LPS-induced downregulation of MRP2 and BSEP in human liver is due to a posttranscriptional process

Maricke G. L. Elferink,1 Peter Olinga,1 Annelies L. Draaisma,1 Marjolijn T. Merema,1 Klaas Nico Faber,2 Maarten J. H. Slooff,3 Dirk K. F. Meijer,1 and Geny M. M. Groothuis1

1Department of Pharmacokinetics & Drug Delivery, University of Groningen, 9713 AV Groningen; 2Division of Gastroenterology and Hepatology, Department of Internal Medicine, University Hospital Groningen, 9713 GZ Groningen; and 3Division of Hepatobiliary Surgery and Liver Transplantation, Department of Surgery, University Hospital Groningen, 9713 GZ Groningen, The Netherlands

Submitted 11 February 2004; accepted in final form 4 June 2004

Elferink, Maricke G. L., Peter Olinga, Annelies L. Draaisma, Marjolijn T. Merema, Klaas Nico Faber, Maarten J. H. Slooff, Dirk K. F. Meijer, and Geny M. M. Groothuis. LPS-induced downregulation of MRP2 and BSEP in human liver is due to a posttranscriptional process. Am J Physiol Gastrointest Liver Physiol 287: G1008–G1016, 2004. First published June 17, 2004; doi:10.1152/ajpgi.00071.2004.—Endotoxin-induced cholestasis in rodents is caused by hepatic downregulation of transporters, including the basolateral Na–dependent taurocholate transporter (ntcp) and the canalicular bile salt export pump (bsep) and multidrug resistance-associated protein 2 (mrp2). Details about the regulation of the human transporter proteins during this process are lacking. We used precision-cut human and rat liver slices to study the regulation of transporter expression during LPS-induced cholestasis. We investigated the effect of LPS on nitrate/nitrite and cytokine production in relation to the expression of inducible nitric oxide synthase, NTCP, BSEP, and MRP2 both at the level of mRNA with RT-PCR and protein using immunofluorescence microscopy. In liver slices from both species, LPS-induced expression of inducible nitric oxide synthase was detected within 1–3 h and remained increased over 24 h. In rat liver slices, this was accompanied by a significant decrease of rat ntcp and mrp2 mRNA levels, whereas bsep levels were not affected. These results are in line with previous in vivo studies and validate our liver slice technique. In LPS-treated human liver slices, NTCP mRNA was downregulated and showed an inverse correlation with the amounts of TNF-α and II-1β produced. In contrast, MRP2 and BSEP mRNA levels were not affected under these conditions. However, after 24-h LPS challenge, both proteins were virtually absent in human liver slices, whereas marker proteins remained detectable. In conclusion, we show that posttranscriptional mechanisms play a more prominent role in LPS-induced regulation of human MRP2 and BSEP compared with the rat transporter proteins.

human liver slices; endotoxin-induced cholestasis; transporter expression; cytokines; immunofluorescence microscopy

During cholestatic liver disorders, alterations in the expression of hepatobiliary transporters have been reported (1, 11, 13, 15, 18, 21, 27, 30, 37, 38, 39, 40). Increasing knowledge about the molecular mechanism behind these alterations is mainly based on studies in rodents (11, 13, 18, 21, 27, 30, 37, 38, 39, 40), whereas only limited information on transporter regulation in human cholestatic liver disease is available (1, 18). For recent reviews concerning transcriptional regulation of canalicular ABC transporters see Refs. 8, 15, 16, 19, and 29.

