Hepatobiliary Transporter Expression in Intercellular Adhesion Molecule 1 Knockout and Fas Receptor-Deficient Mice after Common Bile Duct Ligation Is Independent of the Degree of Inflammation and Oxidative Stress

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ABSTRACT:

Liver injury in intercellular adhesion molecule 1 knockout (ICAM−/−) and Fas receptor-deficient (lpr) mice is markedly reduced after common bile duct ligation (CBDL) due to significantly reduced inflammation and oxidative stress. Liver injury in CBDL rodents is counteracted by adaptive hepatobiliary transporter induction. Since hepatobiliary transporter expression in obstructive cholestasis may be regulated not only by accumulating bile acids but also by inflammatory mediators and oxidative stress, we hypothesized that differences in the inflammatory response may affect hepatobiliary transporter expression in CBDL, which would contribute to reduced liver injury. Therefore, expression of major hepatobiliary transporters (Ntcp, Bsep, Mrp2–4, Ostα/β) was determined by Taqman RT-PCR and Western blotting in sham-operated animals and 3 days after CBDL in wild-type, ICAM−/− and lpr mice of the endotoxin-sensitive C57BL/6 and the endotoxin-resistant C3H/HeJ strains. CBDL resulted in a significant decrease of Ntcp in all genotypes. Canalicul transporters Bsep and Mrp2 were repressed only in the endotoxin-sensitive strain regardless of the genotype. Mrp3 was moderately induced in ICAM−/−, lpr, and endotoxin-resistant mice, whereas Mrp4 was only induced in the endotoxin-resistant strain. Ostβ was massively induced in all CBDL mice, whereas Ostα was reduced. In conclusion, markedly reduced inflammation and oxidative stress in CBDL ICAM−/− and lpr mice does not profoundly affect hepatobiliary transporter expression. Therefore, transporter expression does not account for reduced liver injury in ICAM−/− and lpr mice. Induction of the adaptive transporter response after CBDL is independent of the degree of the inflammatory response. Rather, retention of biliary constituents may determine transporter expression in CBDL.

Cholestasis leads to an accumulation of potentially toxic biliary compounds normally excreted via bile (e.g., bile acids and bilirubin). To reduce toxic cellular burden under cholestatic conditions, hepatocytes are capable of activating a range of defense mechanisms including changes in hepatobiliary transporter expression (Trauner and Boyer, 2004). This has been demonstrated in animal models of cholestasis, where expression of uptake systems for bile acids and organic anions (e.g., Ntcp, Oatp1) is reduced and alternative overflow systems are capable of activating a range of defense mechanisms including changes in hepatobiliary transporter expression (Green et al., 1996; Trauner et al., 1997b, 1998; Zollner et al., 2003a), suggesting a pivotal role for bile acids in mediating adaptive hepatocellular transporter expression.

In addition to bile acids, proinflammatory cytokines and oxidative stress have profound effects on bile secretion and hepatobiliary transporter expression (Green et al., 1996; Trauner et al., 1997b, 1998; Kubitz et al., 1999; Geier et al., 2003, 2005a, b; Siewert et al., 2004; Perez et al., 2006). As such, expression of hepatocellular organic anion uptake systems at the basolateral membrane (i.e., Ntcp, Oatp1, Oatp2, Oatp4) as well as efflux pumps at the canalicular membrane (Bsep, Mrp2) are reduced in lipopolysaccharide-challenged rodents generally in line with changes observed in human cholestatic liver diseases (e.g., primary biliary cirrhosis) (Zollner et al., 2006a). Feeding of cholic acid in mice, which represents the major bile acid retained under cholestatic conditions, led to transporter alterations comparable to those observed in response to CBDL (Fickert et al., 2001; Zollner et al., 2003a), suggesting a pivotal role for bile acids in mediating adaptive hepatocellular transporter expression.

