolla, CA) at 68°C. Probes against Oatp1a1, Mrp2, and GAPDH were labeled by random priming (Prime-It II; Stratagene) using [α - 32 P]dCTP-nucleotides. Hybridization was carried out for 1 hour at 68°C. Blots were washed with 2× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 20 minutes at room temperature, followed by 0.1× SSC/0.1% SDS for 15 minutes at 60°C. The plasmid encoding Oatp1a1 was a generous gift from Dr. B. Hagenbuch (University of Zürich, Switzerland). Dr. D. Keppler (University of Heidelberg, Germany) kindly provided a 347-bp Mrp2 fragment.

Coimmunoprecipitation

A first experiment tested whether HSP70 was attached to the hepatocyte membrane. Therefore, an immunoblot using the monoclonal HSP70 antibody was performed as described.

To test coimmunoprecipitation, a protein A/Sepharose/antibody complex was formed by incubation of 200 μ L antisera against Ntcp, Bsep, Oatp1a1, or Mrp2 with protein A/Sepharose in 1 mL of PBS for 1 hour at room temperature. The protein A/Sepharose/antibody complex was pelleted (6000 $\times$ g, 2 minutes) and resuspended in coimmunoprecipitation (co-IP) buffer consisting of the following (in mmol/L): Tris/HCL, 50 (pH 8.0); NaCl, 150; EDTA, 5; Triton, 1%. One hundred fifty micrograms of protein of LPM of each experimental group were thawed on ice and incubated in 20 μ L co-IP buffer for lysis of membrane proteins. The samples were then centrifuged (14 000 $\times$ g, 30 minutes, 4°C), and the supernatant containing the liberated membrane proteins was transferred to a chilled tube for protein determination.

Then, equal proportions of lysed membrane transporters and the protein A/Sepharose/antibody complex were

Fig 1. Effect of heat stress on biliary BSP in single-pass perfused IPRL. BSP transport (hatched bars) in single pass IPRLs was preserved to some degree by heat stress. BSP studies were performed 24 hours following LPS injection. The percentage of BSP or CT secretion (open bars) was calculated for the time point with the maximum impairment of biliary transport of the marker substrate (ie, 24-hour BSP, 12-hour CT). Values present the mean $\pm$ SEM, $n = 6$ for each group. ** $P < 0.01$ vs control, HS, and HS/LPS; * $P < 0.05$ vs LPS and control.

coincubated in co-IP buffer (total volume of 350 μ L) for 12 hours at 4°C. The protein A/Sepharose/antibody complex with the potentially coimmunoprecipitated heat shock proteins were pelleted again and resuspended in Laemmli buffer, electrophoresed in a 7.5% polyacrylamide gel, and transferred to a nitrocellulose membrane.

In the final step, the transferred proteins were probed with an antibody specifically directed against HSP70. Bands were visualized by chemoluminescence. For methodological reasons, coimmunoprecipitation experiments were not quantified.

Statistical analyses

All values are given as mean $\pm$ SEM. Comparison between groups was performed by 1-way analysis of variance (ANOVA) and the Newman-Keuls method. Values of cytokines were analyzed with Student's *t*-test at comparable time points. *P* values ≤ 0.05 were considered to indicate a statistically significant difference. Densitometry was performed using standard software (QuantityOne; Bio-Rad, Munich, Germany).

RESULTS

Isolated perfused rat liver

Livers were perfused in single-pass fashion allowing a constant concentration of BSP in the in-flow. Because previous studies had shown the greatest impairment of BSP excretion 24 hours after LPS treatment, this time point was used to determine transport function. For BSP

Fig 2. Immunofluorescence imaging of Mrp2 (top row) and Oatp1a1 (bottom row). Semithin cryosections (0.5–1 μ m) were probed simultaneously with a primary antibody against HSP70 (green) and antisera recognizing Mrp2 or Oatp1a1 (both red). The first column (A, E) shows the domain-specific distribution of both transporters at the canalicular or basolateral cell pole, respectively. Heat stress (HS) did not alter intensity level or distribution pattern of both transporters (B, F). In contrast, a markedly decreased intensity of Mrp2 and Oatp1a1 occurred after LPS injection (C, G). Signal intensity of the transporters was preserved after combined heat stress and LPS treatment (HS/LPS) (D, H) (bar = 25 μ m).