Experimental models of cholestasis are mainly based on common bile duct ligation, addition of estrogen, or endotoxin treatment (24). Endotoxin- or sepsis-induced cholestasis is caused by LPS, a component of gram-negative bacterial cell walls. LPS activates macrophages via binding to the CD14 receptor. In the liver, the primary targets of LPS are the Kupffer cells. The activated Kupffer cells start the acute-phase response to prevent tissue damage, to eliminate the infectious agent, and to activate repair processes in the cells. The acute-phase response is initiated by producing proinflammatory cytokines such as TNF-α, II-1β, and II-6 as well as anti-inflammatory cytokines such as IL-10. These cytokines, in turn, bind to receptors on the sinusoidal membrane domain of hepatocytes and initiate a complex pathway of intracellular signal-transduction reactions, leading to a cascade of reactions; among others, the translocation of NF-κB to the nucleus, upregulation of inducible nitric oxide (NO) synthase (iNOS), production of reactive oxygen compounds, lipid peroxidation, and changes in redox potential. Hepatocytes respond to these signals by alterations in gene expression, which also contribute to restoration of homeostasis in the liver cells. Studies with the isolated perfused liver, isolated hepatocytes, and purified membrane vesicles from LPS-treated rodents have demonstrated functional alterations in a variety of hepatocyte transporters. Na–dependent taurocholate transporter (ntcp) is clearly downregulated on endotoxin treatment both at the level of mRNA and at the protein level (11, 13, 27, 37, 38, 40). Multidrug resistance-associated protein 2 (mrp2) is also downregulated at both levels (21, 30, 39, 40). Bile salt export transporter (bsep) has been reported to be downregulated in endotoxemic rat liver (40), but more recent studies indicate that bsep is relatively preserved in different cholestatic models of liver injury, including the endotoxin model (25).

Because several cell types in the liver are involved and cooperate in the defense against the LPS-induced inflammation process, in vitro studies on LPS effects should be preferentially exerted in a system in which all different cell types are present, such as precision-cut liver slices. This in vitro model has been validated in our laboratory using rat and human liver slices (32, 33). During LPS-induced inflammatory reactions in rat liver slices, cell-cell interactions are retained, the Kupffer cells maintain their viability and produce cytokines, which, in turn, cause the upregulation of iNOS in hepatocytes (33). In the
present study, the effect of LPS on the transporters in human liver slices was compared with those in rat liver slices using the same experimental conditions.

MATERIALS AND METHODS

Materials. LPS from *Escherichia coli* (serotype 055:B5) was from Sigma (St. Louis, MO). Williams’ medium E and TRIzol reagent were obtained from GIBCO-BRL (Paisley, Scotland). Six-well culture plates and PCR tubes were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Reverse Transcription System was obtained from Promega and PCR tubes were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Reverse Transcription System was obtained from Promega (Madison, WI), and DNAse treatment and removal reagent DNA-free was from Ambion. SilverStar DNA polymerase was from Eurogentec (Madison, WI), and DNAse treatment and removal reagent DNA-free was from Ambion. SilverStar DNA polymerase was from Eurogentec (Seraing, Belgium). dNTP mix was from MBI Fermentas. Monoclonal antibody to human and rat MRP2 (MIIII-6) was from Alexis. Rabbit anti-BSEP antibodies (K12) were a gift from the Division of Gastroenterology and Hepatology (Dept. of Internal Medicine, University Hospital Groningen, Groningen, the Netherlands) (17). Rabbit anti-Na+/K+ ATPase (K9) were kindly supplied by Dr. B. Stieger (Zürich, Switzerland) (22). Rabbit anti-zonula occludens (ZO)-1 was from Zymed Laboratories (San Francisco, CA). Anti-rabbit IgG (FITC conjugate) and anti-mouse IgG (TRITC conjugate) were from Sigma. Goat anti-rabbit/Alex Flouor 568 was from Molecular Probes (Eugene, OR).

Human liver tissue. Human liver tissue remaining after reduced size or split liver transplantation was used. The donor liver was perfused with cold University of Wisconsin (UW) organ preservation solution in situ before explantation. The livers were stored in cold UW solution on ice until reduction of the liver. The reduction or splitting of the donor organ was performed while it was immersed in UW cooled with ice slush (2). The liver tissue remaining after bipartition was stored again in cold UW solution until the start of the slicing procedure. Total cold preservation time varied from 6 to 13 h. The research protocols were approved by the medical ethical committee of our institution (Academic Hospital, University of Groningen). In Table 1, the sex and ages of the different donors are listed.