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ABBREVIATIONS: ABC, ATP-binding cassette; Bsep (Abcb11), bile salt export pump; CBDL, common bile duct ligation; ICAM-1, intercellular adhesion molecule 1; lpr mice, Fas receptor-deficient mice; Mrp, multidrug resistance-associated protein; Ntcp (Slc10a1), Na+/taurocholate cotransporter; Oatp, organic anion transporter; Ost, organic solute transporter.
TABLE 1
Comprehensive summary of the key findings on hepatic injury and inflammatory gene expression of wild-type, ICAM−/−, and lpr mice from endotoxin-sensitive (C57BL6/6J) and endotoxin-resistant (C3H/HeJ) strains after 3-day CBDL as published in detail previously (Gujral et al., 2004a,b).

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<th>Endotoxin-Sensitive</th>
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<td>Neutrophil extravasation</td>
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(Cremer et al., 1996; Trauner et al., 1997a, 1998; Lee et al., 2000; Geier et al., 2003; Li and Klaassen, 2004). Again, comparable transporter changes were observed in liver biopsies from patients with inflammation-induced cholestasis (Zollner et al., 2001). Inflammation-induced down-regulation of transporters is thought to predispose and prolong cholestatic conditions via the reduction of canalicular bile flow irrespective of the initial etiology. However, the relative contribution of retained endogenous bile acids and inflammatory mediators and oxidative stress in regard to transporter dysregulation and cholestatic liver injury in biliary obstruction is largely undefined.

CBDL-induced cholestatic liver injury is markedly reduced in intercellular adhesion molecule 1 gene knockout (ICAM−/−) and Fas receptor-deficient (lpr) mice, which has been attributed to reduced inflammation, parenchymal neutrophil extravasation, and reactive oxygen formation (Gujral et al., 2003, 2004a,b) (Table 1). Because proinflammatory cytokines are key regulators of hepatobiliary transporter expression, we hypothesized that differences in the regulation of hepatobiliary transport systems as a result of a reduced inflammatory response and reduced oxidative stress may contribute to reduced cholestatic liver injury in CBDL ICAM−/− and lpr mice. This study was therefore designed to compare hepatocellular transporter expression patterns in CBDL ICAM−/−, lpr mice, and wild-type controls.

Materials and Methods

Animals and Experimental Protocol. Experimental protocol (i.e., CBDL for 3 days) and characterization of cholestatic liver injury in male wild-type mice of the endotoxin-sensitive strain, C57BL6/6J, and the endotoxin-resistant strain, C3H/HeJ (Gujral et al., 2004b), ICAM−/− (C57BL6/6J), and Fas receptor-deficient lpr mice (C57BL6/6J and C3H/HeJ) were published previously (Gujral et al., 2004a,b). Aliquots of frozen liver from four to five of these animals were stored in liquid nitrogen and used for further total RNA and protein preparation.

RNA Isolation, Reverse Transcription, and TaqMan Real-Time PCR. Total hepatic RNA from four to five mice was isolated and reverse-transcribed into cDNA as described previously (Fickert et al., 2004). Real-time PCR was performed as described previously (Wagner et al., 2003). Primers and probes used for Taqman RT-PCR are described elsewhere (Wagner et al., 2005).