(hatched bars), it was shown in the LPS group $T_{\max}$ was reduced by 52% compared to controls. HS alone did not influence BSP transport markedly (–9%). Pretreatment with HS prior to LPS injection (HS/LPS) resulted in preservation of BSP transport with a significantly higher excretion of the anion compared to LPS treatment (+55%) (Fig 1, hatched bars).

It should be noted that the nadir of BSP transport occurred later than that of cholytaurine (CT) (data not shown) and that the percentage preservation of BSP transport was lower compared to that of the bile acid (Bolder et al 2002). The impairment of bile acid excretion after LPS injection in comparison to BSP is also shown in Figure 1, open bars.

Tissue distribution

Livers were obtained from rats after treatment with LPS, HS, HS/LPS, and from controls. Oatp1a1, Mrp2, and HSP70 were probed by indirect immunofluorescence using TxR- (for transporters) and Cy2- (for HSP70) conjugated secondary antibodies. With the exception of HSP70, which has been shown previously to be expressed more strongly in the perivenous zone (Bolder et al 2002), no transporter displayed a detectable differential expression along the lobular gradient.

Mrp2: Tissue distribution of Mrp2 was confined to the canalicular membrane as shown in Figure 2A. HS resulted in a slightly increased signal intensity (Fig 2B), whereas Mrp2 staining was decreased in LPS-treated livers (Fig

2C). HS pretreatment followed by LPS injection resulted in a Mrp2 signal that was comparable to that of controls (Fig 2D).

Oatp1a1: Distribution of Oatp1a1 was strictly confined to the basolateral membrane as depicted in Figure 2E. Compared to the HS and control groups shown in Figures 2F and 2E, respectively, LPS injection resulted in a strongly decreased signal (Fig 2G). When HS preceded LPS treatment, signal intensity was markedly higher than in livers subjected to LPS treatment alone (Fig 2H).

HSP70: Enhanced expression of HSP70 was clearly visible in HS livers (Fig 2B,F). HSP70 was also highly expressed after combined HS/LPS treatment (Fig 2D,H) but could not be detected in the control and LPS group (Fig 2A,E and C,G).

Immunoblot analyses

To confirm and quantify differences of anion transporters due to endotoxemia and the potentially protective effect of heat stress, immunoblot analyses with isolated plasma membranes were performed.

Oatp1a1: Results of Oatp1a1 immunoblot analyses are shown in the upper panel of Figure 3. HS alone did not significantly influence the expression of Oatp1a1 in the basolateral membrane (second lane). Twenty-four hours after LPS treatment, a marked reduction of the signal was detected (third lane). When HS pretreatment was applied, the protein signal was similar to that of controls (fourth lane).

Mrp2: Examples of Mrp2 immunoblots are depicted in the lower panel of Figure 3. LPS injection significantly reduced Mrp2 expression compared to the control treatment (third lane). In contrast, a higher signal was detected when HS treatment preceded LPS injection (fourth lane). Solitary heat stress did not influence the Mrp2 levels (second lane).

Time course: To evaluate the levels of anion transporters from 0–108 hours after LPS application time, immunoblots were performed throughout that time course (Fig 4). Following NaCl injection (controls), Oatp1a1 and Mrp2 signals remained constant during the entire period (upper panels). The signal declined after LPS application and reached the lowest level after 24 hours (intermediate panel). A recovery was noted thereafter regaining normal levels at 108 hours (intermediate panels, right lanes). When HS preceded LPS, signals of Oatp1a1 and Mrp2 remained at a higher level compared to LPS alone (lower panels, fourth and fifth lane).

Northern blot analyses

Reduction of encoding mRNA during endotoxemia seems to be a likely hypothesis for decreased expression of hepatocyte anion transporters. To study the levels of mRNA 24 hours and from 0 to 108 hours after LPS application, Northern blot analyses were carried out.