Preparation of liver slices. Male Wistar rats of 280–320 g were used in the experiment. Animals were anesthetized by isofurane and N₂O/O₂. Cores of 8 mm were prepared from the pieces of liver by advancing a Krumdieck slicer. The slicing was performed in ice-cold Krebs-Henseleit buffer (34) supplemented with glucose to a final concentration of 25 mM, saturated with 95% O₂-5% CO₂ (carbogen; pH 7.42). Human and rat liver slices of -200- to 300-μm thickness and a wet weight of 10–40 mg were prepared with the standard settings (cycle speed 40; interrupted mode) of the Krumdieck slicer. After being sliced, the human and rat liver slices were stored in ice-cold UW until the start of the experiment within an hour (32).

Incubation of liver slices. Human and rat liver slices were incubated individually in six-well plates, each slice in 3.2 ml William’s medium E supplemented with d-glucose to a final concentration of 25 mM, 50 μg/ml gentamicin, and saturated with carbogen. The plates were placed in a plastic container, continuously gassed with carbogen, and incubated at 37°C under continuous shaking. Slices were incubated in the presence or absence of 100 μg/ml LPS. In experiments in which the influence of the extra addition of IL-1β was studied, IL-1β was added at the start of the incubations with and without LPS at a final concentration of 1.25 ng/ml. At different time points, both slices and media samples were quickly frozen in liquid nitrogen and stored at −80°C.

ATP determination. Slices were snap-frozen in liquid nitrogen after 3 h of incubation in 1 ml 70% ethanol (vol/vol) and 2 mM EDTA in triplicate. After storage at −80°C and homogenization by sonication, extracts were diluted 10 times with 0.1M Tris·HCl/2 mM EDTA, pH 7.8, to lower the ethanol concentration. ATP content was measured using ATP bioluminescence assay kit CLS II from Boehringer (Mannheim, Germany).

Nitrate/nitrite formation. Nitrate/nitrite (NOₓ) formation was studied by measuring nitrate and nitrite released into the medium. NOₓ concentrations in medium were assayed according to Moshage et al. (28).

Cytokine analysis. TNF-α, IL-1β, IL-6, and IL-10 were analyzed in the culture medium by sandwich ELISA, manufactured by, respectively, Pharmingen (Hamburg, Germany) and R&D Systems (Minneapolis, MN).

RT-PCR. Total RNA was isolated from three combined slices with the use of TRIzol reagent. Single-strand cDNA was synthesized from 3 μg RNA using the reverse transcription system of Promega according to the instructions of the manufacturer. Residual DNA was removed with DNase treatment and removal reagent from Ambion. PCR reactions were conducted with 3 μl of cDNA and 47 μl of reaction mixture containing (in mM) 16 (NH₄)₂SO₄, 67 Tris buffer, pH 8.8, 1.5 MgCl₂, and 0.2 dNTPs, with 0.01% Tween 20, 4% DMSO, 0.1 μl of Taq polymerase, and 1 pmol/μl of each specific primer. Initial denaturation and final extension was 5' at 95°C. The primers were designed using specific primer 3 software (http://www.genome.wi.mit.edu/cgi), and the sequences were analyzed by standard nucleotide BLAST at the National Center for Biotechnology Information. For each primer set, the optimal amount of cycles in the linear region of the log scale was determined by PCR reaction, with increasing cycles at high levels of gene expression for the transporters without LPS induction and for iNOS and the cytokines after LPS induction. The optimal amounts of cycles for rat were iNOS 32, IL-1β 28, TNF-α 30, ntcp 26, bsep 28, and mrp2 24. And for human, the optimal amounts of cycles were iNOS 34, IL-1β 28, TNF-α 32, Ntcp 32, BSEP 32, and MRPII 32. Primer sequences are available on request. To quantify the amplified cDNA, 10 μl of the PCR reaction were loaded on a 2% agarose gel. The DNA bands were visualized with ethidium bromide. The intensity of the PCR product was measured by densitometry with the aid of [image or software reference]. For every PCR reaction, GAPDH and/or 18S RNA was used as an internal control. Changes in mRNA gene expression were calculated relative to the amount present after 1 h of incubation in the absence of LPS (human) or at time 0 (rat).