Preparation of Liver and Analysis of Transporter Protein Levels by Western Blotting. Crude liver membranes were prepared from four to five wild-type and lpr mice of the C57BL6/6J and C3H/HeJ strains as described previously (Fickert et al., 2001; Wagner et al., 2003) whenever sufficient tissue aliquots were available. Transporter protein levels were determined using polyclonal first antibodies against Bsep (dilution, 1:7500; rabbit; kindly provided by Dr. Renxue Wang, British Columbia Cancer Research Center, Vancouver, Canada) (Wang et al., 2001), Ntcp (dilution, 1:2500; rabbit; kindly provided by Dr. Bruno Stieger, University Hospital, Zurich, Switzerland), Mrp2 (dilution, 1:1000; rabbit; kindly provided by Dr. Bruno Stieger, University Hospital, Zurich, Switzerland), Mrp3 (dilution, 1:1000; rabbit; kindly provided by Dr. Dietrich Keppler, Deutsches Krebsforschungszentrum, Heidelberg, Germany) as described previously (Fickert et al., 2001; Wagner et al., 2003). Blots were reprobed with an anti-β-actin antibody (dilution, 1:5000; rabbit; Sigma, Steinheim, Germany) to confirm the specificity of the observed changes in transporter protein levels. Immune complexes were then detected using horseradish-conjugated goat anti-rabbit IgG (F(ab′)2 fragments (dilution, 1:1000; Dako, Glastrup, Denmark) according to the ECL Western blotting detection system (GE Healthcare, Vienna, Austria).

Statistical Analysis. Four to five animals of each group were studied in parallel. Data are reported as means ± S.D. For statistical analysis sham-operated animals were compared with the respective CBDL group using Student’s t test to test for the effects of CBDL. Analysis of variance with Bonferroni post-testing was used in the sham and CBDL groups to test for differences among the respective genotypes within one strain. The SigmaStat statistic program (SPSS Inc., Chicago, IL) was used. A probability value <0.05 was considered significant.

Results

CBDL-Induced Changes in Canalicular Transporter Expression Are Independent of the Inflammatory Response in ICAM−/− and lpr Mice of the Endotoxin-Sensitive C57BL6/6J Strain. CBDL for 3 days resulted in a robust and significant down-regulation of Bsep mRNA to 31%, 24%, and 26% compared with sham-operated controls in wild-type, ICAM−/−, and lpr mice, respectively. No significant differences in the degree of Bsep repression were observed among genotypes (Fig. 1A). Bsep protein levels did not change significantly in CBDL wild-type (1.3-fold compared with wild-type sham) and lpr (1.4-fold compared with lpr sham) (Fig. 1C). Mrp2 mRNA levels also showed a trend for reduction after CBDL to 69%, 50%, and 52% compared with sham-operated controls in wild-type, ICAM−/−, and lpr mice, respectively, although statistical significance was achieved only in lpr mice (Fig. 1B). Mrp2 protein levels tended to be reduced in wild-type mice (70%) and were significantly lower in lpr mice (50%) following CBDL (Fig. 1D). Taken together, these results suggest that the reduced inflammatory response in ICAM−/− and lpr mice has no major impact on canalicular transporter alterations in response to CBDL.

CBDL-Induced Changes in Basolateral Transporter Expression Are Independent of the Inflammatory Response in ICAM−/− and lpr Mice of the Endotoxin-Sensitive C57BL6/6J Strain. Three-day CBDL resulted in a significant repression of Ntcp mRNA to 37%, 29%, and 30% in wild-type, ICAM−/−, and lpr mice, compared with the respective sham-operated controls (Fig. 2A). This was also paralleled at the protein level by a significant 50% Ntcp reduction in CBDL wild-type and lpr mice compared with sham-operated controls of the corresponding genotype (Fig. 2D). Mrp3 mRNA expression was significantly induced after CBDL to 2.3-fold and 2.4-fold in ICAM−/− and lpr mice, respectively, compared with their respective sham-operated controls, whereas wild-type mice showed no significant (1.4-fold) changes. Similar changes were observed at the protein level in CBDL wild-type (1.3-fold) and lpr mice (1.5-fold). This induction was significant in ICAM−/− and lpr mice, probably as a result of a trend for lower baseline expression levels (Fig. 2, B and E). Mrp4 mRNA expression did not change significantly 3 days after CBDL, a finding which again was independent of ICAM and lpr (Fig. 2C). Osteonectin mRNA levels were significantly reduced to 32% as early as 3 days after CBDL in wild-type mice (Fig. 2F). ICAM−/− and lpr mice only showed a nonsignificant trend for reduction (34% and 40% of sham-operated controls, respectively) (Fig. 2F). In contrast, the Ostβ subunit of the heteromorphic organic solute transporter showed a significant 8.5-fold, 5.4-fold, and 18.9-fold induction in 3-day CBDL wild-type, ICAM−/−, and lpr mice, respectively, compared with sham-operated controls of the corresponding genotype (Fig. 2G). Taken together, these results again indicate that the reduced...
inflammatory response to CBDL in ICAM<sup>−/−</sup> and lpr mice has no major impact on basolateral transporter alterations.