Oatp1a1: Levels of Oatp1a1 mRNA are shown in Figure 5, upper panel. A significant reduction compared to controls (first lane) and to HS treatment (second lane) was observed 24 hours after LPS injection (third lane). In contrast to solitary LPS treatment, mRNA levels were higher after HS/LPS treatment (fourth lane).

Mrp2: Levels of Mrp2 are shown in Figure 5, lower panel. It could be shown that LPS injection markedly reduced the signal of Mrp2 mRNA (third lane). HS preceding LPS injection ameliorated the decline of the signal at 24 hours (fourth lane). NaCl (first lane) or HS alone (second lane) did not influence levels of Mrp2 mRNA.

Time course: Levels of transporter mRNA was studied from 0–108 hours after LPS application (Fig 6). A nadir of the mRNA signal of Oatp1a1 (left panels) and Mrp2 (right panels) compared to controls was detected at 24 hours. Recovery of both membrane proteins was observed within 108 hours. Especially for Oatp1a1, recovery was faster in the HS/LPS group (lower panel, 24 hours) compared to the LPS group (intermediate panel).

Coimmunoprecipitation

Oatp1a1, Mrp2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of LPM showed marked expression of HSP70 after HS or HS/LPS (Fig 7A). Although the input samples revealed good levels of HSP70,

Fig 3. Immunoblots of Oatp1a1 and Mrp2. Immunoblot analyses were performed following heat stress (HS), after LPS injection (LPS), or 24 h after a combination of both (HS/LPS). Isolation and purity testing of the membranes was performed as described previously (Bolder et al 1997). Contamination with subcellular fractions, assessed by cytochrome *c* reductase or reduced nicotinamide adenine dinucleotide phosphate did not occur. For immunoblot analyses, 50 μ g (Oatp1a1) or 35 μ g (Mrp2) of protein was loaded. Comparison of blots revealed a marked decrease of signal intensity in the LPS group, whereas a preserved band was detected after heat stress pretreatment (HS/LPS). This course of the protein signal was similar for Oatp1a1 (white) and Mrp2 (hatched). ANOVA, $P < 0.001$. * $P < 0.05$ vs control, LPS, and HS/LPS.

no signal with Oatp1a1 or Mrp2/HSP complexes was visualized (Fig 7B). Therefore, a direct interaction of the anion transporters with HSP70 seems unlikely and the appearance of HSP70 in the input samples has to be attributed to interaction with membrane proteins distinct from the anion transporters.

Ntcp, Bsep: A marked expression of HSP70 after HS or HS/LPS was found when the input samples were probed with a HSP70 antibody. LPS alone did not induce expression of HSP70 (Fig 7A). In the immunoprecipitation experiments, a signal indicating coimmunoprecipitation of HSP70 with Ntcp or Bsep was visualized. This band was absent without HS (Fig 7B).

Immunoelectron microscopy of transporters

Double labeling of sections for HSP70 and transport systems showed that HS-induced expression of HSP70 resulted in moderate label distributed in clusters all over the cytoplasm. Transporter-specific label was confined to the specific membrane domain, with Bsep showing an additional location in subcanalicular vesicles (Gerloff et

Fig 4. Time course of Oatp1a1 and Mrp2 immunoblots. Results of Western blotting ($n = 4$) experiments for Oatp1a1 and Mrp2 from livers harvested after the indicated times following sodium injection (control, white) or after LPS (black) or HS/LPS (hatched) treatment. A total of 50 μ g (Oatp1a1) or 35 μ g (Mrp2) of membrane protein was loaded, respectively. Oatp1a1 ANOVA, $P < 0.001$. LPS, $*P < 0.01$ vs 0 and 6 hours and vs control and HS/LPS at the indicated times. HS/LPS, $**P < 0.05$ vs control 24 hours. Mrp2 ANOVA, $P < 0.01$. LPS, $*P < 0.02$ vs 0 and 6 hours and vs control at the indicated times. HS/LPS, $**P < 0.05$ vs LPS 12, 24 and 36 hours.

al 1998). Neither the basolateral uptake systems Oatp1a1 and Ntcp nor the canalicular Mrp2 showed a spatial association with HSP70. In contrast, Bsep in pericanalicular vesicles, but not in the canalicular membrane, consistently was accompanied by HSP70 with labels no more than 100 nm apart (Fig 8).