Cryosectioning and immunofluorescence staining. Liver slices were embedded in Tissue-Tek, snap-frozen in isopentane (−80°C), and stored at −80°C until use. Cross sections of 5 μm were cut in a cryostat (Leica CM 3050) at −20°C, placed on Superfrost Plus slides (Menzel, Braunschweig, Germany), air dried, and stored at −20°C until being stained. After fixation in acetone for 10 min at room temperature, sections were rehydrated in PBS for 5 min. For colocalization studies of MRP2 and ZO-1, a mixture of the primary antibodies M2-III-6 (mouse anti-MRP2; 1:400 dilution, rabbit anti-ZO-1 (1:400) diluted in PBS/BSA alone, or a single primary antibody for control staining was applied for 16 h in a wet chamber. After being rinsed and washed, the slides were incubated for 1 h with a mixture of FITC anti-mouse IgG

Table 1. Donor characteristics used in this study and NOₓ and cytokine production of liver slices incubated for 24 h with 100 μg/ml LPS

<table>
<thead>
<tr>
<th>Donor Number</th>
<th>Sex</th>
<th>Age, yr</th>
<th>Protein, nmol/mg</th>
<th>NOₓ (24 h), μM</th>
<th>TNF-α (24 h), pg/ml</th>
<th>IL-1β (24 h), pg/ml</th>
<th>IL-6 (24 h), pg/ml</th>
<th>IL-10 (24 h), pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>female</td>
<td>7</td>
<td>8.2</td>
<td>171</td>
<td>110</td>
<td>1,033</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>male</td>
<td>7</td>
<td>4.2</td>
<td>340</td>
<td>363</td>
<td>6,263</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>male</td>
<td>11</td>
<td>7.1</td>
<td>24</td>
<td>77</td>
<td>1,050</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>male</td>
<td>16</td>
<td>6.8</td>
<td>450</td>
<td>289</td>
<td>3,527</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>female</td>
<td>13</td>
<td>4.5</td>
<td>516</td>
<td>505</td>
<td>3,150</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>female</td>
<td>11</td>
<td>15.8</td>
<td>71</td>
<td>ND</td>
<td>391</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>male</td>
<td>34</td>
<td>7.0</td>
<td>18</td>
<td>ND</td>
<td>176</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>female</td>
<td>18</td>
<td>10.0</td>
<td>&lt;5</td>
<td>ND</td>
<td>167</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>male</td>
<td>33</td>
<td>6.5</td>
<td>&lt;5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined; NOₓ, nitrate/nitrite.
and TRITC anti-rabbit IgG (1:400) in PBS with 1% BSA. After a final washing step, the slides were covered with mounting medium (1,4-diazabicyclo[2.2.2]octane (2.5%) in glycerol/PBS (9:1) and a coverslip. For the colocalization of MRP2 and BSEP, the same procedure was used with the rabbit anti-BSEP dilution of 1:200. For NTCP staining, the sections were fixed for 10 min in acetone followed by 10 min in chloroform. Before incubation with rabbit anti-NTCP (1:100 for 60 min at room temperature in 1% BSA/PBS), sections were blocked with 5% BSA/PBS. The second antibody was goat anti-rabbit/Alexa Fluor 568 (1:400).

Confocal laser scanning microscopy. Images were taken with a confocal laser scanning microscope (True Confocal Scanner 4D, Leica, Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB inverted microscope (Leica). Double-label images were taken sequentially at 488 and 562 nm to avoid bleedthrough into the other channel.

Statistics. PCR experiments were performed with five human livers and three rat livers using slices in triplicate from each liver. The three slices were combined for the RNA isolation. NOx and cytokine analyses were done in triplicate. Results were compared using two-tailed unpaired Student’s t-test. A P value <0.05 was considered significant.