**CBDL Alters Basolateral but Not Canalicular Transporter Expression in the Endotoxin-Resistant C3H/HeJ Strain, Independent of the Fas Receptor.** In contrast to the endotoxin-sensitive strain, 3-day CBDL did not significantly alter Bsep mRNA and protein expression levels in endotoxin-resistant mice (Fig. 3, A and C). Also, Mrp2 mRNA and protein levels remained unaffected (Fig. 3, B and D).

At the basolateral membrane, however, CBDL resulted in significant reduction of Ntcp to 30% of wild-type sham-operated controls and to 22% of lpr sham-operated controls (Fig. 4A). This was paralleled by a significant 50% and 70% reduction of Ntcp protein levels (Fig. 4B). Similar to results in endotoxin-sensitive animals, Mrp3 mRNA expression was significantly induced 3.2-fold in wild-type and 5.0-fold in lpr mice of the endotoxin-resistant strain (Fig. 4C). Expression of Ostα/β in lpr mice was comparable to the
expression in endotoxin-sensitive animals. Ostα mRNA levels were significantly reduced after 3-day CBDL to 18% in wild-type mice and tended to decrease to 68% in lpr mice compared with respective sham-operated controls (Fig. 4F). In contrast, Ostβ showed a marked 57-fold induction in wild-type animals and a 36-fold induction in lpr mice, respectively (Fig. 4G). These results therefore indicate that endotoxin-resistant mice lack canalicular transporter changes but show the basolateral alterations also seen in endotoxin-sensitive animals. In both situations, there was no role for Fas receptor as indicated by comparable results in wild-type and Fas receptor-deficient lpr mice.

Discussion

We undertook studies to determine the relative importance of inflammation and oxidative stress for the molecular regulation of hepatobiliary transporter expression in a mouse model of obstructive cholestasis. Cholestatic liver injury after CBDL is reduced in ICAM−/− and lpr mice, as reflected by reduced size of necrotic bile infarcts and significantly lower ALT levels (Gujral et al., 2004a,b). Reduced cholestatic liver injury in these animals can be explained by reduced parenchymal and portal neutrophil extravasation, reduced expression of inflammatory mediators such as cytokines, and markers of oxidative stress following CBDL (Gujral et al., 2004a,b) (Table 1). In addition, cholestatic liver injury and markers of inflammation after CBDL are also reduced in endotoxin-resistant C3H/HeJ mice, a strain with a generally lower inflammatory response to cell injury (Gujral et al., 2004b). Therefore, we applied these mouse models with different degrees of inflammation and oxidative stress in response to biliary obstruction.

The current study shows, however, that differences in the inflammatory response to cholestasis in ICAM−/− and lpr mice are not accompanied by significant differences in hepatobiliary transporter expression in response to CBDL, indicating that retained biliary constituents (e.g., bile acids and bilirubin), which were elevated to similar extents (Gujral et al., 2004a), rather than inflammatory mediators and oxidative stress may determine hepatobiliary transporter expression in cholestasis. However, we found strain-specific differences in transporter expression. As such, endotoxin-resistant animals did not show reduced canalicular Bsep and Mrp2 expressions after CBDL, effects which are thought to be pro-cholestatic (Trauner et al., 1997a; Lee et al., 2000). In addition, only endotoxin-resistant mice displayed Mrp4 induction, and induction of Ostβ was more pronounced compared with endotoxin-sensitive mice. Induction of basolateral Mrp4 and Ostβ is thought to be anti-cholestatic by acting as adaptive bile acid overflow systems under cholestatic conditions, thereby reducing the potentially hepatotoxic intracellular bile acid...
load (Wagner et al., 2003; Boyer et al., 2006). Although factors other than susceptibility to endotoxins might account for observed transporter differences between both strains, it is attractive to speculate that the preservation of canalicualar transporter systems in line with the induction of basolateral overflow pumps could at least in part contribute to reduced cholestatic injury in this mouse strain.