Cytokine determination

From the previous experiments, it seemed possible that heat stress shifted the balance after induction of endotoxemia rather in the anti-inflammatory than to proinflammatory direction. To investigate upstream mediators of the inflammatory cascade, $\text{TNF}\alpha$ and IL-10 were analyzed in the first 48 hours after LPS application.

TNF α : The level of $\text{TNF}\alpha$ peaked 1 hour after LPS injection and returned to its normal range within 2 hours after LPS injection. HS/LPS treatment resulted in markedly lower $\text{TNF}\alpha$ levels. The course of $\text{TNF}\alpha$ following LPS injection or after HS/LPS treatment is depicted in Figure 9, upper panel.

IL-10: The course of IL-10 showed much lower levels in

the HS/LPS group than in the LPS group. The course of IL-10 is shown in Figure 9, lower panel.

DISCUSSION

Effect of heat stress on hepatocyte transport function

Protective effects of heat stress have been confirmed in sepsis models with intact animals (Villar et al 1994; Ribeiro et al 1995) and isolated organs (Bolder et al 2002). Our data provide evidence for a protective effect of heat stress against the impairment of organic anion transport caused by endotoxemia in rats. With the focus on hepatocyte function, we have shown that the heat stress limits liver damage and preserves organ function. It is likely that the limited damage may improve outcome with regard to cell function, organ function, morbidity, and even mortality.

Our previous findings showed preserved levels of Ntcp and Bsep coinciding with preserved transport of cholytaurine, a common natural bile acid. In the present experiments, BSP transport was impaired by 52% and transport reduction occurred later than that of bile acids

Fig 5. Northern blot analyses following heat stress and LPS treatment. Steady-state mRNA levels of Oatp1a1 (white) and Mrp2 (hatched) in livers obtained from control rats after LPS injection (LPS) or after heat stress (HS) and after HS with LPS application (HS/LPS) ($n = 4$). A total of 20 μ g was probed for the transporters as described. The Mrp2 transporter hybridized with 2 bands (8.3 and 5.5 kb) as reported previously. mRNA of both transporters was preserved after HS pretreatment compared to LPS alone. ANOVA, $P < 0.001$. * $P < 0.05$ vs control, LPS, and HS/LPS.

(Bolder et al 2002). Likewise, Oatp1a1 and Mrp2 levels followed a slower course with a nadir 24 hours after LPS treatment. Heat stress alone did not have a significant effect on BSP transport. In contrast, heat stress prior to LPS preserved BSP transport to a marked degree. In previous kinetic experiments using isolated basolateral and canalicular membranes, it was shown that in this model of endotoxemia only $V_{\max}$ but not K_m was altered for CT and BSP (Bolder et al 1997). Together with our results from immunoblot and Northern blot experiments, a direct connection of preserved transcription and higher levels of the respective membrane transporters are more likely than a change in affinity for BSP. Nevertheless, the magnitude of protection was lower than that for cholytaurine. This may be due to the fact that percentual accumulation of BSP in IPRLs is higher than that of CT and may therefore cause more irreversible damage. In addition, BSP requires conjugation with glutathione for transport by Mrp2 (Combes 1965). Because endotoxemia im-

poses oxidative stress, a reduced concentration of intracellular glutathione might result in a lower conjugation rate. However, this could modulate the functional reserve for BSP excretion but would not contradict our findings of an earlier recovery of Mrp2 transcription.

Potential mechanisms involved

Evidence from previous studies suggests that HSPs exert protective effects against many conditions involving cellular stress (Kluger et al 1975; Hightower and White 1981; Hotchkiss et al 1993; Ribeiro et al 1995). HSPs act as molecular chaperones. Many stimuli have been shown to induce HSP70. Nevertheless, LPS alone did not induce the synthesis of HSP70 as shown by immunofluorescence, despite the oxidative insult imposed by endotoxemia. These findings of nonactivation of inducible HSPs by LPS are in parallel to other experiments in rats and mice and may explain the higher mortality of endotoxemia without heat stress preconditioning (Morikawa et al 1998; Dokladny et al 2001).