RESULTS

LPS-induced inflammatory responses in rat liver slices are in line with in vivo studies. Incubations of rat liver slices with 100 μg/ml LPS resulted in an average NOx concentration of 82 μM (n = 3) at 24 h of incubation. iNOS, TNF-α, and IL-1β expression at the level of mRNA were measured using the RT-PCR technique. All three genes were significantly upregulated vs. their respective controls following LPS treatment (Fig. 1, A-C). iNOS mRNA expression was not measurable in fresh liver slices, but it appeared clearly after 3 h of incubation, both with and without LPS. Therefore, induction of INOS was arbitrarily expressed relative to the expression level at 3 h in the absence of LPS (Fig. 1, A-C). TNF-α induction was expressed relative to the expression level at 1 h in the absence of LPS (Fig. 1B) and IL-1β to the expression level at time 0 (Fig. 1C). The highest induction levels for iNOS were observed after 8 and 16 h, for TNF-α at 5 h, and for IL-1β at 5 and 8 h. iNOS, TNF-α, and IL-1β expression slightly increased during control incubations, which is tentatively attributed to the unavoidable presence of trace amounts of LPS in the control incubations. Previous experiments showed that 10,000-fold lower LPS concentrations (10 ng/ml) already gave rise to elevated NOx production (33). The same samples were used for the analysis of the expression of ntcp, bsep, and mrp2 (Fig. 1, D-F). Induction of the carriers was expressed relative to the levels in fresh tissue slices at time 0. Although ntcp was clearly downregulated starting at 8 h of incubation in the presence of LPS, downregulation was only significant at 24 h due to the simultaneous decrease of the controls (Fig. 1D). On the other hand, bsep was not affected by incubation with or without LPS (Fig. 1E). The mrp2 mRNA in the presence of LPS was downregulated, whereas the control slices perfectly retained their original levels. A significant downregulation of mrp2 was observed at 16 and 24 h (Fig. 1F). Downregulation of ntcp and
The human liver slices, cytokine production varied considerably. (data not shown). In contrast to the rat liver slices (33), in which not only the overshoot of cytokines and NOx produced in the incubation medium but also the release into the medium. In Table 1, the individual NOx release is significant after 16 h of incubation (data not shown). In contrast to the rat liver slices (33), in human liver slices, cytokine production varied considerably.

The first five livers of this list were used for the RT-PCR experiments, and livers 6–9 were used for immunohistochemical analysis. The average values for TNF-α, IL-1β, IL-6, and IL-10 release of human livers (1–5) are given in Fig. 2. TNF-α production was significant after 3 h of exposure to LPS, and the level remained constant up to 24 h. IL-1β, IL-6, and IL-10 were significantly increased after 5 h, and the amount increased up to 24 h.

iNOS, TNF-α, and IL-1β were also measured at the level of mRNA (Fig. 3, A–C). Similar to the rat, iNOS mRNA could only be visualized with RT-PCR after 3 h of incubation with or without LPS. Therefore, the induction level is expressed relative to the level at 3 h in the absence of LPS (Fig. 3A). TNF-α was rapidly induced and had already reached its highest level after 1 h. After 3 h, TNF-α mRNA slowly decreased until levels were not significant above the controls at 8 h of incubation (Fig. 3B). IL-1β mRNA was highest at 3 h of incubation and remained relatively constant during the course of the experiment (Fig. 3C). In the same cDNA samples, mRNA levels of the transporters NTCP, BSEP, and MRP2 were measured (Fig. 3, D–F). NTCP in human liver slices remained constant in the control incubations but was downregulated in the presence of LPS, already visible at 16 h but only significant after 24 h (Fig. 3D). BSEP and MRP2 mRNA were not significantly affected by LPS. BSEP levels showed a tendency to increase in time, both in the presence of LPS and in the control samples, but MRP2 was relatively constant during the 24 h of incubation (Fig. 3, E and F). Downregulation of NTCP and simultaneous maintenance of MRP2 expression levels were confirmed with real-time PCR experiments for three of five human livers, in which SYBRgreen was used to detect the PCR product (data not shown).