Differences in the degree of inflammation and oxidative stress within genotypes, however, did not markedly affect canalicualar and basolateral transporter expression in wild-type, ICAM−/−, and lpr mice after CBDL. In general, canalicualar bile acid transporters such as Bsep and Mrp2 are down-regulated by proinflammatory cytokines and induced by bile acids (Trauner and Boyer, 2003). Bsep expression during longer-standing obstructive cholestasis is, however, relatively well preserved compared with other membrane transporters in animal models as well as in human cholestatic liver disease (Lee et al., 2000; Zollner et al., 2001, 2003b; Wagner et al., 2003). The preserved Bsep expression may operate as an adaptive mechanism under cholestatic conditions and may still promote the biliary excretion of accumulating bile acids (Lee et al., 2000). Also similar to Bsep, Mrp2 expression did not markedly differ among the genotypes after CBDL. In experimental models of inflammation, lipopolysaccharide treatment profoundly reduced Mrp2 expression levels (Trauner et al., 1997a; Geier et al., 2003). Reduced expression and binding activity of transactivating nuclear receptors (Kim et al., 2003; Fang et al., 2004) as well as post-transcriptional mechanisms (e.g., retrieval from the canalicualar membrane) (Kubitz et al., 1999) may be responsible for transporter reduction in inflammation. On the other hand, activation of nuclear receptors such as farnesoid X receptor by bile acids results in increased Mrp2 expression (Fickert et al., 2001; Schuetz et al., 2001; Zollner et al., 2003a). The findings of the current study indicate that the decrease of Mrp2 in endotoxin-sensitive animals cannot be counteracted by accumulating bile acids despite differences in the inflammatory response to cholestatic injury. Resistance to endotoxin, however, prevented Mrp2 down-regulation.

Down-regulation of the major bile acid uptake system Ntcp is a common protective mechanism of liver cells under cholestatic conditions in rodents and human (Gartung et al., 1996; Trauner et al., 1998; Fickert et al., 2001; Zollner et al., 2001, 2003b). In contrast to canalicualar bile acid transporters, proinflammatory cytokines as well as bile acids are able to down-regulate Ntcp. In our experiments, Ntcp was down-regulated independent of the degree of inflammation and oxidative stress, suggesting that retention of bile acids may account for protective Ntcp repression in obstructive cholestasis (Geier et al., 2005b; Zollner et al., 2005). This finding is in line with our previous observation that Kupffer cell depletion and cytokine antagonists in inflammatory-competent mice would have suggested a compromised transporter regulation, as is shown for the acute phase responses, and, thus, less effective nuclear receptor stimulation (Beigneux et al., 2002; Kim et al., 2003).

In summary, we herein demonstrate that reduced inflammation and oxidative stress in ICAM−/− and lpr mice do not markedly affect hepatobiliary transporter expression in obstructive cholestasis. Rather, the retention of biliary constituents such as bile acids and bilirubin may determine expression of hepatic transport proteins in CBDL. Moreover, differences in hepatobiliary transporter expression cannot sufficiently explain reduced cholestatic liver injury observed in ICAM−/− and lpr mice after CBDL. In contrast, differences in neutrophil accumulation, cytokine levels, and oxidative stress modify the extent of cholestatic liver injury in these models.

References


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