The HSP effect is conferred by interaction between HSPs and proteins losing their quaternary structure, thereby reducing the possibility of target molecules to become degraded (Buchner 1996; Hightower and Hendershot 1997). In our experiments, coimmunoprecipitation was shown for Ntcp and Bsep but not for Oatp1a1 and Mrp2. This result could explain preserved transport for bile acids but not for the glutathione conjugate of BSP. Therefore, a more upstream mechanism is likely to account for the protected BSP transport.

A candidate mechanism may be the reduced level of $\text{TNF}\alpha$ due to heat stress. $\text{TNF}\alpha$ is a key mediator in endotoxemia conferring proinflammatory effects (Green et al 1996). Studies of Trauner et al showed that $\text{TNF}\alpha$ induced down-regulation of 2 critical transcription factors (FbB BP1 and HNF1) for Ntcp and Bsep expression (Trauner et al 1998). Further studies using HNF1-deficient ($\text{Tef1}^{-/-}$) mice reported 3–10 times elevated serum levels of bile acids and bilirubin consistent with a lack of Ntcp and Oatp transporters 1a1, 1a4, 1b2 (Shih et al 2001). Jung et al broadened the view of hepatocyte nuclear factor (HNF) as an important transcription factor regulating organic anion and bile acid transport by interacting with human Oatp promotor sequences (Jung et al 2001). Given the finding of reduced HNF1/promotor interaction during endotoxemia, a comparable mechanism following injection of proinflammatory cytokines was recently shown for Oatp1a1 and in part for Mrp2 (Geier et al 2005). Therefore, reduced $\text{TNF}\alpha$ levels observed after heat stress could result in preserved binding of HNF1 and earlier recovery of mRNA and protein signals of Oatp1a1 and Mrp2. In addition, increased levels of IL-10, a natural antagonist of proinflammatory mediators, were

Fig 6. Time course of Oatp1a1 and Mrp2 mRNA levels. The figure depicts Oatp1a1 signals and the 5.5-kB band of Mrp2 obtained from Northern blot experiments after sodium injection (control, white), LPS treatment (black), or HS/LPS treatment (hatched). A nadir of the signal was detected 24 hours following LPS injection. Some protection and an enhanced recovery of both signals was obtained after heat stress pretreatment (HS/LPS). Oatp1a1 ANOVA, $P < 0.001$. LPS, $*P < 0.01$ vs 0, 6, and 36 hours. HS/LPS, $*P < 0.01$ vs 0, 6, 24, and 36 hours. $**P < 0.01$ vs LPS 12 hours. Mrp2 ANOVA, $P < 0.004$. LPS, $*P < 0.02$ vs 0, 6, and 36 hours. HS/LPS, $**P < 0.02$ vs 0, 6, and 36 hours. $\ddagger P < 0.02$ vs LPS 24 hours.

found after heat stress pretreatment. Application of IL-10 reduced LPS-induced injury in rodent models of liver failure (Arai et al 1995; Minter et al 2001), colitis (Lindsay et al 2001), and alveolitis (Huaux et al 1999). Down-regulation of monocyte response to LPS seems to be one mechanism of IL-10 counteracting inflammation. On a molecular level, IL-10 inhibits NF- κ B activity by preventing the nuclear shift of transactivating p65 subunits or allowing the nuclear translocation of p50 subunits unable to activate transcription in monocytic cells (Randow et al 1995; Driessler et al 2004). This modulation of the inflammatory cascade could result in a shift to an earlier recovery of transporter signals. As our study focused on changes of hepatocyte function additional effects may gain importance in a model using a whole organ. Therefore, local perfusion disturbances, due to endothelia damage and consecutive hypoxia, cannot be ruled out.