Downregulation of NTCP in human liver slices correlates with IL-1β and TNF-α production. Cytokine release in response to LPS varied considerably in the different human liver slices (Table 1). In Fig. 4, NTCP mRNA levels, after 24-h exposure to LPS, are plotted as a function of IL-1β (Fig. 4A) and TNF-α (Fig. 4B) production. NTCP downregulation was most pronounced in the livers, with the highest IL-1β and TNF-α production having a correlation of 0.8 and 0.6, respectively (Fig. 4). Moreover, the NTCP signal further decreased if an extra amount of 1.25 ng/ml IL-1β was added simultaneously with LPS at the start of the experiment (data not shown). This observation suggests that IL-1β is involved in the downregulation of NTCP.
downregulation of NTCP. Also, TNF-α has been mentioned as an important mediator in endotoxin-induced cholestasis in several studies (13, 27, 37, 41). Yet, in the absence of LPS, neither IL-1β nor TNF-α had an effect on expression of the carriers, indicating that downregulation of NTCP is caused by multiple factors (data not shown). IL-6 and IL-10 production did not correlate with NTCP downregulation. The correlation coefficient in both cases was 0.2.

**MRP2 and BSEP downregulation in human liver is due to a posttranscriptional process.** Although human MRP2 was not downregulated at the level of mRNA on LPS treatment, impairment of MRP2 in humans is expected on the basis of jaundice in patients with cholestatic diseases. To study the localization and semiquantitative protein expression of the transporters with the use of immunofluorescence microscopy, four additional human livers were treated with LPS (Table 1, livers 6–9). The data shown in Figs. 5 and 6 are from liver 8 and are representative of all livers examined. As a marker for the canalicular membrane ZO-1, a component of the tight junction was chosen (36, 42). For the localization of MRP2, ZO-1 and MRP2 were stained simultaneously. In the first 5 h of LPS incubation, no changes in MRP2 staining could be observed.

---

**Fig. 3.** The effect of LPS on the mRNA levels of iNOS, TNF-α, IL-1β, NTCP, BSEP, and MRP2 in human liver slices. Human liver slices were incubated with (filled bars; 100 μg/ml) and without LPS (open bars) during 24 h. mRNA levels of iNOS (A), TNF-α (B), IL-1β (C), NTCP (D), BSEP (E), and MRP2 (F) were measured at indicated time points with RT-PCR as described in MATERIALS AND METHODS. Values are the means ± SE of 5 independent experiments. *P < 0.05.

**Fig. 4.** Correlation between IL-1β and TNF-α release and NTCP mRNA levels in human liver slices after 24 h of LPS incubation. Human liver slices were incubated with and without LPS (100 μg/ml) during 24 h. IL-1β release and NTCP mRNA levels were measured as described. The individual data of 5 human livers are plotted.
After 8 h, some decrease in MRP2 staining was visible. After 16 h, the decrease was more prominent, and after 24 h, MRP2 was essentially absent in the canalicular membrane. During this process, MRP2 immunofluorescence remained localized in the bile canaliculi, as indicated by ZO-1, and intracellular vesicles containing MRP2, as described in rat liver on LPS treatment (21), were not observed. In Fig. 5, A-D, immunostaining of MRP2 and ZO-1 after 1 and 24 h of incubation with and without LPS is shown. According to ZO-1 staining, canalicular structures remained intact during 24 h of incubation with and without LPS (Fig. 5, C and D). In the presence of LPS, only small traces of MRP2 are visible after 24 h (Fig. 5D), whereas in the control slices, the amount of MRP2 was not significantly decreased (Fig. 5C). In the next experiment, MRP2 was stained together with BSEP. Figure 6, E-J, shows immunostaining of MRP2 and BSEP in control slices and slices incubated with LPS for 24 h. BSEP completely colocalized and disappeared together with MRP2 (compare Fig. 6, E-G and H-J). The sinusoidal membrane protein NTCP was also downregulated in human liver slices (Fig. 6, A-D). In control slices, a slight decrease in NTCP protein was seen after 24 h of incubation (Fig. 6B). In the presence of LPS, NTCP protein loss was more dramatic (Fig. 6C). If extra amounts of TNF-α and IL-1β were added to the incubation medium together with LPS, NTCP was no longer detectable (Fig. 6D).

LPS induced downregulation of MRP2, BSEP, and NTCP at the protein level in human liver. Because mRNA levels of MRP2 and BSEP were not affected, protein expression for these two proteins appears to be regulated by a posttranscriptional process.

Rat liver slices were immunostained for mrp2 and ZO-1 using exactly the same procedure and antibodies for comparison with the human data. Samples with and without LPS incubation were taken at 2, 4, 16, and 24 h. The overall picture...
closely resembles the human data. Rat mrp2 gradually disappears from the canalicular membrane, resulting in almost complete loss of mrp2 after 24 h of LPS incubation. Figure 5, E-H, shows the results obtained after 2 and 24 h. Interestingly, in the rat slices, we could detect small dots with mrp2 staining delocalized from the canalicular membrane (Fig. 5F, arrow).

Finally, we examined rat liver slices that had been treated with a combination of the inflammation inhibitor dexamethasone and LPS. Dexamethasone counteracted the effect of LPS completely. After 24 h of incubation in the presence of dexamethasone and LPS, mrp2 immunostaining was not significantly altered with respect to the control (data not shown). The immunohistochemical data obtained with rat liver slices completely reflect earlier studies performed with the rat liver perfusion technique (21).

**DISCUSSION**

In the present study, we showed that regulation of gene expression in response to LPS is different for rat and human: in rat liver, mrp2 mRNA is downregulated, whereas in human liver, MRFP2 mRNA levels remain unchanged during 24 h of incubation with LPS. In contrast to the mRNA levels, both MRFP2 and BSEP protein are removed almost
completely from the canicular membrane in the presence of LPS, indicating that these two proteins are regulated posttranscriptionally.

The present data obtained with rat liver slices are in line with in vivo data. Just as in rat liver, incubation of human liver slices with LPS resulted in cytokine release and NOx production. However, the magnitude of the responses was very variable. Such high variations are usually observed in human livers when employed for studying drug metabolism (3, 9, 31, 32). This variation could be inherent to intrinsic individual differences but also to the variation in liver quality of the different donors (9). In the present study, we only analyzed those livers with an ATP content of 2.0 nmol/mg protein or more after 3 h of incubation. In general, high ATP content corresponds with good morphology of the slices. Human livers with low ATP content showed relatively low or no cytokine and NO release, indicating that these livers with low viability also showed a low LPS response. However, NOx production in human liver is unpredictable and does not implicate low viability. High IL-1β production was positively correlated with high TNF-α production. No correlation of IL-1β levels with IL-6 or IL-10 production or NOx release could be observed (Table 1).

It is highly unlikely that these variations are inherent to the slice technology. In the first place, rat liver slices do not show this variation. Second, human liver slices retain the RNA levels of NTCP, MRP2, and BSEP during 24 h of incubation in the absence of LPS, irrespective of their reaction on LPS treatment (Fig. 3, D-F). In addition, the continuous exposure of the slices to LPS resulted in continuously high TNF-α levels and continuously increasing NOx, IL-1β, IL-6, and IL-10 levels up to 24 h. It is important to note that NTCP levels in human liver slices are much better maintained compared with those in cultured hepatocytes (23).

The cold preservation time (6–13 h) of the human transplantation livers used in this study is not expected to be of influence on transporter expression. The membrane transport capacity of hepatocytes isolated from livers preserved during 6–39 h was not significantly different from those isolated from nonpreserved healthy human liver tissue obtained from patients undergoing partial hepatectomy (32).