Based on the results of electron microscopy and of coimmunoprecipitation studies, it seems likely that at least Bsep, the predominant mediator of bile acid excretion, interacts directly with HSP70, whereas no such effect was found for the BSP transporters. It was noted that immunoelectron microscopy did not show association of Ntcp and HSP70. This is in contrast to the coimmunoprecipitation of liberated Ntcp from plasma membranes. From these results, we conclude that interaction of HSP70 and canalicular transporters occurs only in pericanalicular vesicles but not when the protein is integrated into the plasma membrane. This gives evidence for various lines of protection of Bsep, which may be on the protein and on the mRNA level (Lee et al 2000). This might result in a better protection of bile acid efflux transporters compared to Ntcp the bile acid uptake system preventing hepatocyte accumulation of potentially toxic bile acids. On

Fig 7. Coimmunoprecipitation of bile acid and organic anion transporters and HSP70. Panel A depicts immunoblots using liver plasma membranes (LPM). Blots were processed as described and probed with a monoclonal antibody against HSP70. It was clearly shown that heat stress is required to induce HSP70 as control preparations or solitary LPS injection did not result in HSP70 induction. Panel B gives the results after liberation of membrane proteins from LPM and consecutive precipitation of protein or protein/HSP70 complexes. The protein complexes were precipitated with anti-transporter antibodies linked to protein A/Sepharose. After blotting the precipitated complexes, a second antibody against HSP70 was applied to investigate coimmunoprecipitation of the HSP and the transport protein. The autoantibody preparations coincubated the protein A/Sepharose complexes linked to anti-transporter antibodies together with the HSP70 antibody but without plasma membrane proteins. No interaction of HSP and the protein A/Sepharose complexes was detected. Signals of transporters present in precipitated complexes were found for Ntcp and Bsep but not for Oatp1a1 and Mrp2.

the other hand, no protection on the protein level was found for both anion transporters. This could explain why recovery of BSP transport was slower and less complete than that of CT transport.

Medical implications

In humans, induction of hyperthermia has been used to treat localized infections, but could not be applied in generalized sepsis (Trautmann et al 1938). Therefore, the most promising treatment of septic cholestasis remains the elimination of the septic source (Gallinaro and Polk Jr 1991).

Nevertheless, it should be noted that development of fever and expression of HSPs in comparison to hypothermia is correlated to a more favorable outcome in sep-

tic patients (Clemmer et al 1992). Consequently, suppression of fever results in elevated lethality (Bernard et al 1997). Therefore, the role of drugs reducing the threshold of HSP induction seems worthwhile to explore. Experiments using the antiulcer drug geranygeranylactone has been shown to enhance HSP synthesis in response to heat stress (Yamagami et al 2000). This resulted in enhanced survival in a rat liver transplantation model or maintained contractility of diaphragm muscle strips in a rat sepsis model (Fudaba et al 2001; Masuda et al 2003).

From our results, we conclude that heat stress is a powerful modulator of hepatocyte protein expression on the transcriptional and posttranscriptional level. Induction of HSPs or mimicking the heat stress reaction by minimally invasive measures might prove worthwhile to explore.

Fig 8. Immunoelectron microscopy of rat livers after HS and HS/LPS treatment. Micrographs show representative portions of the basolateral or canalicular membrane domains, respectively. Transport systems were probed with 10-nm gold and HSP70 with small 5-nm gold (arrows). HSP70, which is expressed after HS treatment, is distributed in clusters in the cytoplasm. In this location, Bsep colocalizes—at least spatially—with HSP70. Bsep is the only transporter that, in addition to the canalicular membrane, is expressed in pericanalicular vesicles. In this location, Bsep associates with HSP70 that can be found within a distance of less than 100 nm. Bar = 500 nm.

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Fig 9. Time course of TNF α and IL-10 after LPS treatment and heat stress (HS) preconditioning. TNF α levels were highly elevated by LPS treatment, whereas HS stress pretreatment led to a less-pronounced increase of this proinflammatory factor (upper panel). In contrast, HS seemed to induce IL-10 secretion and an increase of this anti-inflammatory cytokine (lower panel). $n = 6$ in each group. ** $P < 0.001$ and * $P < 0.01$.

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