Both in rat and human liver slices, the NTCP gene is clearly downregulated. From both species, the genes have been cloned and their respective promoters have been analyzed (4, 6, 10, 20, 35, 38). Although the rat and human gene show a homology of 77%, the 5′-flanking promoter regions differ considerably (35). The effect of endotoxin on the rat ntcp gene has been investigated in different promoter studies (4, 38). Ntcp downregulation by endotoxin is mediated at the level of transcription through reduction of the nuclear binding activity of hepatocyte nuclear factor 1 (HNF1) and retinoic X receptor (RXR)/retinoic acid receptor (RAR). Evidence has been presented that IL-1β is responsible for the suppression of nuclear levels of RXRα/RARα (4, 5). More recently, the involvement of IL-1β in endotoxin-induced downregulation of mmp2 and ntcp has been confirmed in in vivo studies in the rat. Although HNF1 and RXR/RAR have been identified as important for in vitro regulation of these transporter genes, their role in vivo could not be confirmed in these experiments (12). In the present study, we hypothesized that in human liver downregulation of NTCP is also mediated by IL-1β. This idea is supported by the following observations: human liver slices with the highest IL-1β show the highest degree of NTCP downregulation. Moreover, if an extra amount of IL-1β is added together with LPS, NTCP expression levels were further decreased or even completely abolished. TNF-α could also be involved in the downregulation of human NTCP (Fig. 4B). To get more solid information about the differential effects of cytokines on human NTCP mRNA expression, more experiments are needed. A suitable approach could be the selective inactivation of cytokines with specific inhibitors during LPS challenge, as has been described by Geier et al. (12).

In contrast to the rat studies, MRP2 in human liver appeared not downregulated at the level of mRNA after LPS treatment. For the rat, it has been proposed that both ntcp and mmp2 are downregulated via suppression of RXR/RAR by IL-1β (14). In recent studies in mice, it was also shown that cytokines (IL-6 or IL-1β) suppress mmp2 at the level of mRNA in endotoxemia (14). In humans, this regulatory pathway apparently is not present for MRP2.

Downregulation of bsep by LPS in the rat in vivo was much less pronounced compared with ntcp or mmp2 (25, 40). In accordance with this observation, BSEP expression at the level of mRNA in human liver was not affected.

In this study, we show that downregulation of MRP2 in humans occurs via posttranscriptional mechanisms. This is confirmed by studies in liver biopsies from patients with inflammation-induced icteric cholestasis (43). mRNA levels of MRP2 in these liver biopsies remained unchanged compared with control liver biopsies, whereas MRP2 protein levels studied with immunostaining were significantly reduced. More recently, it was observed that citalopram- and dothiepin-induced cholestasis in two patients was accompanied by hepatocellular relocation of MRP2 to the basolateral membrane, whereas expression was sustained (26).

Localization studies of rat mmp2 have indicated that LPS can induce the translocation of mmp2 from the canicular membrane into cytoplasmic membrane vesicles (7, 21). Most probably, the same mechanism is involved in the disappearance of MRP2 from the canaliculus in human liver. However, intracellular vesicles containing MRP2 were not observed in human liver. This might reflect a species difference for translocation and/or degradation of MRP2 between rat and human. Alternatively, this might be due to a detection problem caused by relative low quantities of human MRP2 in the membrane vesicles or by low affinity of the antibody used.

The in vitro slice technology offers the possibility of a direct comparison between human and rat transporter gene regulation at the same experimental conditions. In the near future, we will focus on the role of key regulatory transcription factors, which play a role in the downregulation of NTCP and the trafficking pathway and mechanism of degradation of MRP2 and BSEP after being removed from the canicular membrane.

ACKNOWLEDGMENTS

We thank J. Bijzet for help with the IL-6 analysis.

GRANTS

This work was supported by the Netherlands Organization for Scientific Research (NWO; Grant NWO-902.23.191).

REFERENCES

1. Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, and Suchy FJ. Human bile salt export pump promoter is


